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November 2019

Joaquin Sorolla y Bastida (1863–1923). *Court of the Dances, Alcázar, Sevilla*, 1910. Oil on canvas, 37.5 × 25 in. / 95.2 × 63.5 cm. Digital image courtesy of the Getty's Open Content Program, The J. Paul Getty Museum, Los Angeles, CA.

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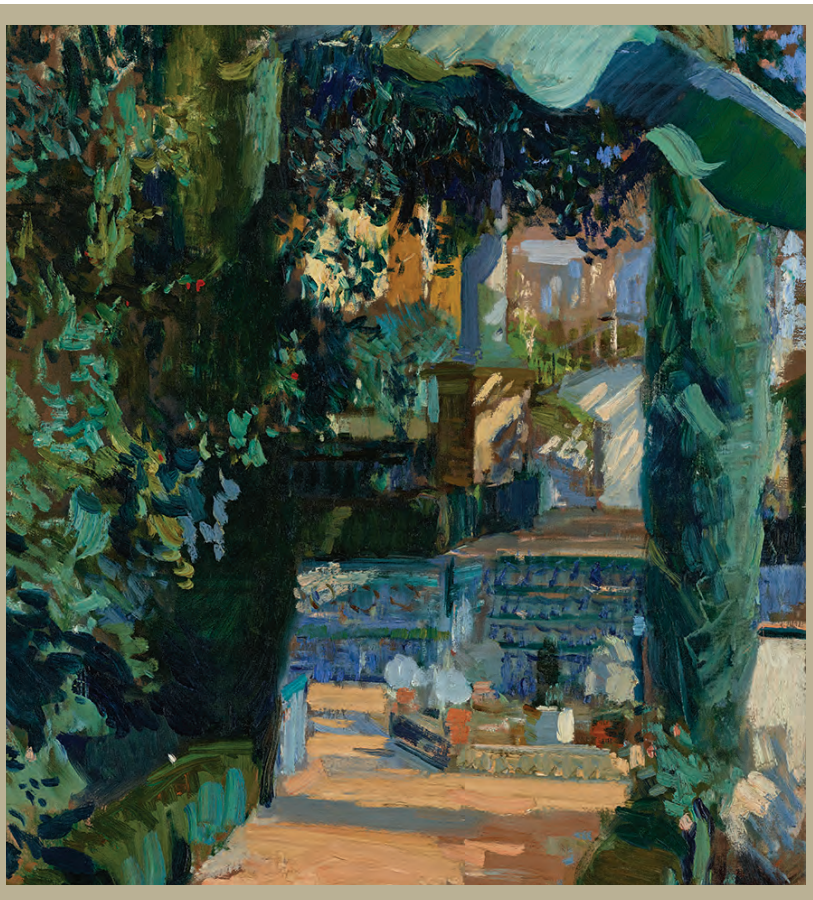

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Joaquin Sorolla y Bastida (1863–1923). *Court of the Dances, Alcázar, Sevilla*, 1910 (detail). Oil on canvas, 37.5 × 25 in. / 95.2 × 63.5 cm. Digital image courtesy of the Getty's Open Content Program, The J. Paul Getty Museum, Los Angeles, CA.

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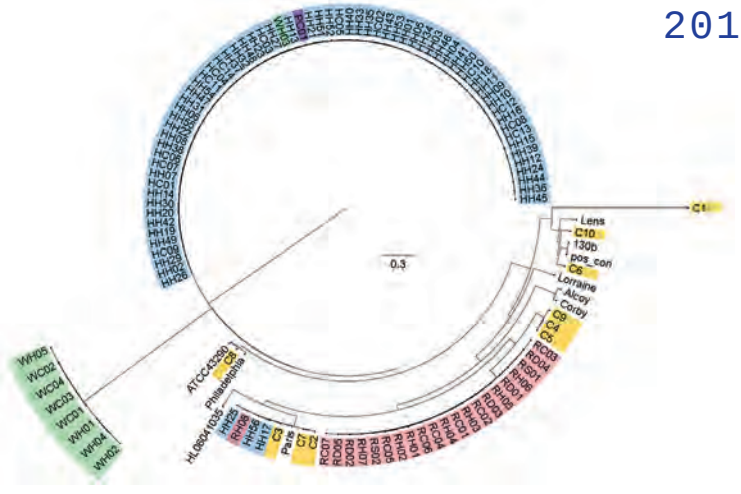

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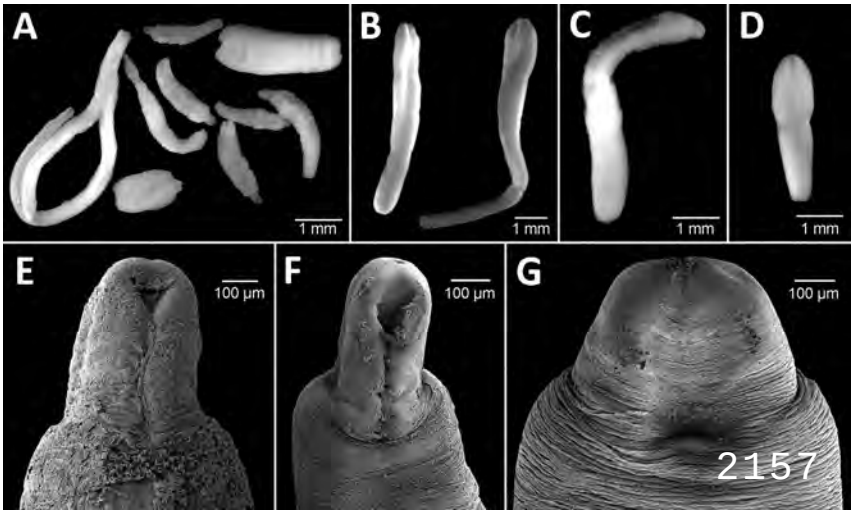

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Vaccine-Derived Poliovirus Infection among Patients with Primary Immunodeficiency and Effect of Patient Screening on Disease Outcomes, Iran

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Patients with immunodeficiency-associated vaccine-derived poliovirus (iVDPV) are potential poliovirus reservoirs in the posteradication era that might reintroduce polioviruses into the community. We update the iVDPV registry in Iran by reporting 9 new patients. In addition to national acute flaccid paralysis surveillance, cases were identified by screening nonparalyzed primary immunodeficiency (PID) patients. Overall, 23 iVDPV patients have been identified since 1995. Seven patients (30%) never had paralysis. Poliovirus screening accelerated the iVDPV detection rate in Iran after 2014. The iVDPV infection rate among nonparalyzed patients with adaptive PID was 3.1% (7/224), several folds higher than previous estimates. Severe combined immunodeficiency patients had the highest risk for asymptomatic infection (28.6%) compared with other PIDs. iVDPV2 emergence has decreased after the switch from trivalent to bivalent oral poliovirus vaccine in 2016. However, emergence of iVDPV1 and iVDPV3 continued. Poliovirus screening in PID patients is an essential step in the endgame of polio eradication.

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During the 3 decades since the establishment of the Global Polio Eradication Initiative (GPEI), the use of oral poliovirus vaccine (OPV) and inactivated poliovirus vaccine (IPV) has led to a >99.99% decrease in the incidence of wild-type poliomyelitis (≈350,000 cases in 1988 to 32 cases in 2018) and eradication of wild poliovirus type 2 (WPV2) (*1*–*4*). No report on wild poliovirus type 3 has occurred since 2012. WPV1 is still circulating in some areas of Pakistan, Afghanistan, and Nigeria (*5*,*6*). OPV has been the backbone of eradication strategy as an inexpensive and easily accessible tool (*7*). However, its widespread use has been associated with some adverse events, including vaccine-associated paralytic poliomyelitis and the emergence of vaccine-derived polioviruses (VDPVs) (*8*). To date, most VDPV case-patients excreted serotype 2 viruses in stool (*9*,*10*). Accordingly, the World Health Organization (WHO) changed the worldwide immunization schedules to end the administration of trivalent OPV (tOPV), which includes serotypes 1, 2, and 3, and introduce bivalent OPV (bOPV), which includes serotypes 1 and 3, alongside ≥1 dose of IPV (all 3 serotypes) (*11*,*12*).

The genome of OPV strains is susceptible to spontaneous mutations because of its unstable structure, and the emergence of VDPVs threatens the whole eradication program (*13*). By definition, VDPV serotypes 1 and 3 have >1% divergence in the viral protein (VP) 1 coding region of their original OPV strain. VDPV2 is defined when the OPV strain serotype 2 attains >0.6% VP1 nucleotide divergence (*10*,*14*). Patients with primary immunodeficiencies (PIDs) are susceptible to not clearing the vaccine strains after receipt of OPV, which provides an environment for prolonged virus replication and genomic changes. These

[1]These first authors contributed equally to this article.

patients have an ≈3,000-fold increased risk for onset of prolonged immunodeficiency-associated VDPV (iVDPV) infection and vaccine-associated paralysis (*2*).

In previous studies, we reported the largest series of patients who had paralysis because of iVDPV infection (*1*). Given the implementation of poliovirus screening programs on PID patients, the number of nonparalyzed iVDPV excretors has increased substantially in recent years (*9*). In this study, we update our registry of patients in Iran with iVDPV infection. In addition to describing important clinical and virologic properties of newly identified patients, our findings underscore the importance of poliovirus screening programs in increasing the iVDPV detection rate.

Study Design

We collected data on patients with iVDPV shedding up to the end of 2018 by consulting a national registry of patients with acute flaccid paralysis (AFP) and by screening the stool of nonparalyzed PID patients for poliovirus infection. All AFP case-patients in Iran are routinely examined for shedding of polioviruses. Stool specimens were collected within 14 days after paralysis onset.

In our screening program, virologic data of patients were collected prospectively during 2014–2018. Patients with an established PID diagnosis recorded in our national PID registry and without any paralytic symptom were considered eligible (*15*,*16*). Inclusion criteria were not restricted to patients' age or any specific PID type. We also included patients with innate immunodeficiencies because these patients might be exposed to iVDPV-excreting patients during hospital stays. Patients consented to and were recruited for 1-time poliovirus screening at admission to the healthcare facilities related to our registry network as well as for routine follow-up visits, intravenous immunoglobulin (IVIG) infusion, or infection control. Our method for stool sampling has been described previously (*15*). Patients were screened only once unless they had positive results in their first encounter, in which case they continued monthly stool testing until the infection cleared. In each encounter, 2 stool specimens were collected and tested over a 4-day period, depending on the proximity of the patient to the study site.

All stool specimens were processed at Iran's National Polio Laboratory in Tehran by using cell culture and real-time reverse transcription PCR, in accordance with WHO protocol (*17*). Poliovirus isolates were sent to the US Centers for Disease Control and Prevention (Atlanta, Georgia, USA) for sequencing of the VP1 genomic region.

For our analysis, we defined VP1 divergence rate as the maximum value (percentage) of VP1 divergence from the original Sabin strain, divided by the total duration (years) of virus replication. As an index for a timely detection of infection, we calculated iVDPV detection speed as the duration of observed iVDPV excretion divided by the total time of iVDPV replication (*9*). To calculate these parameters, we considered the time interval between the first OPV administration and the last iVDPV isolation as the approximate duration of virus replication (*9*).

We extracted clinical and immunologic data from our national PID registry database (*16*,*18*). We categorized patients into a combined immunodeficiency (CID) group (including severe CID [SCID], less severe CID [e.g., major histocompatibility complex class II deficiency], and CID with syndromic features); a predominantly antibody deficiency (PAD) group (including agammaglobulinemia [autosomal and X-linked] and hypogammaglobulinemia); or an innate immunodeficiency group (e.g., neutropenia, chronic granulomatous disease, and complement deficiency), on the basis of the diagnostic criteria of the European Society for Immunodeficiencies (https://esid.org/Working-Parties/Registry-Working-Party/Diagnosis-criteria). We performed genetic evaluation on genomic DNA extracted from whole blood, as described previously (*19*,*20*).

For patients with classical clinical profiles suggestive of a specific CID and agammaglobulinemia, we performed Sanger sequencing on the most likely genes. For patients with failed Sanger sequencing or with clinical characteristics of hypogammaglobulinemia resembling several genetic defects, we performed targeted next-generation sequencing and whole-exome sequencing by using a pipeline described previously (*19*). We reevaluated the pathogenicity of all disease-attributable gene variants by using the updated guideline for interpretation of molecular sequencing by the American College of Medical Genetics and Genomics as described previously (*19*). We performed monthly collection and analysis of stool specimens until the clearance of infection or death. By definition, clearance of iVDPV was achieved when stool was poliovirus-negative for 2 consecutive months (*1*).

We used Stata 14 software (https://www.stata.com) for descriptive and analytical statistics, the Shapiro-Wilk test to assess the normality of distributions, independent *t*-tests for parametric and Mann-Whitney U tests for nonparametric assessment of associations, and the Pearson χ^2 test for categorical parameters. We considered a p value <0.05 statistically significant.

Results

Symptomatic iVDPV-Excreting Patients

We identified 16 patients who had AFP and concurrent iVDPV shedding during 1995–2016 (Appendix Table, https://wwwnc.cdc.gov/EID/article/25/11/19-0540-App1.pdf). Patients 1–14 have been thoroughly described in previous reports (*1*,*21*). Two new cases with paralysis were identified after 2014.

The first paralysis patient (patient 17) was a girl who had right leg paralysis at 6 months of age. PID was suspected on the basis of a history of recurrent respiratory tract infections and axillary bacillus Calmette-Guérin lymphadenitis. She was diagnosed with T-B-NK+ SCID. iVDPV serotype 2 with 9 VP1 nucleotide substitutions was isolated from stool. The last stool specimen collected 4 months after paralysis was positive for iVDPV2 and had 14 nucleotide substitutions. Her parents and monozygotic twin sister, who also had SCID and had received 3 OPV doses, had poliovirus-negative stool. The patient died at age 1 year.

The second paralysis patient (patient 21) was a 14-month-old boy who had seizure and muscle weakness in the neck, trunk, lower limbs, and right upper limb. Low serum immunoglobulins and B-lymphopenia evinced the diagnosis of agammaglobulinemia. iVDPV serotype 2 was isolated from stool. Stool collected 3 months later was poliovirus-negative. The patient is now in good health and is receiving regular IVIG, having only residual paralysis in his right leg.

Asymptomatic iVDPV-Excreting Patients

Since the beginning of our poliovirus screening program among PID patients, a total of 266 nonparalyzed patients (175 boys and 91 girls) have been evaluated. Median age (interquartile range [IQR]) was 5.0 (1.3–11.0) years in all patients, 7.5 (3.0–18.5) years in patients with PAD, 1.4 (0.9–6.0) years in patients with CID, and 4.5 (1.0–5.2) years in patients with innate immunodeficiencies (Figure 1). A total of 602 stool specimens were collected from these patients; 58 specimens were collected from iVDPV-excreting patients during their shedding periods. The most frequent adaptive PID was CVID (76 patients [28.6%]), followed by agammaglobulinemia (51 patients [19.2%]) and SCID (21 patients [7.9%]). Forty-two (15.79%) patients had defective innate immunity (Table).

Four (1.5%) patients were excreting nonpolio enteroviruses, and 12 (4.5%) were excreting Sabin-like (SL) polioviruses only. These patients were retested in the second encounter after 1 month; all had negative results. Seven patients (2.6%) were found to shed iVDPVs and did not have symptoms of paralysis. Of note, 6 of these patients were detected among SCID case-patients (6/21 [28.6%]) and the other among agammaglobulinemia case-patients (1/51 [2%]). In a previous study, we briefly reported 3 of these patients (patients 16, 18, and 19), who were identified among 102 patients screened during January 2014–November 2015 (*15*). We continued our poliovirus screening program to include 164 additional cases. Four patients (patients 15, 20, 22, and 23) were found to have nonparalytic iVDPV shedding after November 2015.

Patient 15 was a boy with T-B-NK+ SCID identified at 8 months of age. He shed iVDPV1 for several consecutive months and ultimately cleared the infection after a successful hematopoietic stem cell transplantation (HSCT) at age 1 year. Detailed characteristics of this patient have been discussed in a separate study (*22*).

Patient 16 was a girl with several hospitalizations for fever, bacillus Calmette-Guérin adenitis, and recurrent urinary infections. At 8 months old, SCID was diagnosed and confirmed by finding a homozygous nonframeshift trinucleotide deletion in a class II major histocompatibility complex transactivator gene. iVDPV2 with 12 nucleotide substitutions were isolated from

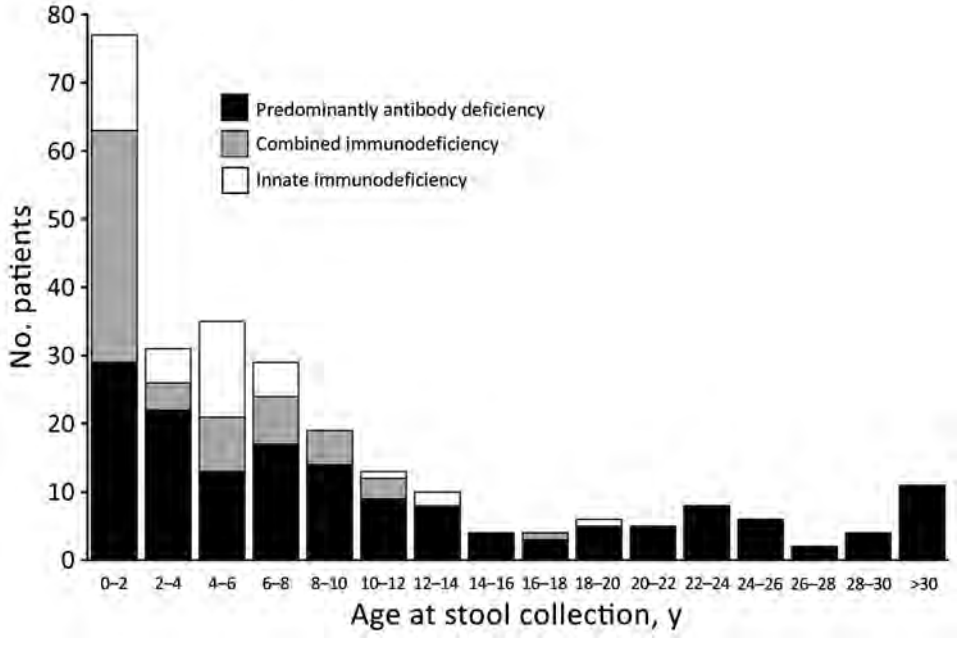

Figure 1. Patient ages at the time of first stool screening in study of vaccine-derived poliovirus infection among patients with primary immunodeficiency, by category of primary immunodeficiencies, Iran, 1995–2018.

Table. Age, PID type, and stool screening results in nonparalyzed PID patients in study of vaccine-derived poliovirus infection among patients with PID, Iran, 1995–2018*

Immunodeficiency	No. patients (%), N = 266	Age at stool collection, y, median (IQR)	Stool screening result
Predominantly antibody deficiencies			
AGG	51 (19.17)	5.0 (2.5–10.7)	1 iVDPV, 3 SL, 1 NPEV
CVID	76 (28.57)	12.5 (7.0–20.7)	2 SL, 3 NPEV
HIGM	17 (6.39)	6.8 (3.2–9.5)	Negative
HGG	18 (6.77)	3.0 (1.2–7.0)	1 SL
Combined immunodeficiencies			
SCID	21 (7.89)	0.9 (0.7–1.2)	5 iVDPVs, 1 SL
Less severe CIDs	21 (7.89)	1.5 (1.0–4.8)	1 iVDPV, 3 SL
CID with syndromic features	20 (7.52)	6.5 (5.0–8.7)	Negative
Innate immunity defects	42 (15.79)	4.5 (1.0–5.2)	2 SL

*AGG, agammaglobulinemia; CID, combined immunodeficiency; CVID, common variable immunodeficiency; HGG; unspecified hypogammaglobulinemia; HIGM, hyper-immunoglobulin M syndrome; IQR, interquartile range; iVDPV, immunodeficiency-associated vaccine-derived poliovirus; MHC2, major histocompatibility class 2 deficiency; NPEV, nonpolio enterovirus; PID, primary immunodeficiency; SL, Sabin-like poliovirus; SCID, severe combined immunodeficiency.

her stool when she was 10 months old. During >3 years of continued shedding, her poliovirus evolved to have 37 VP1 nucleotide substitutions. Despite regular IVIG administration, she still had the infection at the latest follow-up testing. She never had paralysis and is now is a candidate for HSCT.

Patient 18 was an infant boy with oral candidiasis, cutaneous manifestations, and failure to thrive. A presumptive diagnosis of SCID was confirmed by detecting a homozygous mutated adenosine deaminase gene. Stool was positive for an iVDPV2 with 6 VP1 nucleotide substitutions. The patient died at 3.5 months of age from a severe respiratory infection.

Patient 19 was a girl admitted to a peripheral hospital for chronic diarrhea, oral aphthous ulcers, and failure to thrive at 5 months of age. However, her PID was not detected, and she received her fourth OPV dose at 6 months of age. After further workup at 11 months of age, we established a final diagnosis of SCID by detecting a homozygous missense mutation in a recombination activating gene 1. iVDPV2 was isolated from stool. Despite appropriate medical therapy, she died less than 1 month later.

Patient 20 was a boy with agammaglobulinemia. A stool specimen collected at 13 months of age was positive for iVDPV2 with 18 nucleotide substitutions and for SL type 3 virus (SL3). iVDPV2 shedding continued for 3 months and had 20 VP1 nucleotide substitutions. Specimens collected at 18 months of age only showed iVDPV3 with 22 nucleotide substitutions; no poliovirus type 2 was detected. iVDPV3 evolved to have 24 nucleotide substitutions until the patient was 21 months of age. Stool specimens tested 6 months later were negative for any iVDPV serotype.

Patient 22 was a boy who had received 4 doses of bOPV (serotypes 1 and 3) and IPV. At 7 months of age, CID was diagnosed and later confirmed by detecting a homozygous single nucleotide deletion in the regulatory factor X associated ankyrin-containing protein gene. His initial stool specimen was positive for SL3 and had 9 VP1 nucleotide substitutions. Serial stool testing showed a continuous poliovirus replication for 4 months and a gain of 12 VP1 mutations, which exceeded the cutoff level for our definition for iVDPV3. Stool collected 5 months later was poliovirus-negative.

Patient 23 was a boy, the fourth child born to consanguineous parents in rural areas. There was a history of 2 siblings' death from Kostmann syndrome and autosomal-recessive polycystic kidney disease. Immunodeficiency was not detected at birth, and he had received 2 doses of bOPV until 4 months of age, when he had severe pneumonia and SCID. Diagnosis was confirmed by finding a mutated recombination activating gene 2. At 6 months of age, iVDPV1 with 10 VP1 mutations were isolated from his stool. He never had paralysis but died from pneumonia within a few weeks.

Iran's iVDPV Registry

Since the establishment of Iran's iVDPV registry, a total of 23 iVDPV-excreting patients have been identified; 7 (30%) never had paralysis. Nineteen (82.6%) of the 23 patients were boys. PID type was confirmed by molecular studies in 10 patients. Thirteen (56%) patients had CID, and 9 (39%) had PAD. SCID was the most frequent PID type (11 cases [48%]), followed by agammaglobulinemia (8 cases [35%]). One patient had an undetermined PID type. One patient had unspecified hypogammaglobulinemia. The mean (± SD) age at the time of first iVDPV isolation was 10.82 (± 5.18) months for all patients. This age was older in patients who had paralysis (11.8 ± 5.6 months), compared with nonparalyzed patients (8.6 ± 3.1 months). However, the observed difference was not statistically significant ($p = 0.17$).

Eight patients (89%) with PAD and 7 patients (54%) with CID had paralysis ($p = 0.083$). Thirteen patients died. Eleven patients (47.8%) cleared the infection from stool during the follow-up period. Of note, 8 (89%) PAD patients cleared the infection, compared with only 3 (23%) CID patients ($p = 0.002$).

Twenty-six iVDPVs were isolated from 23 patients. Three patients simultaneously shed 2 different serotypes (patients 5, 9, and 20). iVDPV2 was the most frequent serotype (69%), followed by serotype 1 (19%) and serotype 3 (12%). The median (IQR) of maximum VP1 divergence was 1.7% (1.3%–2.3%) and of viral replication time was 12.4 (10.7–19.2) months. The median viral evolution rate was 1.66% (1.14–2.2%) per year. We observed no difference in evolution rate between different groups of patients on the basis of PID category and paralysis occurrence ($p>0.1$). The mean of iVDPV detection speed was higher in nonparalyzed patients than in those with paralysis (0.24 vs. 0.17). However, this difference was not statistically significant ($p = 0.433$).

Discussion

In this study, we describe a comprehensive registry of 23 iVDPV-excreting patients identified in Iran since 1995, as an update to our previous report (*1*). Although most patients initially had paralysis, more than one quarter were asymptomatic and were only identified by poliovirus screening. The rate of asymptomatic iVDPV infection in our national study (2.6% overall; 3.1% in adaptive immunity defects) was several folds higher than this rate in a previous multinational report (0.8%) (*15*), despite a comparable proportion of SCID in both studies. Other studies also reported detection rates several times lower than what we observed in nonparalyzed PID patients (*23*–*25*). These differences can be caused by different proportions of PID types among these studies or the predominance of certain genetic defects or other predisposing factors in different countries.

Paralysis was observed more frequently in patients with PAD than with CID, supporting new evidence that hypothesizes the role of cell-mediated reactions in the progression to paralysis (*9*). PAD patients were also more likely to clear the infection. Immune mechanisms contributing to these observations have been discussed in recent studies; cytotoxic interactions can damage poliovirus-infected motor neurons in the anterior horn of the spinal cord (leading to paralysis) and enterocytes in the gastrointestinal tract (leading to infection clearance) (*4*,*9*,*22*).

The predominance of iVDPV2 is consistent with previous studies (*9*,*10*). Although the global switch to bOPV can reduce the emergence of VDPV2 in OPV-using countries (*26*), bOPV still carries the risk for VDPV emergence from 2 other serotypes (*22*). Gradual evolution of iVDPV3 from SL3 in patient 22 is an illustration of this risk and shows the importance of serial stool testing in immunodeficient SL excreters. According to WHO, iVDPV3 was the most frequent serotype (7/14 iVDPVs) detected worldwide during January 2017–June 2018 (*10*). Asymptomatic shedding of any iVDPV serotype endangers the whole polio eradication program.

A thorough nucleotide analysis revealed that the VP1 evolution rate occasionally exceeded the estimated rate of 1%–2% per year (e.g., patients 5, 8, 10, 15, 18, and 23 had rates >2% per year). Several hypotheses, including random or selected mutations along with genomic recombination, have been proposed for this observation (*1*,*2*,*9*,*27*). Some patients shed multiple variants simultaneously, suggesting that genomic recombination can occur during replication of SL viruses. Patient 20 was initially excreting iVDPV2 along with SL3; after a few months, only iVDPV3 was detected in his stool. This rapid evolution in the VP1 region to reach iVDPV definition could be explained by crossover of genetic material between the preexisting iVDPV2 and concurrently evolving SL3. The underlying mechanism for iVDPV2 clearance and the predominance of iVDPV3 is unclear in this case, which could be attributable to random chance or host–pathogen interactions.

Patient 17 and her monozygotic twin sister both had SCID. Patient 17 was the only member of the family who shed iVDPV. We suppose that the complete polio immunization history in healthy parents has prevented iVDPV transmission to the sisters. The twin sister had a high risk for iVDPV infection both from OPV administration and transmission from an infected sibling. We cannot rule out a previous iVDPV infection and subsequent spontaneous clearance in the uninfected twin. Also, a risk for future transmission would persist in this situation. Although person-to-person transmission of SL can cause immunity in healthy persons, it might lead to prolonged iVDPV infection in immunodeficient contacts. This risk is high given that PID patients are frequently admitted for IVIG administration or infection control and can transmit the virus to other patients. We advocate regular poliovirus screening of all PID patients, especially in those with CID. More frequent screening could be an appropriate strategy in patients who have direct or indirect contact with known iVDPV excretors. Intensified precautions to prevent transmission by the oral–fecal route should be implemented for poliovirus-excreting (SL or iVDPV) patients during hospital admissions.

The fundamental prerequisite for our registry was the implementation of the poliovirus screening program among PID patients. This framework provided the opportunity to diagnose asymptomatic iVDPV excretors and caused a rapid rise in the iVDPV detection rate after 2014 (Figure 2). This acceleration was most dramatic among patients with CID, especially SCID, which increases susceptibility to prolonged and asymptomatic infections (*12*). The rate of asymptomatic shedding was several folds higher in SCID patients than patients with any other PID type; this finding supports the notion that CID patients should receive particular attention in the polio endgame.

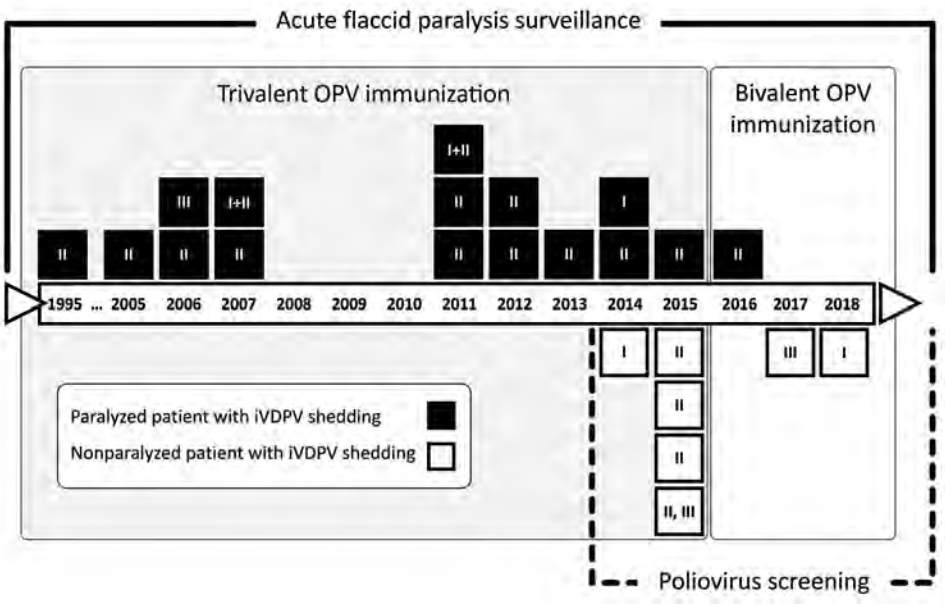

Figure 2. Timeline of Iran's registry of iVDPV infection, showing the number of patients identified after acute flaccid paralysis or through screening, 1995–2018. The iVDPV detection rate was initially accelerated after implementation of the poliovirus screening program. The switch in vaccination schedule from trivalent to bivalent oral poliovirus vaccine was applied in 2016, leading to a decrease in iVDPV serotype 3 emergence. Two patients excreted iVDPVs with combined serotypes 1 and 2. One patient excreted 2 distinct iVDPVs (serotypes 2 and 3). Numbers in each square indicate the iVDPV serotype. iVDPV, immunodeficiency-associated vaccine-derived poliovirus; OPV, oral poliovirus vaccine.

Poliovirus screening programs would likely increase the speed of iVDPV detection speed. Although not statistically significant (probably because of the limited study population), this index was lower in patients identified after paralysis than in asymptomatic patients. This trend is consistent with a recent systematic review suggesting that paralysis might be a late manifestation of iVDPV infection (*9*) and highlights the importance of early poliovirus screening for timely diagnosis and treatment in PID patients, mainly in those with adaptive immunity defects (because we did not identify any paralytic patients with defective innate immunity). Although implementing screening might impose higher costs on the health systems, the eradication goal might not be accomplished without a reasonable expenditure on such programs. A case in point is the environmental detection of vaccine-related polioviruses with some features of iVDPV in communities that have not reported any case of wild or vaccine-related poliomyelitis for decades after their switch to IPV (e.g., Finland, Israel, and Switzerland) (*28*–*30*).

For patient 19, PID remained undiagnosed for a long time after its initial manifestations, leading to inappropriate administration of the fourth OPV dose. In addition, patient 23 did not undergo neonatal PID testing and received OPV despite a family history suggestive for primary immunodeficiency. These occurrences might be attributable to insufficient vigilance of primary healthcare providers to signs and risk factors associated with PID. In many countries, neonatal PID screening is in place for early diagnosis of SCIDs and agammaglobulinemia, which assists the physicians in modifying patients' vaccination scheduling and medical management appropriately (*31*). In addition to implementation of screening programs, improving the awareness of healthcare providers about PID manifestations and the risks associated with OPV administration should be considered as a pivotal part of child healthcare and the polio eradication endgame.

Successful HSCT appears to lead to clearance of iVDPV infection (*22*). Clearing poliovirus infection early after HSCT in patient 15 could be attributed to the recovery phase of innate and cell-mediated immune reconstitution (*22*). Despite appropriate medical therapy, patient 16 never cleared iVDPV2 after >3 years of continuous shedding. HSCT remains the only possible solution for her immunodeficiency and poliovirus infection.

Adjunct to appropriate changes in vaccination strategy, developing new antiviral drugs would be warranted for termination of chronic infections and outbreak control. Pocapavir (V-073) and V-7404 have been suggested as new agents against poliovirus, available for limited, compassionate use for PID patients who are at increased risk for poliovirus infection and are not suitable candidates for HSCT (*15*,*32*). However, the efficacy and safety of these drugs in immunodeficient patients with an established iVDPV infection have not yet been proven.

In conclusion, the emergence of VDPVs remains a serious obstacle in the pathway of polio eradication. Although the recent change in vaccination strategy can prevent the emergence of new cases, unidentified iVDPV excretors still carry the risk for reintroducing viruses from any serotype into the community. Serial screening of PID patients for poliovirus infection is an essential step in the transition to a polio-free world.

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Dr. Shaghaghi is a research scientist at Johns Hopkins Hospital, Baltimore, Maryland, USA. His primary research interests are immunodeficiency and immunooncology.

References

1. Shaghaghi M, Shahmahmoodi S, Abolhassani H, Soleyman-Jahi S, Parvaneh L, Mahmoudi S, et al. Vaccine-derived polioviruses and children with primary immunodeficiency, Iran, 1995–2014. Emerg Infect Dis. 2016;22:1712–9. http://dx.doi.org/10.3201/eid2210.151071
2. Kew OM, Sutter RW, de Gourville EM, Dowdle WR, Pallansch MA. Vaccine-derived polioviruses and the endgame strategy for global polio eradication. Annu Rev Microbiol. 2005;59:587–635. http://dx.doi.org/10.1146/annurev.micro.58.030603.123625
3. Morales M, Tangermann RH, Wassilak SG. Progress toward polio eradication—worldwide, 2015–2016. MMWR Morb Mortal Wkly Rep. 2016;65:470–3. http://dx.doi.org/10.15585/mmwr.mm6518a4
4. Chevalier G, Suberbielle E, Monnet C, Duplan V, Martin-Blondel G, Farrugia F, et al. Neurons are MHC class I-dependent targets for CD8 T cells upon neurotropic viral infection. PLoS Pathog. 2011;7:e1002393. http://dx.doi.org/10.1371/journal.ppat.1002393
5. Hagan JE, Wassilak SG, Craig AS, Tangermann RH, Diop OM, Burns CC, et al.; Centers for Disease Control and Prevention (CDC). Progress toward polio eradication—worldwide, 2014–2015. MMWR Morb Mortal Wkly Rep. 2015;64:527–31.
6. Zaffran M, McGovern M, Hossaini R, Martin R, Wenger J. The polio endgame: securing a world free of all polioviruses. Lancet. 2018;391:11–3. http://dx.doi.org/10.1016/S0140-6736(17)32442-X
7. Plotkin S. History of vaccination. Proc Natl Acad Sci U S A. 2014;111:12283–7. http://dx.doi.org/10.1073/pnas.1400472111
8. Burns CC, Diop OM, Sutter RW, Kew OM. Vaccine-derived polioviruses. J Infect Dis. 2014;210(Suppl 1):S283–93. http://dx.doi.org/10.1093/infdis/jiu295
9. Shaghaghi M, Soleyman-Jahi S, Abolhassani H, Yazdani R, Azizi G, Rezaei N, et al. New insights into physiopathology of immunodeficiency-associated vaccine-derived poliovirus infection; systematic review of over 5 decades of data. Vaccine. 2018;36:1711–9. http://dx.doi.org/10.1016/j.vaccine.2018.02.059
10. Jorba J, Diop OM, Iber J, Henderson E, Zhao K, Sutter RW, et al. Update on vaccine-derived polioviruses—worldwide, January 2017–June 2018. MMWR Morb Mortal Wkly Rep. 2018;67:1189–94. http://dx.doi.org/10.15585/mmwr.mm6742a5
11. World Health Organization. Polio eradication and endgame strategic plan 2013–2018 [cited 2019 Feb 20]. http://polioeradication.org/who-we-are/strategic-plan-2013-2018
12. Driss N, Mellouli F, Ben Yahia A, Touzi H, Barbouche MR, Triki H, et al. Sequential asymptomatic enterovirus infections in a patient with major histocompatibility complex class II primary immunodeficiency. J Clin Microbiol. 2014;52:3486–9. http://dx.doi.org/10.1128/JCM.01122-14
13. Khetsuriani N, Prevots DR, Quick L, Elder ME, Pallansch M, Kew O, et al. Persistence of vaccine-derived polioviruses among immunodeficient persons with vaccine-associated paralytic poliomyelitis. J Infect Dis. 2003;188:1845–52. http://dx.doi.org/10.1086/379791
14. Centers for Disease Control and Prevention (CDC). Update on vaccine-derived polioviruses—worldwide, April 2011–June 2012. MMWR Morb Mortal Wkly Rep. 2012;61:741–6.
15. Aghamohammadi A, Abolhassani H, Kutukculer N, Wassilak SG, Pallansch MA, Kluglein S, et al.; JMF Centers Network Investigators and Study Collaborators. Patients with primary immunodeficiencies are a reservoir of poliovirus and a risk to polio eradication. Front Immunol. 2017;8:685. http://dx.doi.org/10.3389/fimmu.2017.00685
16. Abolhassani H, Kiaee F, Tavakol M, Chavoshzadeh Z, Mahdaviani SA, Momen T, et al. Fourth update on the Iranian National Registry of Primary Immunodeficiencies: integration of molecular diagnosis. J Clin Immunol. 2018;38:816–32. http://dx.doi.org/10.1007/s10875-018-0556-1
17. Dowdle WR, De Gourville E, Kew OM, Pallansch MA, Wood DJ. Polio eradication: the OPV paradox. Rev Med Virol. 2003;13:277–91. http://dx.doi.org/10.1002/rmv.401
18. Aghamohammadi A, Mohammadinejad P, Abolhassani H, Mirminachi B, Movahedi M, Gharagozlou M, et al. Primary immunodeficiency disorders in Iran: update and new insights from the third report of the national registry. J Clin Immunol. 2014;34:478–90. http://dx.doi.org/10.1007/s10875-014-0001-z
19. Abolhassani H, Aghamohammadi A, Fang M, Rezaei N, Jiang C, Liu X, et al. Clinical implications of systematic phenotyping and exome sequencing in patients with primary antibody deficiency. Genet Med. 2019;21:243–51. http://dx.doi.org/10.1038/s41436-018-0012-x
20. Abolhassani H, Chou J, Bainter W, Platt CD, Tavassoli M, Momen T, et al. Clinical, immunologic, and genetic spectrum of 696 patients with combined immunodeficiency. J Allergy Clin Immunol. 2018;141:1450–8. http://dx.doi.org/10.1016/j.jaci.2017.06.049
21. Shaghaghi M, Parvaneh N, Ostad-Rahimi P, Fathi SM, Shahmahmoodi S, Abolhassani H, et al. Combined immunodeficiency presenting with vaccine-associated paralytic poliomyelitis: a case report and narrative review of literature. Immunol Invest. 2014;43:292–8. http://dx.doi.org/10.3109/08820139.2013.859156
22. Shaghaghi M, Irannejad M, Abolhassani H, Shahmahmoodi S, Hamidieh AA, Soleyman-Jahi S, et al. Clearing vaccine-derived poliovirus infection following hematopoietic stem cell transplantation: a case report and review of literature. J Clin Immunol. 2018;38:610–6. http://dx.doi.org/10.1007/s10875-018-0521-z
23. Li L, Ivanova O, Driss N, Tiongco-Recto M, da Silva R, Shahmahmoodi S, et al. Poliovirus excretion among persons with primary immune deficiency disorders: summary of a seven-country study series. J Infect Dis. 2014;210(Suppl 1):S368–72. http://dx.doi.org/10.1093/infdis/jiu065
24. Mohanty MC, Madkaikar MR, Desai M, Taur P, Nalavade UP, Sharma DK, et al. Poliovirus excretion in children with primary immunodeficiency disorders, India. Emerg Infect Dis. 2017;23:1664–70. http://dx.doi.org/10.3201/eid2310.170724
25. Galal NM, Meshaal S, ElHawary R, Nasr E, Bassiouni L, Ashghar H, et al. Poliovirus excretion following vaccination with live poliovirus vaccine in patients with primary

immunodeficiency disorders: clinicians' perspectives in the end-game plan for polio eradication. BMC Res Notes. 2018;11:717. http://dx.doi.org/10.1186/s13104-018-3822-7
26. Garon J, Seib K, Orenstein WA, Ramirez Gonzalez A, Chang Blanc D, Zaffran M, et al. Polio endgame: the global switch from tOPV to bOPV. Expert Rev Vaccines. 2016;15:693–708. http://dx.doi.org/10.1586/14760584.2016.1140041
27. Odoom JK, Yunus Z, Dunn G, Minor PD, Martín J. Changes in population dynamics during long-term evolution of Sabin type 1 poliovirus in an immunodeficient patient. J Virol. 2008;82: 9179–90. http://dx.doi.org/10.1128/JVI.00468-08
28. Roivainen M, Blomqvist S, Al-Hello H, Paananen A, Delpeyroux F, Kuusi M, et al. Highly divergent neurovirulent vaccine-derived polioviruses of all three serotypes are recurrently detected in Finnish sewage. Euro Surveill. 2010;15:19566.
29. Zurbriggen S, Tobler K, Abril C, Diedrich S, Ackermann M, Pallansch MA, et al. Isolation of sabin-like polioviruses from wastewater in a country using inactivated polio vaccine. Appl Environ Microbiol. 2008;74:5608–14. http://dx.doi.org/10.1128/AEM.02764-07
30. Shulman LM, Manor Y, Sofer D, Handsher R, Swartz T, Delpeyroux F, et al. Neurovirulent vaccine-derived polioviruses in sewage from highly immune populations. PLoS One. 2006;1:e69. http://dx.doi.org/10.1371/journal.pone.0000069
31. Puck JM; SCID Newborn Screening Working Group. Population-based newborn screening for severe combined immunodeficiency: steps toward implementation. J Allergy Clin Immunol. 2007;120:760–8. http://dx.doi.org/10.1016/j.jaci.2007.08.043
32. McKinlay MA, Collett MS, Hincks JR, Oberste MS, Pallansch MA, Okayasu H, et al. Progress in the development of poliovirus antiviral agents and their essential role in reducing risks that threaten eradication. J Infect Dis. 2014;210(Suppl 1):S447–53. http://dx.doi.org/10.1093/infdis/jiu043

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etymologia

Serratia marcescens [sə-ra′-she-ə mar-ces′-cens]

Gianluca Nazzaro

Serratia marcescens, which can cause nosocomial outbreaks and urinary tract and wound infections, is abundant in damp environments. It can be easily found in bathrooms, including shower corners and basins, where it appears as a pink–orange–red discoloration, due to the pigment known as prodigiosin. *Serratia* was discovered in Italy in 1819 when it affected polenta in a small town near Padua.

Bartolomeo Bizio, a Venetian pharmacist, studied the mode of transmission of the red substance and named this microorganism *Serratia* in honor of Serafino Serrati, who ran the first steamboat on the Arno River in 1795, anticipating the discovery of Robert Fulton in 1807. The word *marcescens* was chosen from Latin for the species name meaning to decay, reflecting the rapid deterioration of the pigment. *Serratia marcescens* was later renamed *Monas prodigiosus* in 1846, then *Bacillus prodigiosus*, before the original name was restored in the 1920s in recognition of the work of Bizio.

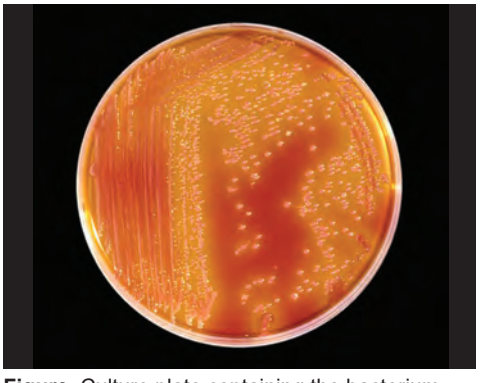

Figure. Culture plate containing the bacterium *Serratia marcescens*. The colonies are red because of a pigment (prodigiosin) produced by this organism. Source: Centers for Disease Control and Prevention, 1985.

Sources

1. Nazzaro G, Veraldi S. *Serratia marcescens*: an Italian story. Int J Dermatol. 2017;56:795–6. https://doi.org/10.1111/ijd.13632
2. Sehdev PS, Donnenberg MS. Arcanum: the 19th-century Italian pharmacist pictured here was the first to characterize what are now known to be bacteria of the genus *Serratia*. Clin Infect Dis. 1999;29:770, 925. https://doi.org/10.1086/520431
3. Veraldi S, Nazzaro G. Skin ulcers caused by *Serratia marcescens*: three cases and a review of the literature. Eur J Dermatol. 2016;26:373–6.

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Comparison of Whole-Genome Sequences of *Legionella pneumophila* in Tap Water and in Clinical Strains, Flint, Michigan, USA, 2016

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During the water crisis in Flint, Michigan, USA (2014–2015), 2 outbreaks of Legionnaires' disease occurred in Genesee County, Michigan. We compared whole-genome sequences of 10 clinical *Legionella pneumophila* isolates submitted to a laboratory in Genesee County during the second outbreak with 103 water isolates collected the following year. We documented a genetically diverse range of *L. pneumophila* strains across clinical and water isolates. Isolates belonging to 1 clade (3 clinical isolates, 3 water isolates from a Flint hospital, 1 water isolate from a Flint residence, and the reference Paris strain) had a high degree of similarity (2–1,062 single-nucleotide polymorphisms), all *L. pneumophila* sequence type 1, serogroup 1. Serogroup 6 isolates belonging to sequence type 2518 were widespread in Flint hospital water samples but bore no resemblance to available clinical isolates. *L. pneumophila* strains in Flint tap water after the outbreaks were diverse and similar to some disease-causing strains.

Legionnaires' disease is a severe form of pneumonia caused by inhalation of virulent species of aerosolized *Legionella* bacteria. In January 2016, the Michigan Department of Health and Human Services (MDHHS) and the Genesee County Health Department publicly announced 2 Legionnaires' disease outbreaks in Genesee County, Michigan, USA (*1*,*2*). The first outbreak occurred from June 2014 through March 2015 and the second from May 2015 through October 2015; a total of 90 cases and 12 deaths were documented (*1*–*3*). From April 2014 through October 2015, the city of Flint, in Genesee County, switched its drinking water source from Detroit Water and Sewer Department (DWSD), which used corrosion control, to the corrosive Flint River, without implementing federally mandated corrosion control; this new water source led to elevated lead in tap water over a prolonged period, now called the Flint water crisis (*4*). This disruption in water quality likely also stimulated the growth of *L. pneumophila*, the species most frequently identified as the causative agent of Legionnaires' disease (*5*,*6*), in Flint's distribution and plumbing systems (*7*).

Our prior work associated the Legionnaires' disease outbreaks with factors known to be conducive to *Legionella* growth: elevated iron (a consequence of corroded iron water mains), reduced free chlorine disinfectant residuals, and elevated water temperatures (*7*,*8*). Later, Zahran and colleagues reported that the odds of Flint residents being referred for Legionnaires' disease treatment while the Flint River was the source of tap water increased 6.3-fold and confirmed our report of associations with low chlorine residuals (*9*), but the odds analysis, which was based on the use of referral date rather than symptom onset date, excluded many healthcare-associated cases (*10*). Furthermore, during the second outbreak, *Legionella* spp. and *L. pneumophila* genes were found to be higher in the tap water of large buildings in Flint than in other water systems in US areas not experiencing outbreaks (*8*). Conversely, levels of the *mip* gene, which is specific to *L. pneumophila*, were largely below detection in Flint single-family residences, at least during the later stages of the water crisis when they were measured (2015–2016) (*8*). Large buildings with extensive plumbing networks, such as hospitals, are generally more susceptible to *Legionella* growth than are simpler plumbing systems characteristic of single-family homes (*11*); however, residences

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are also of interest for *Legionella* growth, given concerns about the high rate of sporadic Legionnaires' disease (*12*) and potential for exposure in the home.

Our study objective was to use next-generation DNA sequencing to compare *L. pneumophila* isolated from Flint tap water after the second Legionnaires' disease outbreak with tap water isolates from neighboring drinking water systems outside of Flint that were never served by Flint River water and clinical strains received during the second outbreak at a regional reference laboratory in Genesee County. Within Flint, *Legionella* isolates were obtained from the tap water of a hospital, a large public building, and single-family residences several months after the water source was switched back to DWSD. In addition to serogroup testing, we used whole-genome sequencing to compare isolates in terms of sequence type (ST), average nucleotide identity, and single-nucleotide polymorphisms (SNPs).

Materials and Methods

Water Sample Collection and *Legionella* Isolation

After Flint resumed purchasing water with corrosion control from the original supplier, DWSD, water sampling campaigns were conducted 5 months (March 7–9, 2016), 8 months (June 21–27, 2016), and 10 months (August 15–16, 2016) later. Samples were collected from residences, small businesses, a large public building, and a hospital in Flint; as controls, samples were collected from buildings located outside of Flint that used DWSD or well water (Table 1). The March 2016 campaign targeted sampling of residences, small businesses, a large public building, and a hospital; samples were collected from hot (flushed for 30 seconds) and cold (stagnant) taps at each location. Samples were collected from the kitchen sink in homes and from restrooms in public buildings. The June 2016 campaign extensively sampled homes as part of a water heater cleaning campaign; the following samples were collected before and after a cleaning protocol: hot and cold stagnant kitchen tap samples, a stagnant shower sample of blended hot and cold water, a hot flushed kitchen tap sample, the water heater drain valve, and a flushed cold water sample from the outside hose bib or nearest tap to the service entry point. The August 2016 campaign targeted sampling from hot (flushed 30 seconds) and cold (stagnant) water taps from homes and small businesses. *Legionella* was cultured according to standard methods (*13*), and colonies were streaked to isolation.

Clinical Isolates

MDHHS provided 11 clinical isolates from de-identified Legionnaires' patients who received a diagnosis in 2015; however, 1 isolate could not be cultured and was deemed nonviable. When we initiated this study, we assumed that all 11 isolates originated from patients with some history of exposure in Flint or Genesee County during the Flint water crisis. However, we later learned that the commonality among clinical isolates was that they had been submitted to a Genesee County laboratory for analysis during the second outbreak and that 3 of the 11 isolates originated from patients who resided and received treatment outside of Genesee County (J. McFadden, MDHHS, pers. comm., 2017 Feb 1). Because the clinical isolates in this study were de-identified, comparison with the water isolates is described in terms of "*L. pneumophila* known to be capable of causing LD." We also included publicly available DNA sequence information from clinical reference strains in the analysis (Appendix 1 Table 2, https://wwwnc.cdc.gov/EID/article/25/11/18-1032-App1.pdf).

Whole-Genome Analysis of *L. pneumophila* Isolates

Whole-genome sequencing was conducted by MicrobesNG (https://microbesng.uk) on an Illumina MiSeq (https://www.illumina.com) with 2 × 250-bp paired-end reads and Nextera library preparation (Illumina). Sequencing was performed for a representative subset of each building type and water source, including 103 water isolates and the 10 available clinical *L. pneumophila* isolates (Appendix 1 Table 1). To verify DNA integrity, DNA extracts were quantified via a Qubit 2.0 fluorometer (https://www.thermofisher.com) and analyzed via gel electrophoresis. Positive (*L. pneumophila* strain 130b) and negative (*Stenotrophomonas maltophilia*) control strains were also sequenced, and 3 clinical strains were sequenced in duplicate on 2 MiSeq runs to evaluate run-to-run variation (Appendix 1 Figures 1–3). On average, 806,825 reads were obtained per isolate (range 280,380–2,031,828 reads). Reads were trimmed by using Trimmomatic (*14*), and de novo assemblies were generated by using SPAdes (*15*).

Genome sequences are available in GenBank under BioProject PRJNA453403. *Legionella* species assignments were determined via blastn (https://blast.ncbi.nlm.nih.gov) for isolate 16S rRNA gene sequences. Average nucleotide identity was calculated as previously described (*16*), and SNPs were identified by using kSNP3.0 (*17*). We also included 9 *L. pneumophila* reference strains (Appendix 1 Table 2). We performed sequence-based typing targeting the *flaA*, *pilE*, *asd*, *mip*, *mompS*, *proA*, and *neuA* alleles (*18*) by using the mompS tool (*19*).

Serogroup Analysis

We identified *L. pneumophila* isolates belonging to serogroup 1 via detection of the *wzm* gene (*20*) in whole genome sequences. We verified DNA sequence-based classifications and determined unknown serogroups by using direct fluorescent antibody staining with fluorescein isothiocyanate–conjugated antibodies (m-TECH, http://www.4m-tech.com).

Table 1. Total number of buildings sampled, number of samples collected, and number of isolates analyzed for *Legionella*, Flint, Michigan, USA*

Water sample source	March 2016			June 2016			August 2016		
	No. buildings or samples	No. (%) positive	No. isolates analyzed	No. buildings or samples	No. (%) positive	No. isolates analyzed	No. buildings or samples	No. (%) positive	No. isolates analyzed
Flint residences	**5**	**0**		**32**†	**2 (6)**		**10**‡	**2 (20)**	
Hot (flushed)	5	0	0	62	2 (3)	3	14	1 (7)	1
Hot (stagnant)	NS			62	2 (3)	4	NS		
Cold (flushed)	NS			61	1 (2)	2	NS		
Cold (stagnant)	5	0	0	61	1 (2)	4	11	4 (36)	1
Water heater drain valve	NS			62	1 (2)	5	NS		
Shower (hot and cold)	NS			62	1 (2)	2	3	1 (33)	1
Hospitals	**1**	**1 (100)**		**NS**			**NS**		
Hot (flushed)	19	16 (84)	56	NS			NS		
Cold (stagnant)	19	6 (32)	14	NS			NS		
Buildings receiving DWSD water	**4**	**0**		**NS**			**8**	**0**	
Hot (flushed)	4	0	0	NS			8	0	0
Cold (stagnant)	4	0	0	NS			8	0	0
Flint large buildings	**2**	**1 (50)**		**NS**			**NS**		
Hot (flushed)	5	0	0	NS			NS		
Cold (stagnant)	5	1 (20)	1	NS			NS		
Buildings receiving well water	**1**	**1 (100)**		**NS**			**NS**		
Hot (flushed)	4	4 (100)	5§	NS			NS		
Cold (stagnant)	3	2 (67)	4¶	NS			NS		
Flint small businesses	**6**	**0**		**NS**			**8**	**0**	
Hot (flushed)	6	0	0	NS			8	0	0
Cold (stagnant)	6	0	0	NS			8	0	0

*Positive samples indicate presumptive *L. pneumophila* identified by performing culture according to the method described in (*12*). Unless otherwise noted, identification as *L. pneumophila* was confirmed by using whole-genome sequencing. Boldface indicates total buildings sampled. Blank cells indicate that data were not reported when applicable samples were not collected. NS, no samples of this type were collected.
†1 of the 32 homes was also sampled in March 2016.
‡5 of the 10 homes were also sampled in March 2016; 1 of the 10 was sampled in June 2016 (but not in March 2016; samples from this house were positive on both dates).
§4 of 5 isolates were a non–*L. pneumophila* species, according to whole-genome sequencing.
¶4 of 4 isolates were a non–*L. pneumophila* species, according to whole-genome sequencing.

Results

Legionella Isolate Characterization

Of the 515 total water samples collected and from which *L. pneumophila* isolation was attempted (Table 1), 43 samples (8%) were positive for *Legionella*. Of these, 22 (58%) of 38 hospital samples from March 2016, eight (2%) of 370 residence samples from June 2016 (positives originating from 2 separate residences), and 6 (21%) of 28 residence samples from August 2016 (positives originating from different taps in a single residence) were positive for culturable *L. pneumophila*. No isolates were obtained from businesses receiving DWSD water, but 6 (86%) of 7 taps at the school serviced by well water were identified as positive (although 5 of these were later determined to be *Legionella* species other than *L. pneumophila*).

16S rRNA genes mined from whole-genome sequences indicated that all clinical and water isolates, except for 8 of the 9 well water isolates, were *L. pneumophila*. The positive control strain was correctly identified as *L. pneumophila*; SNP analysis further classified it according to its known provenance (130b), and the negative control strain was also confirmed to be *S. maltophilia* (i.e., not *Legionella*). Serogrouping via presence of the *wzm* gene for serogroup 1 and direct fluorescent antibody staining for other serogroups indicated that all *L. pneumophila* isolates belonged to serogroups 1 and 6 (Table 2).

L. pneumophila isolates from clinical and water samples belonged to several STs (Table 2). Of serogroup 1 isolates, all belonged to STs 1, 44, 159, 192, 211, 213, or 222 or to a previously uncharacterized ST that we submitted to the European Working Group for *Legionella* Infections database (http://www.ewgli.org) and that has now been designated as ST2513. Serogroup 6 isolates all belonged to a previously uncharacterized ST that has now been designated as ST2518. Most hospital isolates belonged to ST2518, and isolates originating from residential tap water belonged primarily to ST192. Only ST1 was represented by both clinical and water isolates, specifically, 3 clinical isolates, 3 isolates from hospital tap water, and 1 isolate from residential tap water.

When classified according to SNP similarity, isolates formed distinct clades that were generally consistent with the ST classification (Figure). The ST1 clade varied by 2–1,062

Table 2. Summary of *Legionalla pneumophila* isolates from Flint, Michigan, USA, 2016

ST	Serogroup	Isolate origin*
1	1	3 hospital water (HH17, HH25, HH56), 1 residence water (RH08), 3 clinical (C2, C3, C7)
44	1	1 clinical (C6)
159	1	1 clinical (C1)
192	1	19 residence water (RC01, RC02, RC03, RC04, RC06, RC07, RD01, RD02, RD03, RD04, RD05, RH02, RH03, RH04, RH05, RH07 RH07, RS01, RS02)
211	1	1 clinical (C8)
213	1	2 clinical (C4, C5)
222	1	1 clinical (C9)
2513†	1	1 clinical (C10)
2518†	6	66 hospital water (HC01, HC02, HC03, HC04, HC05, HC06, HC07, HC08, HC09, HC10, HC11, HC12, HC13, HC14, HH01, HH02, HH03, HH04, HH05, HH06, HH07, HH08, HH09, HH10, HH11, HH12, HH13, HH14, HH15, HH16, HH18, HH19, HH20, HH21, HH22, HH23, HH24, HH26, HH27, HH29, HH30, HH31, HH32, HH33, HH34, HH35, HH36, HH37, HH38, HH39, HH40, HH41, HH42, HH43, HH44, HH45, HH46, HH47, HH48, HH49, HH50, HH51, HH52, HH53, HH54, HH55), 1 public building (PC01), 1 well water (WH03)
ND	ND	HH28, RC05, RH01, RS03, WC01, WC02, WC03, WC04, WH01, WH02, WH04, WH05

*First letter of isolate name indicates building type/location: H, hospital; R, residence; W, school using well water; P, large public building. Second letter indicates sample collection location; H, hot water tap; C, cold water tap; D, water heater drain valve; S, shower. Numerals 1–10 indicate clinical strains. ND, not determined because of lack of *L. pneumophila*–specific alleles or insufficient genome coverage; ST, sequence type. †New sequence types from this study submitted to European Working Group for Legionella Infections database (http://www.ewgli.org).

SNPs, and isolates varied from the reference Paris strain by 371–505 SNPs. In particular, clinical isolate C3 shared the highest degree of similarity with Flint tap water isolates (38–46 SNPs). Some degree of variation is expected to be associated with variability in sequencing accuracy because the 3 clinical isolates that were sequenced in duplicate on multiple MiSeq lanes differed from their replicate by 0–10 SNPs. Several other distinct clades emerged in which water isolates were grouped primarily by building type. A large clade of ST2518 isolates included most samples from the hospital, 1 sample from well water, and 1 sample from a large public building. Another clade contained only isolates originating from Flint residence water samples belonging to ST192. The SNP results were confirmed by phylogenetic analysis and average nucleotide identity comparison (Appendix 1 Figures 1–3; Appendix 2, https://wwwnc.cdc.gov/EID/article/25/11/18-1032-App2.xlsx).

The STs of 8 isolates derived from well water could not be determined because *L. pneumophila*–specific alleles were absent, suggesting that the isolates were mistakenly phenotypically characterized as *L. pneumophila* on the basis of colony morphology. Average nucleotide identity values comparing these isolates with the positive control *L. pneumophila* strain (130b) were 62.645%–62.969%, whereas average nucleotide identity values of a single species are generally >95% (*21*). These 8 isolates seem to be most closely related to *L. taurinensis*, *L. rubrilucens*, or *L. erythra*, because the 16S rRNA genes extracted from these genomes shared >99% nt similarity to all 3 species.

Discussion

When considered per capita, the Legionnaires' outbreaks in Genesee County are among the largest in US history. However, to our knowledge, few clinical sputum isolates were collected or preserved from these outbreaks; for most cases, only urine-antigen testing was conducted. A common problem in the United States is reliance on urine-antigen testing and lack of collection of clinical *Legionella* isolates; these practices unfortunately limit the ability to track sources of infection, learn from past outbreaks, and prevent future outbreaks (*22*,*23*). Among the clinical sputum isolates that were sent to Genesee County laboratories during the outbreaks, none were from patients residing in homes serviced by Flint water (S. Lyon-Callo, MDHHS, pers. comm., 2018 Apr 5); thus, direct examination of potential residential exposure is not possible from this study. Given that 68% of patients' residences were confirmed to not have been serviced by Flint water (*3*), the potential exists that a portion of the remaining 32% had some residential exposure in Flint.

Another challenge of tracking sources of Legionnaires' disease is limited availability of water isolates. Given that the outbreaks were not publicly announced until 3 months after the conclusion of the second outbreak (January 2016), few environmental specimens were collected or preserved when the outbreaks were occurring. Analysis of any other water isolates that might exist from the time of the crisis would be valuable for learning more about this outbreak. MDHHS reported that 106 environmental *Legionella* specimens were retained at a Flint hospital but were not submitted to the State Health Department as had been requested (Sarah Lyon-Callo, MDHHS, pers. comm., 2019 Apr 30). Thus, a more definitive study of environmental sources of the outbreaks is not possible without a wider collection of clinical and environmental isolates.

Our study provides a survey of the landscape of genetic diversity among *Legionella* isolates collected from tap water from a range of building types served by the Flint drinking water distribution system over the 1-year period after the switch back to DWSD water. We compared these isolates with clinical isolates and with isolates from tap water of neighboring water systems never served by the Flint River or DWSD. Although it was not possible to collect water isolates during the actual outbreaks, previous studies have demonstrated that a single strain of

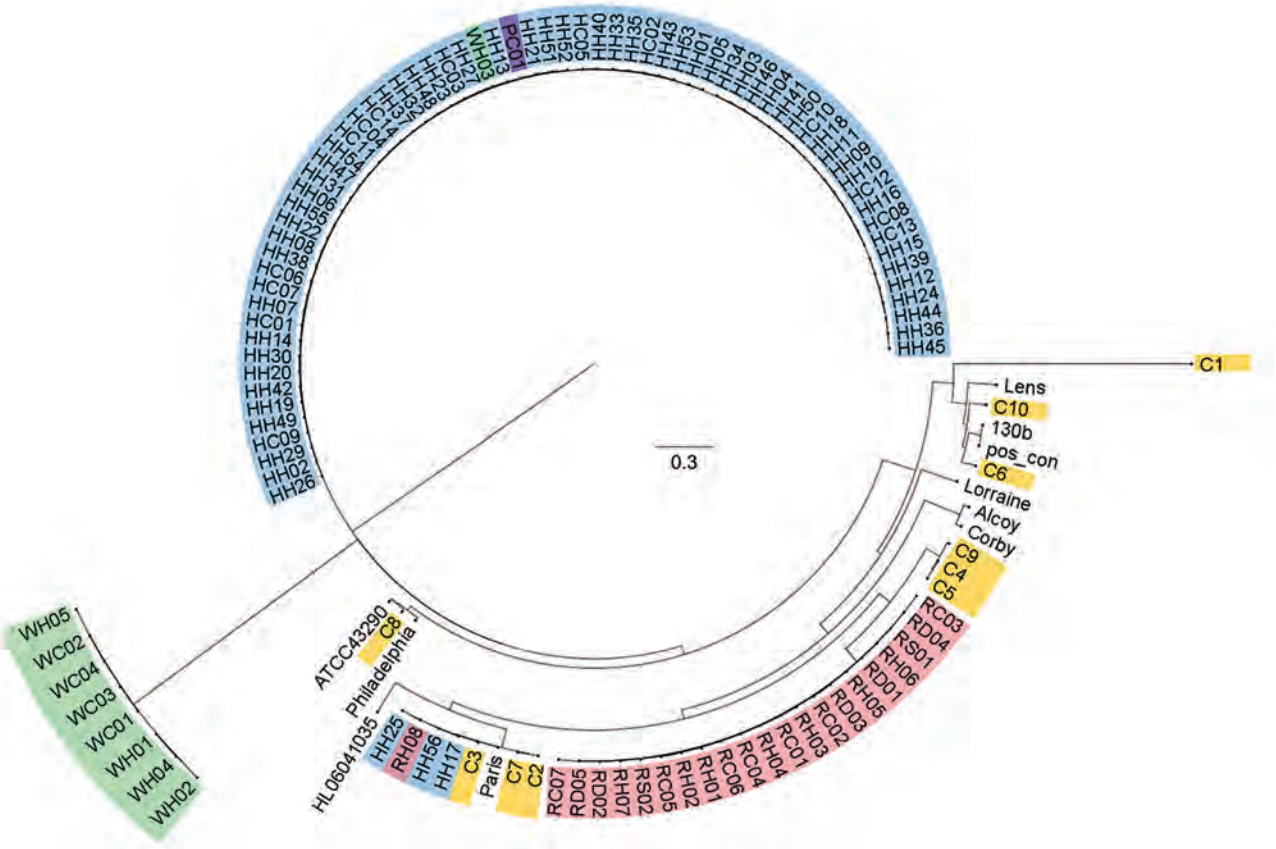

Figure. Single-nucleotide polymorphism (SNP) analysis of isolates from study of *Legionella pneumophila* in tap water, Flint, Michigan, USA. Analysis was conducted in kSNP3.0 (https://sourceforge.net/projects/ksnp/) and visualized by using FigTree 14.3 (https://github.com/rambaut/figtree/releases/tag/v1.4.3). Isolate sources: yellow, clinical samples; blue, hospital water; red, residence water; purple, public building water; green, buildings supplied by well water. With the exception of buildings supplied by well water, all buildings were serviced by Flint municipal water. Reference strains are detailed in Appendix 1 Table 2 (https://wwwnc.cdc.gov/EID/article/25/11/18-1032-App1.pdf). Scale bar indicates nucleotide substitutions per site.

L. pneumophila can colonize buildings and persist over multiple years (*24–26*). Thus, it is reasonable to assume that water isolates collected in 2016 were probably representative of strains colonizing building water systems over the previous months or even years.

Our study provides reasonable evidence that plumbing served by the Flint drinking water system was colonized by strains of *L. pneumophila* capable of causing Legionnaires' disease, particularly serogroup 1 and ST1. Although no epidemiologic links have been made between clinical cases and cooling tower exposures in these outbreaks, direct or indirect use of tap water (e.g., via feed to cooling towers) is possible. High degrees of similarity (2–1,062 SNPs) were noted between the ST1 isolates of clinical and water origin, a finding that was consistent with phylogenetic and average nucleotide identity analysis (Appendix 1 Figures 1–3; Appendix 2). The highest degree of similarity between clinical and water isolates was between C3 and RH08 (38 SNPs), HH25 (40 SNPs), HH17 (45 SNPs), and HH56 (46 SNPs). C2 differed from water isolates by 1,053–1,062 SNPs, and C7 differed from water isolates by 1,041–1,049 SNPs. With the exception of 1 isolate (WH03), isolates obtained from tap water from buildings never served by the Flint River were markedly distinct from those originating from residences or hospitals in Flint as well as the clinical isolates. The low number of SNPs between replicate genomes sequenced in this study (0–10) suggests that the extent to which technical variation in whole-genome sequencing contributed to observed sequence variation is low. Previous studies have documented that although some Legionnaires' disease outbreaks are characterized by *L. pneumophila* clinical strains that differ by as few as <5 SNPs, other outbreaks may differ by as many as 418 core SNPs (*27*). Thus, the SNP variability between water and clinical strains of ST1 in this study, particularly C3, is comparable to the documented range of variation in other outbreaks. In contrast, clinical strain C2 varied from the Paris reference strain by only 505, C3 by 371, and C7 by 491 SNPs. Therefore, ascertaining what level of SNP divergence between strains is demonstrative of a common source or virulent strain is

challenging. Given the well-established pathogenicity of the Paris strain, the results are also suggestive of genomic similarity among virulent strains of *Legionella*. Regardless, the similarity between C3 and strains isolated from Flint tap water samples (38–46 SNPs) is notable.

ST1 water isolates were collected from taps of a hospital and a residence, indicating that this ST seems to have been somewhat widespread in the water distribution system, spanning multiple Flint buildings. However, the presence of several distinct phylogenetic clades of *L. pneumophila* isolated from Flint water systems further demonstrates that a single strain of *L. pneumophila* did not dominate the system citywide. We hypothesize that this finding is likely the result of conditions favorable to *Legionella* growth, which we previously documented in the Flint system (*7*), facilitating the proliferation of multiple strains of *L. pneumophila* in different buildings and parts of the system. Similarly, the broad distribution of clinical isolates across 7 STs supports the hypothesis that any waterborne exposures that resulted in Legionnaires' disease could hypothetically have originated from a diverse array of *L. pneumophila* strains and exposure sources.

All clinical isolates characterized in this study belonged to *L. pneumophila* serogroup 1, which is identified as the cause of ≥57% of reported Legionnaires' cases in the United States (*6*). ST1 (belonging to serogroup 1) has been widely implicated in Legionnaires' outbreaks worldwide, including outbreaks in France (*28*), China (*29*), Germany (*30*), Canada (*31*), and the United States (*32*). In the United States, ST1 is thought to be both the most common cause of sporadic Legionnaires' disease cases and the most common waterborne ST found in potable and nonpotable water (*32*). ST1 isolates are highly conserved at the nucleotide level (*33*), making it challenging to link clinical cases with environmental sources because of the prevalence of ST1 and lack of genetic variability.

Water isolates belonging to serogroup 6, all classified as ST2518, were widespread in samples collected from a Flint hospital in March 2016. A study of *L. pneumophila* isolates collected from Flint tap water in September and October 2016 also found that serogroup 6 isolates were widespread in residential premise plumbing water samples, although these isolates all belonged to STs 367 and 461 (*34*). Byrne and colleagues found that serogroup 6 strains were at least as infectious for macrophages as a known virulent laboratory strain, emphasizing the potential for Legionnaires' disease to be caused by strains other than serogroup 1 (*34*), although more research is needed to confirm the relevance of serogroup 6 strains for human infectivity. In our study, none of the clinical strains available for analysis were serogroup 6.

It is noteworthy that 19% of hot water and 12% of cold water taps were positive for culturable *L. pneumophila*. Although *L. pneumophila* typically multiplies at 25°C–37°C (*35*) and prospers in hot water plumbing systems (*36*), it has also been widely documented in cold water taps; one molecular analysis–based study found that as many as 47% of surveyed cold water taps were positive for genes specific to *L. pneumophila* serogroup 1 (*37*).

When MDHHS recently conducted an epidemiologic characterization of the Genesee County Legionnaires' disease cases recorded in 2014 and 2015, although a lack of clinical isolates hampered a comprehensive investigation, they found that exposures that occurred at 1 Flint hospital potentially explained most cases (*1–3*). Our study provides complementary whole-genome sequencing–based characterization of clinical isolates and tap water *L. pneumophila* isolates collected after the Flint outbreaks. Notably, we found a high degree of similarity between 4 water isolates originating from Flint tap water and 3 regional clinical strains known to cause Legionnaires' disease. Our study also established that a variety of *L. pneumophila* strains were culturable from Flint tap water and that they tended to cluster genetically by residence versus hospital origin. Likewise, we found notable diversity among clinical strains, spanning 7 STs. Thus, multiple *L. pneumophila* strains were associated with the Flint 2014–2015 Legionnaires' outbreaks, potentially resulting from multiple sources of exposure, although further epidemiologic investigation is needed to identify whether multiple sources were involved and whether there were any common sources of exposure. Although we did not intend for this study to provide an epidemiologic analysis of precise sources of *Legionella* exposure for Legionnaires' patients, our publicly available data could support such studies in the future.

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About the Author

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References

1. Michigan Department of Health and Human Services. Legionellosis outbreak—Genesee County, June, 2014–March, 2015 full analysis. 2016 [cited 2018 Jun 22]. https://www.michigan.gov/documents/mdhhs/6-14_to_3-15_Legionellosis_Report_Full_Analysis_Results_511708_7.pdf
2. Michigan Department of Health and Human Services. Legionellosis outbreak—Genesee County, May 2015–October 2015 full analysis. 2016 [cited 2018 Jun 22]. https://gchd.us/wp-content/uploads/2016/08/Updated-LD-Report-4-11-16.pdf
3. Michigan Department of Health and Human Services. Epidemiology of Legionnaires' disease in Genesee County, Michigan, 2014–2017 chart book. 2018 [cited 2018 Jun 22]. https://content.govdelivery.com/attachments/MIDHHS/2018/05/29/file_attachments/1014713/GC%2BLegionella%2BChartBook%2BFINAL.pdf
4. Pieper KJ, Martin R, Tang M, Walters L, Parks J, Roy S, et al. Evaluating lead in water levels during the Flint Water Crisis and associated remediation interventions. Environ Sci Technol. 2017;52:8124–32. https://doi.org/10.1021/acs.est.8b00791
5. Dooling KL, Toews K, Hicks LA, Garrison LE, Bachaus B, Zansky S. Active Bacterial Core Surveillance for Legionellosis—United States, 2011–2013. MMWR Morb Mortal Wkly Rep. 2015;64:2011–3.
6. Marston BJ, Lipman HB, Breiman RF. Surveillance for Legionnaires' disease. Risk factors for morbidity and mortality. Arch Intern Med. 1994;154:2417–22. https://doi.org/10.1001/archinte.1994.00420210049006
7. Rhoads WJ, Garner E, Ji P, Zhu N, Parks J, Schwake DO, et al. Distribution system operational deficiencies coincide with reported Legionnaires' disease clusters in Flint, Michigan. Environ Sci Technol. 2017;51:11986–95. https://doi.org/10.1021/acs.est.7b01589
8. Schwake DO, Garner E, Strom OR, Pruden A, Edwards MA. *Legionella* DNA markers in tap water coincident with a spike in Legionnaires' disease in Flint, MI. Environ Sci Technol Lett. 2016;3:311–5. https://doi.org/10.1021/acs.estlett.6b00192
9. Zahran S, McElmurry SP, Kilgore PE, Mushinski D, Press J, Love NG, et al. Assessment of the Legionnaires' disease outbreak in Flint, Michigan. Proc Natl Acad Sci U S A. 2018;115:E1730–9. https://doi.org/10.1073/pnas.1718679115
10. Michigan Department of Health and Human Services. MDHHS response to Flint Area Community Health and Environment Partnership, Proceedings of the National Academy of Sciences Article. February 5, 2018 [cited 2018 Jun 22]. https://www.michigan.gov/documents/mdhhs/MDHHS_Response_to_FACHEP_Proceedings_of_the_National_Academy_of_Sciences_Article_FINAL_613088_7.pdf
11. Sabria M, Yu VL. Hospital-acquired legionellosis: solutions for a preventable infection. Lancet Infect Dis. 2002;2:368–73. https://doi.org/10.1016/S1473-3099(02)00291-8
12. Hicks LA, Garrison LE, Nelson GE, Hampton LM; Centers for Disease Control and Prevention (CDC). Legionellosis—United States, 2000–2009. MMWR Morb Mortal Wkly Rep. 2011;60:1083–6.
13. Centers for Disease Control and Prevention, Procedures for the recovery of *Legionella* from the environment [cited 2018 Jun 22]. https://www.epa.gov/sites/production/files/2018-11/documents/cdc_legionella_method.pdf
14. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20. https://doi.org/10.1093/bioinformatics/btu170
15. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77. https://doi.org/10.1089/cmb.2012.0021
16. Goris J, Konstantinidis KT, Klappenbach JA, Coenye T, Vandamme P, Tiedje JM. DNA–DNA hybridization values and their relationship to whole-genome sequence similarities. 2007; 57:81–91.
17. Gardner SN, Slezak T, Hall BG. kSNP3.0: SNP detection and phylogenetic analysis of genomes without genome alignment or reference genome. Bioinformatics. 2015;31:2877–8. https://doi.org/10.1093/bioinformatics/btv271
18. Gaia V, Fry NK, Afshar B, Lu PC, Etienne J, Peduzzi R, et al. Consensus sequence-based scheme for epidemiological typing of clinical and environmental isolates of *Legionella pneumophila*. J Clin Microbiol. 2005;43:2047–52.
19. Gordon M, Yakunin E, Valinsky L, Chalifa-Caspi V, Moran-Gilad J; ESCMID Study Group for Legionella Infections. A bioinformatics tool for ensuring the backwards compatibility of *Legionella pneumophila* typing in the genomic era. Clin Microbiol Infect. 2017;23:306–10. https://doi.org/10.1016/j.cmi.2017.01.002
20. Mérault N, Rusniok C, Jarraud S, Gomez-Valero L, Cazalet C, Marin M, et al.; DELPH-I Study Group. Specific real-time PCR for simultaneous detection and identification of *Legionella pneumophila* serogroup 1 in water and clinical samples. Appl Environ Microbiol. 2011;77:1708–17. https://doi.org/10.1128/AEM.02261-10
21. Rodriguez-R LM, Konstantinidis KT. Bypassing cultivation to identify bacterial species: culture-independent genomic approaches identify credibly distinct clusters, avoid cultivation bias, and provide true insights into microbial species [cited 2019 Sep 4]. https://www.asmscience.org/content/journal/microbe/10.1128/microbe.9.111.1
22. Berkelman RL, Pruden A. Prevention of Legionnaires' disease in the 21st century by advancing science and public health practice. Emerg Infect Dis. 2017;23:1905–7. https://doi.org/10.3201/eid2311.171429
23. Phin N, Parry-Ford F, Harrison T, Stagg HR, Zhang N, Kumar K, et al. Epidemiology and clinical management of Legionnaires' disease. Lancet Infect Dis. 2014;14:1011–21. https://doi.org/10.1016/S1473-3099(14)70713-3
24. Perola O, Kauppinen J, Kusnetsov J, Kärkkäinen UM, Lück PC, Katila ML. Persistent *Legionella pneumophila* colonization of a

hospital water supply: efficacy of control methods and a molecular epidemiological analysis. APMIS. 2005;113:45–53. https://doi.org/10.1111/j.1600-0463.2005.apm1130107.x
25. Rangel-Frausto MS, Rhomberg P, Hollis RJ, Pfaller MA, Wenzel RP, Helms CM, et al. Persistence of *Legionella pneumophila* in a hospital's water system: a 13-year survey. Infect Control Hosp Epidemiol. 1999;20:793–7. https://doi.org/10.1086/501586
26. Scaturro M, Fontana S, Dell'eva I, Helfer F, Marchio M, Stefanetti MV, et al. A multicenter study of viable PCR using propidium monoazide to detect *Legionella* in water samples. Diagn Microbiol Infect Dis. 2016;85:283–8. https://doi.org/10.1016/j.diagmicrobio.2016.04.009
27. Raphael BH, Baker DJ, Nazarian E, Lapierre P, Bopp D, Kozak-muiznieks NA, et al. Genomic resolution of outbreak-associated *Legionella pneumophila* serogroup 1 isolates from New York State. Appl Environ Microbiol. 2016;82:3582–90.
28. Ginevra C, Jacotin N, Diancourt L, Guigon G, Arquilliere R, Meugnier H, et al. *Legionella pneumophila* sequence type 1/Paris pulsotype subtyping by spoligotyping. J Clin Microbiol. 2012;50:696–701. https://doi.org/10.1128/JCM.06180-11
29. Qin T, Zhang W, Liu W, Zhou H, Ren H, Shao Z, et al. Population structure and minimum core genome typing of *Legionella pneumophila*. Sci Rep. 2016;6:21356. https://doi.org/10.1038/srep21356
30. Borchardt J, Helbig JH, Lück PC. Occurrence and distribution of sequence types among *Legionella pneumophila* strains isolated from patients in Germany: common features and differences to other regions of the world. Eur J Clin Microbiol Infect Dis. 2008;27:29–36. https://doi.org/10.1007/s10096-007-0392-3
31. Reimer AR, Au S, Schindle S, Bernard KA. *Legionella pneumophila* monoclonal antibody subgroups and DNA sequence types isolated in Canada between 1981 and 2009: Laboratory Component of National Surveillance. Eur J Clin Microbiol Infect Dis. 2010;29:191–205. https://doi.org/10.1007/s10096-009-0840-3
32. Kozak-Muiznieks NA, Morrison SS, Mercante JW, Ishaq MK, Johnson T, Caravas J, et al. Comparative genome analysis reveals a complex population structure of *Legionella pneumophila* subspecies. Infect Genet Evol. 2018;59:172–85. https://doi.org/10.1016/j.meegid.2018.02.008
33. Mercante JW, Caravas JA, Ishaq MK, Kozak-Muznieks NA, Raphael BH, Winchell JM. Genomic heterogeneity differentiates clinical and environmental subgroups of Legionella pneumophila sequence type 1. PLoS One. 2018;13:e0206110
34. Byrne BG, McColm S, McElmurry SP, Kilgore PE, Sobeck J, Sadler R, et al. Prevalence of infection-competent serogroup 6 *Legionella pneumophila* within premise plumbing in southeast Michigan. MBio. 2018;9:e00016–8. https://doi.org/10.1128/mBio.00016-18
35. Wadowsky RM, Wolford R, McNamara AM, Yee RB. Effect of temperature, pH, and oxygen level on the multiplication of naturally occurring *Legionella pneumophila* in potable water. Appl Environ Microbiol. 1985;49:1197–205.
36. Rhoads WJ, Ji P, Pruden A, Edwards MA. Water heater temperature set point and water use patterns influence *Legionella pneumophila* and associated microorganisms at the tap. Microbiome. 2015;3:1–13.
37. Donohue MJ, O'Connell K, Vesper SJ, Mistry JH, King D, Kostich M, et al. Widespread molecular detection of *Legionella pneumophila* serogroup 1 in cold water taps across the United States. Environ Sci Technol. 2014;48:3145–52. https://doi.org/10.1021/es4055115

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Clinical and Molecular Epidemiology of Invasive Group B *Streptococcus* Disease among Infants, China

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Invasive group B *Streptococcus* (GBS) remains a leading cause of illness and death among infants globally. We conducted prospective and retrospective laboratory-based surveillance of GBS-positive cultures from infants ≤3 months of age in 18 hospitals across China during January 1, 2015–December 31, 2017. The overall incidence of GBS was 0.31 (95% CI 0.27–0.36) cases/1,000 live births; incidence was 0–0.76 cases/1,000 live births across participating hospitals. The case-fatality rate was 2.3%. We estimated 13,604 cases of GBS and 1,142 GBS–associated deaths in infants ≤90 days of age annually in China. GBS isolates were most commonly serotype III (61.5%) and clonal complex 17 (40.6%). Enhanced active surveillance and implementation of preventive strategies, such as maternal GBS vaccination, warrants further investigation in China to help prevent these infections.

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One aim of the United Nations Children's Fund Sustainable Development Goals is to end preventable deaths among newborns and children <5 years of age by 2030 (*1*). Invasive group B *Streptococcus* (GBS), the gram-positive *Streptococcus agalactiae* bacterium, is a leading cause of illness and death among infants, including those in high-income countries. After a series of systematic reviews and a meta-analysis, a compartmental-model simulation estimated ≈319,000 GBS cases, including ≈90,000 deaths, worldwide in 2015 (*2*). Furthermore, conservative estimates for GBS-associated stillbirths were 57,000, and 33,000 invasive disease episodes occurred in pregnant and peripartum women (*2*). The overall global incidence of invasive GBS disease among infants ≤3 months of age in 2015 was estimated to be 0.49 (95% CI 0.43–0.56) cases/1,000 live births; the case-fatality rate was estimated at 8.4% (95% CI 6.6%–10.2%) (*3*). Two studies from China estimated the GBS incidence rate was 0.18–0.32 cases/1,000 live births (*3*). Nevertheless, China had the second highest absolute number of GBS cases among infants globally with 25,000 (uncertainty range 0–59,000) (*2*).

In 2015, China had 12.4% (17.8 million) of the 143 million global births. Systematic reviews noted the paucity of data on GBS from Asia, including China, as a major data gap (*2–6*), highlighting the need for prospective

[1]These authors contributed equally to this article.

population-based studies. A previous study by our group reported a GBS incidence rate of 0.55 (95% CI 0.44–0.69) cases/1,000 live births in 3 hospitals in the Guangdong Province of southern China during 2011–2014 (*7*). To improve on the generalizability of our earlier study, we undertook a multicenter population-based study at 18 sentinel hospitals across 16 provinces in China. The objectives of our study were defining the epidemiology of invasive group B streptococcal disease in infants ≤3 months of age in China and evaluating the molecular epidemiology of invasive disease strains by serotyping and multilocus sequence typing (MLST).

Methods

Study Design and Population

We conducted a prospective population- and laboratory-based surveillance study for GBS in 18 urban tertiary hospitals located in 16 provinces of China during May 5, 2016–December 31, 2017. We defined cases for this study as illness among infants ≤3 months of age with GBS isolated from a normally sterile site, including blood, cerebrospinal fluid, soft tissues, or peritoneal or pleural fluids. We classified GBS cases as early-onset disease (EOD) for cases occurring within 0–6 days of birth and late-onset disease (LOD) for cases occurring within 7–90 days of birth (*3*). We provided sentinel hospitals with a clinical protocol to identify GBS cases. Attending physicians assessed patients to make the clinical diagnosis of invasive group B streptococcal disease. According to our protocol, blood cultures were taken before antimicrobial drug therapy for infants with clinical symptoms or signs of suspected sepsis, including but not limited to fever, breathing problems, heart rate or blood pressure abnormalities, reduced movement, fussiness, cyanosis, seizures, or limpness or stiffness. Upon laboratory confirmation of GBS culture from ≥1 normally sterile site, the investigator contacted the parents or guardians for consent for inclusion in the study. We acquired clinical data from the hospital information system of each site.

We also conducted a retrospective study to identify GBS cases for January 1, 2015–May 4, 2016, by using laboratory-based passive surveillance. We searched electronic information systems in laboratories for reports of GBS isolated from a normally sterile specimen. We counted infants only once, regardless of the number of positive specimens. We collected GBS isolates stored in local sites and abstracted clinical data of cases from the hospital information system.

To obtain a representative sample of China, we conducted our study in sentinel hospitals from each region of China: northeast, north, west, east, central, and south. Assuming 550,000 live births from selected hospitals during the 3-year study period and an incidence rate of 0.25 cases/1,000 live births, we expected to see 137 GBS cases in sentinel hospitals and ≥130 GBS cases born outside of study hospitals but seeking care in a study hospital, for a total sample size ≥267.

Participating hospitals met the following inclusion criteria (*8*): large, urban, tertiary-care center; adequate research capabilities and facilities to conduct the study, including laboratory facilities and the ability to identify, process, and store GBS isolates; investigators willing to devote time to the study; and location, to ensure ≥1 hospital from each region. Trained site investigators in each participating hospital collected clinical data by using a standardized case report form. To estimate incidence rates by site, we obtained data on the number of live births from the information department of each participating hospital.

Ethics Approvals

The Medical Ethics Committee of Guangzhou Women and Children's Medical Center, Guangzhou, China, served as the central institutional review board for all facilities and approved this study (approval no. 2016050405). Each participating hospital had the option of using this approval or obtaining approval at their institution. For the prospective component of the study, we obtained written informed consent from parents or guardians of infants with invasive GBS disease. For the retrospective study, the review board waived the need for informed consent. We registered this study in the US National Library of Medicine clinical trials database (http://clinicaltrials.gov) on June 13, 2016, under registration no. NCT02812576.

Laboratory Methods

Each local hospital laboratory performed GBS isolation, cultivation, and identification by using the following protocol. Sterile samples were inoculated in French (bioMérieux, https://www.biomerieux.com) or BACTEC (Becton Dickinson, https://www.bd.com) culture bottles and analyzed with VITEK 2 COMPACT (bioMérieux) or BD Phoenix 100 (Becton Dickinson). GBS strains were grown at 37°C in 5%–10% CO_2 in trypticase soy agar supplemented with 5% sheep's blood for 18–24 h, according to the manufacturer's instructions. All GBS isolates were stored at –70°C and shipped on dry ice in standardized skim milk–tryptone-glucose-glycerol storage medium to the laboratory of Guangzhou Women and Children's Medical Center, which is certified by Joint Commission International (https://www.jointcommission.org), for further analysis.

Molecular Subtyping

We used multiplex PCR for Lancefield serotyping on all isolates (*9*), and tested 20% of randomly selected

isolates by Strep-B-Latex (Statens Serum Institute, https://en.ssi.dk) rapid latex agglutination test kit to cross-check the PCR results (*9*,*10*). PCR assays included probes for serotypes Ia, Ib, II, III, IV, V, VI, VII, VIII, and nontypeable. We performed MLST by amplifying and sequencing the internal fragments of 7 housekeeping genes, *adhP*, *pheS*, *atr*, *glnA*, *sdhA*, *glcK*, and *tkt*. We assigned an allele number to each fragment according to its sequence, then assigned each isolate a sequence type (ST) according to the allelic profile of the 7 amplicons. We compared alleles and STs of all GBS isolates with those in the *S. agalactiae* MLST database (http://pubmlst.org/sagalactiae). We assigned GBS isolates to the same group when they shared identical alleles at 6 of the 7 loci with ≥1 other member of the group. We also assigned isolates to different clonal complexes (CCs).

Data Collection and Statistical Analysis

We calculated the incidence rate by dividing the number of confirmed GBS cases in infants born in the participating hospitals by the number of live births in that hospital during the study period. To calculate the case-fatality rate, we divided the number of deaths among GBS cases by the total number of enrolled cases in each hospital.

Sensitivity Analysis

We used the estimated average incidence from this study to calculate the expected number of cases based on the national number of births in China in 2016. We also calculated the expected number of GBS cases at each site by using the highest incidence rate of each site to adjust for possible underestimates. We also performed sensitivity analysis by using the global case-fatality rate of 8.4% (*3*) to estimate the number deaths due to invasive group B streptococcal disease in infants ≤90 days of age in China.

We calculated 95% CI by using the Wilson interval method and analyzed categorical data by using χ^2 or Fisher exact test. We evaluated incidence trends by using Cochran-Armitage trend test and considered $p<0.05$ statistically significant. We performed all analyses by using SAS version 9.4 (SAS Institute, https://www.sas.com) and used BioNumerics 5.1 (Applied Maths, http://www.applied-maths.com) to create minimum spanning trees.

Results

For the full study period, January 1, 2015–December 31, 2017, we identified 304 cases of invasive group B streptococcal disease in infants from 18 hospitals, 146 (48.0%) EOD and 158 (52.0%) LOD; 123 (64 EOD and 59 LOD) were identified during the retrospective study and 181 (82 EOD and 99 LOD) during the prospective study. Of the 146 EOD cases, 82 (56.2%) were identified ≤24 hours after birth (Table 1; Appendix Table 1). The median length of hospitalization was 16 (interquartile range 12–26) days; infants with LOD spent more days in the hospital than did those with EOD (Table 1). Of 304 infants with GBS, 7 (2.3%) died, including 3 (3.4%) of 87 with meningitis (Appendix Table 3). Case-fatality rates were similar in the prospective (2.4%) and retrospective periods (2.2%). Attending physicians reported that 17 (5.6%) case-patients exhibited neuropathy at discharge.

Most (83%) case-patients, received an initial combination therapy of 2 antimicrobial drugs, including 54.2% who received empiric treatment with third-generation cephalosporins. *S. agalactiae* was 100% susceptible to cephalosporins, ampicillin, vancomycin, meropenem, and linezolid. We observed high prevalence of reduced susceptibility to tetracycline (80.1%), erythromycin (78.3%), and clindamycin (68.2%).

Invasive GBS Disease Incidence Rates

Of 304 GBS cases, 199 occurred in infants born at sentinel hospitals, 115 EOD and 84 LOD cases. Sentinel hospitals reported 634,531 live births during the study period, 388,005 during the prospective period and 246,526 during the retrospective period (Table 2; Figure 1). The calculated overall incidence of invasive GBS disease was 0.31 (95% CI 0.27–0.36) cases/1,000 live births, and incidence was similar between the prospective (0.31 cases/1,000 live births) and retrospective periods (0.32 cases/1,000 live births) (Appendix Table 2). The incidence of EOD was 0.18 (95% CI 0.15–0.22) cases/1,000 live births and LOD was 0.13 (95% CI 0.11–0.16) cases/1,000 live births. We did not see seasonal variation in incidence rates (Figure 2).

Guangdong Women and Children's Hospital in the south reported the highest incidence rate, 0.76 (95% CI 0.54–1.08) cases/1,000 live births, along with Hubei Maternal and Child Health Hospital in the central region (0.56 [95% CI 0.41–0.77] cases/1,000 live births), and Guangzhou Women and Children's Medical Center in the south (0.54 [95% CI 0.39–0.75] cases/1,000 live births) (Figure 3, panel A). Three participating hospitals reported no invasive GBS cases (Table 2). The incidence rate by region was highest in the central region (0.53 [95% CI 0.41–0.68] cases/1,000 live births), the south (0.48 [95% CI 0.38–0.60] cases/1,000 live births), and the northeast (0.39 [95% CI 0.24–0.63] cases/1,000 live births) and was lowest in the west (0.01 [95% CI 0–0.07] cases/1,000 live births) (Figure 3, panel B).

Our sensitivity analysis assumed an incidence rate of 0.76 cases/1,000 live births, the highest incidence rate reported by Guangdong Women and Children's Hospital in our study (Table 2). Under this assumption, we estimated the annual number of cases of GBS in infants ≤3 months of age in China would be 13,604 among the country's birth cohort of 17.9 million. Using the global case-fatality rate of 8.4%, we estimate China would have 1,142 GBS-associated deaths annually (*3*).

Table 1. Characteristics of infants ≤3 months of age with invasive GBS disease, by disease onset, China, January 1, 2015–December 31, 2017*

Characteristics	Early-onset disease, n = 146	Late-onset disease, n = 158	Total, n = 304
Median age at diagnosis, mo (IQR)	0 (0–1)	21(15–46)	8 (0–22)
Sex			
M	74 (50.7)	64 (40.5)	138 (45.4)
F	72 (49.3)	94 (59.5)	166 (54.6)
Birth weight, g			
Median (IQR)	3,175 (2,700–3,500)	3,185 (2,800–3,500)	3,185 (2,725–3,500)
1,500–2,500	26 (17.8)	21(13.3)	47 15.5)
<1,500	5 (3.4)	8 (5.1)	13 (4.3)
Site of delivery			
Sentinel hospital	115 (78.8)	84 (53.2)	199 (65.5)
Other hospital	31 (21.2)	74 (46.8)	105 (34.5)
Median gestational age, wk (IQR)	39 (36–40)	39 (37–40)	39 (37–40)
<34	17 (11.6)	13 (8.2)	30 (9.9)
34–37	22 (15.1)	19 (12.0)	41 (13.5)
≥37	107 (73.3)	126 (79.8)	233 (76.6)
Delivery type			
Vaginal	97 (66.4)	94 (59.5)	191 (62.8)
C-section	40 (27.4)	62 (39.2)	102 (33.6)
Forceps	7 (4.8)	1 (0.6)	8 (2.6)
Unknown	2 (1.4)	1 (0.6)	3 (1.0)
Specimen type			
Blood	107 (73.3)	78 (49.4)	185 (60.9)
Cerebrospinal fluid	5 (3.4)	19 (12.0)	24 (7.9)
Blood and cerebrospinal fluid	34 (23.3)	61 (38.6)	95 (31.2)
Bacterial infections†			
Sepsis	122 (83.6)	121 (76.6)	243 (79.9)
Pneumonia‡	76 (52.1)	50 (31.7)	126 (41.4)
Meningitis	18 (12.3)	69 (43.7)	87 (28.6)
Sepsis and pneumonia	62 (42.5)	35 (22.2)	97 (31.9)
Sepsis and meningitis	7 (4.8)	43 (27.2)	50 (16.4)
Pneumonia and meningitis	2 (1.4)	2 (1.3)	4 (1.3)
Clinical symptoms†			
Fever	38 (26.0)	121 (76.6)	159 (52.3)
Breathing problems	83 (56.9)	38 (24.1)	121 (39.8)
Cyanosis	32 (21.9)	10 (6.3)	42 (13.8)
Seizures	4 (2.7)	20 (12.7)	24 (7.9)
Limpness or stiffness	5 (3.4)	10 (6.3)	15 (4.9)
Poor feeding	13 (8.9)	27 (17.1)	40 (13.2)
Irritability	4 (2.7)	15 (9.5)	19 (6.3)
Median length of hospitalization, d (IQR)	15 (12–19)	18 (13–32)	16 (12–26)
Discharge outcome			
Recovered	105 (71.9)	100 (63.3)	205 (67.4)
Transferred to other hospitals	4 (2.7)	13 (8.2)	17 (5.6)
Died	4 (2.7)	3 (1.9)	7 (2.3)
Abnormal neurology at discharge	5 (3.4)	12 (7.6)	17 (5.6)
Condition improved	12 (8.2)	16 (10.1)	28 (9.2)
Discharge requested	16 (11.0)	14 (8.9)	30 (9.9)

*Values are no. (%) except as indicated. GBS, group B *Streptococcus*; IQR, interquartile range.
†Cases could have >1.
‡As assessed by attending physician by using criteria from Zhu Futang Practice of Pediatrics (*11*).

Serotype Distribution

We typed 244 available isolates and identified 6 serotypes, Ia, Ib, III, IV, V, and VI. Serotype III (61.5%) was most pervasive, along with Ib (28.7%), and we saw fewer instances of Ia (5.7%) and V (2.9%), and 1 case (0.4%) each of serotypes IV and VI, as well as 1 nontypeable isolate (Figure 4, panel A). The relative distribution of serotypes differed between EOD and LOD (p = 0.004) (Figure 4, panel A); however, serotypes III and Ib were still most pervasive. Overall, the serotype distribution was similar across regions in China (Figure 4, panel B). The proportion of cases caused by serotype Ib was higher in the north and northeast than other regions (p = 0.002). Approximately two thirds of meningitis cases and 61.9% of sepsis cases were caused by serotype III, compared with 52.0% of pneumonia cases (p = 0.113). In contrast, 40% of diagnosed pneumonia was caused by serotype Ib compared with 30% of either sepsis or meningitis cases (p = 0.112).

MLST Analysis

We performed MLST on all 244 serotyped isolates and identified 27 different sequence types (STs); 83.2% of

Table 2. Live births and incidence of invasive GBS disease among infants ≤3 months of age, by region and sentinel hospital, China, January 1, 2015–December 31, 2017*

Region	Births	Case-patients born in sentinel hospitals	Expected cases/ region†	Hospital‡	Live births	GBS cases			GBS case-patients born in study hospitals			Expected cases/ hospital†
						Total	EOD	LOD	Total	EOD	LOD	
NE	41,088	16 (0.39)	31	SY§	32,666	34	16	18	16 (0.49)	11 (0.34)	5 (0.15)	25
				HY§	8,422	0	0	0	0	0	0	6
North	115,310	21 (0.18)	88	BJ	43,099	3	3	0	3 (0.07)	3 (0.07)	0	33
				TJ	42,320	7	5	2	6 (0.14)	5 (0.12)	1 (0.02)	32
				TS	29,891	23	7	16	12 (0.40)	4 (0.13)	8 (0.27)	23
East	127,474	28 (0.22)	97	NJ	70,153	16	4	12	14 (0.20)	4 (0.06)	10 (0.14)	53
				ZJ	57,321	14	12	2	14 (0.24)	12 (0.21)	2 (0.03)	44
Central	108,175	57 (0.53)	82	CS	40,521	19	15	4	19 (0.47)	15 (0.37)	4 (0.10)	31
				HB	67,654	38	17	21	38 (0.56)	17 (0.25)	21 (0.31)	51
South	157,949	76 (0.48)	120	GZ	66,477	74	18	56	36 (0.54)	12 (0.18)	24 (0.36)	51
				GD	40,628	36	24	12	31 (0.76)	23 (0.57)	8 (0.20)	31
				GX	50,844	13	10	3	9 (0.18)	8 (0.16)	1 (0.02)	39
West	84,535	1 (0.01)	64	NX§	8,795	2	1	1	0	0	0	7
				XJ	26,545	1	1	0	1 (0.04)	1 (0.04)	0	20
				XJ1§	8,777	2	0	2	0	0	0	7
				XJ2§	7,374	0	0	0	0	0	0	6
				CQ	33,044	0	0	0	0	0	0	25

*Values are no. (no./1,000 live births) except as indicated. BJ, Tsinghua University Hospital; CQ, Chongqing Health Center for Women and Children; CS, Changsha Hospital for Maternal and Child Health; EOD, early-onset disease; GBS, group B *Streptococcus*; GD, Guangdong Women and Children's Hospital; GX, The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region; GZ, Guangzhou Women and Children's Medical Center; HB, Hubei Maternal and Child Health Hospital; HY, The First Affiliated Hospital, Harbin Medical University; LOD, late-onset disease; NE, northeast; NJ, Nanjing Maternity and Child Health Care Hospital; NX, General Hospital of Ningxia Medical University; SH, Children's Hospital of Fudan University; SY, Shengjing Hospital, China Medical University; TJ, Tianjin Central Hospital of Gynecology Obstetrics; TS, Tangshan Maternal and Child Health Care Hospital; XJ, Maternal and Child Health Care Hospital of Uygur Autonomous Region; XJ1, The First Affiliated Hospital of Xi'an Jiaotong University; XJ2, The Second Affiliated Hospital of Xi'an Jiaotong University; ZJ, Women's Hospital, Zhejiang University.
†Estimated using highest incidence of 0.76/1,000 live births reported from this study.
‡Maternal and child hospitals, except where indicated. One hospital, Children's Hospital of Fudan University, participated in the study but is omitted from this table because it does not have an obstetrics department, it had no infants with GBS born in it, and it was not included in calculation of incidence rates.
§General hospital.

which were ST17, ST19, ST10, ST12, and ST23. ST17 was most prevalent (89/244, 36.5%), along with ST19 (34/244, 13.9%), ST10 (33/244, 13.5%), and ST12 (32/244, 13.1%). Of ST17 isolates, 98% were serotype III, as were 88.2% of ST19 isolates; 2.2% of ST17 and 2.9% of ST19 were serotype Ib; and 8.8% of ST19 isolates were serotype V. Most ST10 (93.4%) and ST12 (93.8%) isolates were serotype Ib and 66.7% of ST23 isolates were serotype Ia.

Twenty-seven STs clustered into 10 CCs (Figure 5). The most prevalent was CC17 (99/244 isolates, 40.6%), along with CC19 (47/244, 19.3%), CC12 (35/244, 14.3%), CC10 (32/244, 13.1%), and CC23 (18/244, 7.4%). Most CC17 and CC19 isolates were serotype III (137/150, 91.3%), and 90.0% of serotype Ib isolates were CC12 and CC10. CC17 and CC19 were dominant in the south, whereas CC10 and CC12 were common in the north and northeast (Figure 6). The proportion of CC17 isolates was higher in LOD (73/138, 52.9%) than in EOD (26/106, 24.5%) cases ($p<0.0001$). Strains belonging to CC19 and CC12 were less common in LOD than in EOD cases: CC19 in 14.5% (20/138) of LOD cases and 25.5% (27/106) of EOD cases ($p = 0.031$), and CC12 in 10.1% (14/138) of LOD and 19.8% (21/106) of EOD cases ($p = 0.033$).

Discussion

GBS is associated with severe neonatal infection in some hospitals in China (*7*,*12*–*16*), but a paucity of generalizable data is available across the country. We conducted a large, multicenter study to evaluate the clinical and molecular epidemiology of invasive group B streptococcal disease among infants ≤3 months of age in China. We reported an overall GBS incidence rate of 0.31 (95% CI 0.27–0.36) cases/1,000 live births, which is lower than the estimated worldwide incidence rate of 0.49 (95% CI 0.43–0.56) cases/1,000 live births, but similar to estimates from elsewhere in Asia (0.30 [95% CI 0.43–0.56] cases/1,000 live births) (*3*). We estimated the incidence rates of EOD to be 0.18 cases/1,000 live births and LOD to be 0.13 cases/1,000 live births, which also are lower than estimated from a previous meta-analysis. That report estimated the global EOD incidence rate as 0.41 (95% CI 0.36–0.47) cases/1,000 live births and the LOD incidence rate as 0.26 (95% CI 0.21–0.30) cases/1,000 live births. The number of cases and birth cohort was one third more during the prospective than retrospective period, possibly because China implemented a 2-child policy in October 2015 (*17*). The incidence of invasive GBS was similar between the prospective and retrospective periods.

Despite implementing standard study and laboratory protocols at each participating hospital, we found

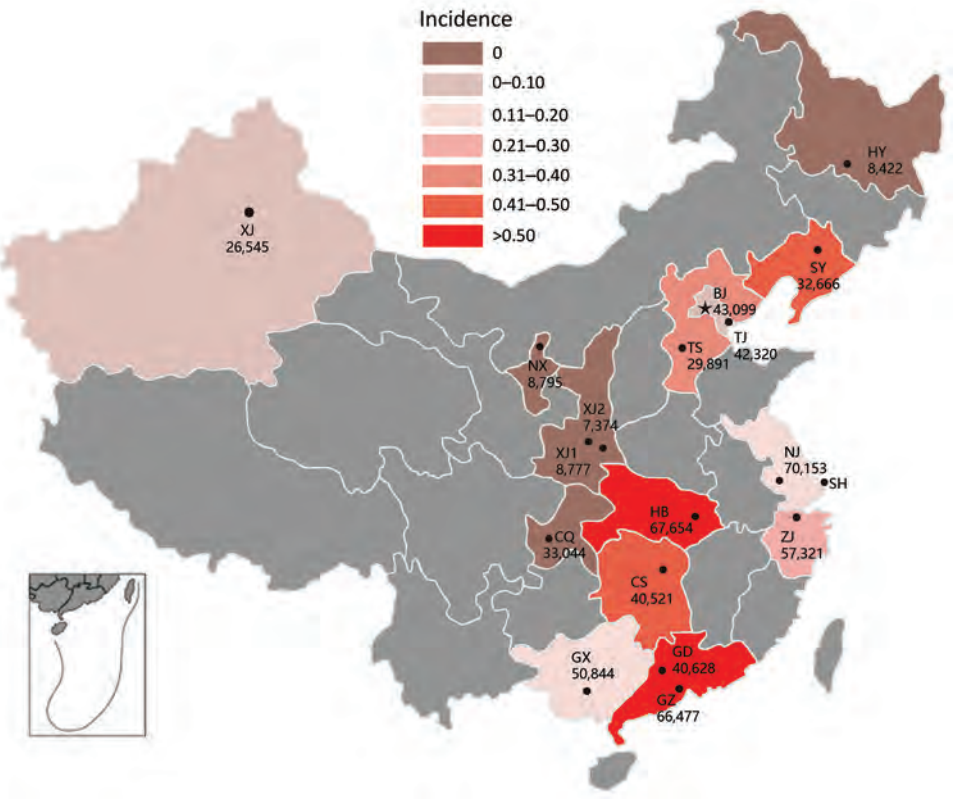

Figure 1. Incidence rate (cases/1,000 live births) of invasive group B streptococcal disease among infants ≤3 months of age by province, China. Number of live births per participating hospital is provided. Gray shaded areas did not participate in this study. Inset shows South China Sea Islands. BJ, Tsinghua University Hospital; CQ, Chongqing Health Center for Women and Children; CS, Changsha Hospital for Maternal and Child Health; GD, Guangdong Women and Children's Hospital; GX, The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region; GZ, Guangzhou Women and Children's Medical Center; HB, Hubei Maternal and Child Health Hospital; HY, The First Affiliated Hospital, Harbin Medical University; NJ, Nanjing Maternity and Child Health Care Hospital; NX, General Hospital of Ningxia Medical University; SH, Children's Hospital of Fudan University; SY, Shengjing Hospital, China Medical University; TJ, Tianjin Central Hospital of Gynecology Obstetrics; TS, Tangshan Maternal and Child Health Care Hospital; XJ, Maternal and Child Health Care Hospital of Uygur Autonomous Region; XJ1, The First Affiliated Hospital of Xi'an Jiaotong University; XJ2, The Second Affiliated Hospital of Xi'an Jiaotong University; ZJ, Women's Hospital, Zhejiang University.

substantial variability in the incidence rates between hospitals and regions in China. GBS incidence rates varied from 0 to 0.76 cases/1,000 live births at participating hospitals and from 0.01 to 0.53 cases/1,000 live births across regions. Similar geographic heterogeneity in incidence has been reported from South Africa, ranging from 0.03 to 2.72 cases/1,000 live births across 9 provinces (*18*). The authors of that study attributed the differences in incidence rates to biases in case detection, with varying thresholds for investigating and laboratory capacity for detecting GBS across the provinces, rather than actual differences (*18*).

Elucidating the reasons for the regional differences in incidence of GBS in China could inform strategies

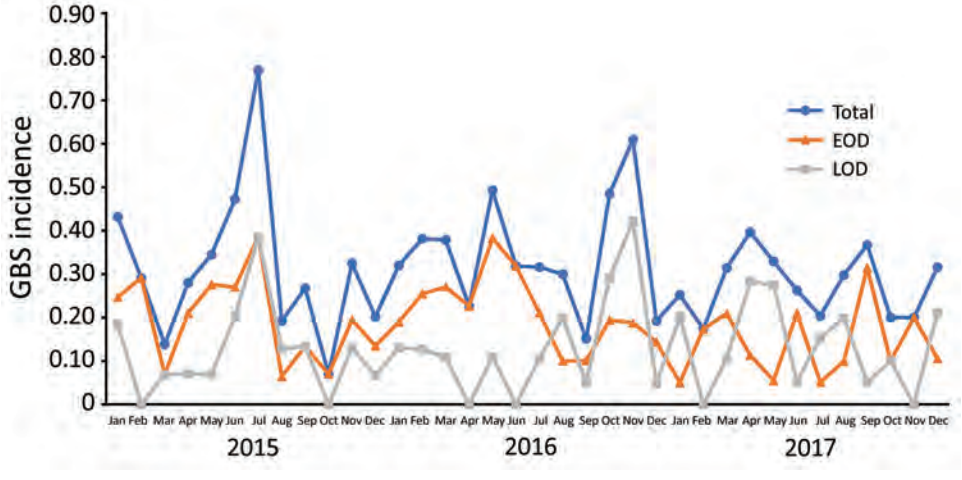

Figure 2. Incidence rate of invasive GBS disease (no. cases/1,000 live births) among infants ≤3 months of age in participating regions of China, 2015–2017. EOD, early-onset disease; GBS, invasive group B *Streptococcus*; LOD, late-onset disease.

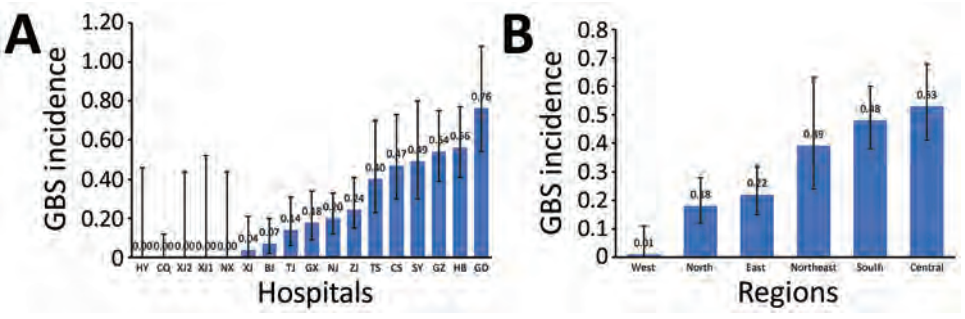

Figure 3. Incidence rate of invasive GBS disease (no. cases/1,000 live births) among infants ≤3 months of age, China, 2015–2017. A) Incidence by study hospital. B) Incidence by region. Error bars indicate 95% CIs. BJ, Tsinghua University Hospital; CQ, Chongqing Health Center for Women and Children; CS, Changsha Hospital for Maternal and Child Health; EOD, early-onset disease; GBS, invasive group B *Streptococcus*; GD, Guangdong Women and Children's Hospital; GX, The Maternal and Child Health Hospital of Guangxi Zhuang Autonomous Region; GZ, Guangzhou Women and Children's Medical Center; HB, Hubei Maternal and Child Health Hospital; HY, The First Affiliated Hospital, Harbin Medical University; LOD, late-onset disease; NJ, Nanjing Maternity and Child Health Care Hospital; NX, General Hospital of Ningxia Medical University; SH, Children's Hospital of Fudan University; SY, Shengjing Hospital, China Medical University; TJ, Tianjin Central Hospital of Gynecology Obstetrics; TS, Tangshan Maternal and Child Health Care Hospital; XJ, Maternal and Child Health Care Hospital of Uygur Autonomous Region; XJ1, The First Affiliated Hospital of Xi'an Jiaotong University; XJ2, The Second Affiliated Hospital of Xi'an Jiaotong University; ZJ, Women's Hospital, Zhejiang University

hospitals might use to better identify such cases and improve care for patients with invasive GBS disease. We have several hypotheses for the differences in incidence rates we observed. Prior GBS research experience by staff in hospitals located in south and central China compared with those in other regions could have raised attending clinicians' awareness of the need to investigate for GBS and given laboratory personnel more experience in microbiological identification of GBS. In addition, clinicians had discretion to conduct passive surveillance investigations, which could vary between personnel and study sites. Our study included only 5 general hospitals and 3 of these were located in the west, a region where we found a low GBS incidence rate. Typically, general hospitals have lower birth rates (Table 2), and some do not treat infants. For instance, the Maternal and Child Health Care Hospital of Uygur Autonomous Region has a policy to transfer all sick infants >28 days of age to the local children's hospital for treatment. In addition, parents usually prefer to seek medical care for their infants at a children's hospital rather than a general hospital. Therefore, our study might have missed some GBS cases born in general hospitals, resulting in lower incidence rates in these hospitals and the western region. Other explanations for regional differences include differences in maternal GBS colonization rates, strain virulence, challenges in accessing health services, and inadequate sterile sample collection capacity or improper methods. We will conduct further investigations on hospitals reporting no GBS cases without obvious reasons.

Our study identified a lower fatality rate (2.3%) than observed globally in a meta-analysis (8.4%; 95% CI 6.6%–10.2%), which could reflect early detection of suspected GBS and timely management with antimicrobial drugs. Also, we found that only 5.6% of surviving infants across all participating hospitals had neurologic sequelae at discharge. However, this value could be an underestimate because we did not use a standard assessment across sites and sequelae might not be obvious in infants. Further investigation in longer term follow up studies in China are warranted to quantify the residual burden of neurologic sequelae in infants with invasive GBS disease.

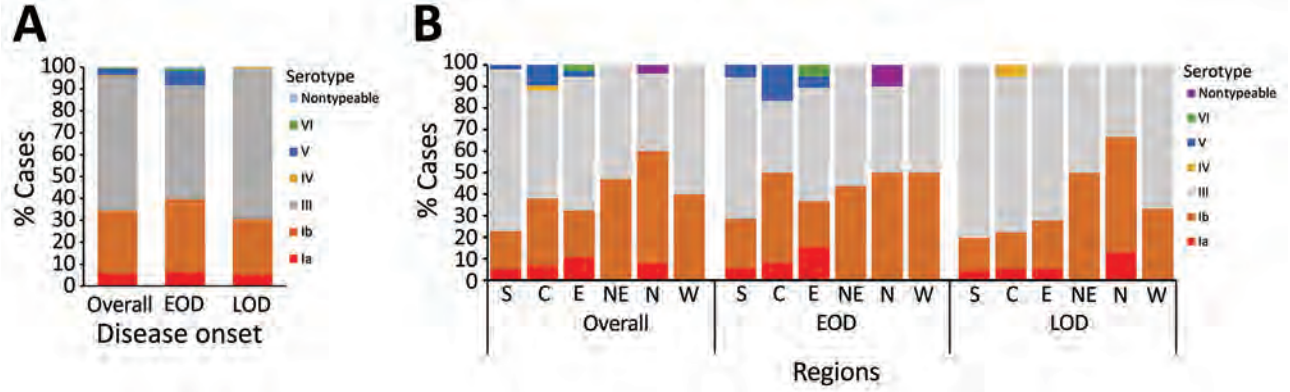

Figure 4. Distribution of invasive group B *Streptococcus* serotypes by disease onset (A) and region (B), China, 2015–2017. C, central; E, east; EOD, early-onset disease; LOD, late-onset disease; N, north; NE, northeast; S, south; W, west.

Given that maternal GBS colonization is the primary risk factor for EOD, the US Centers for Disease Control and Prevention issued guidelines recommending intravenous intrapartum antibiotic prophylaxis (IAP) during labor to prevent EOD in infants born to women colonized with GBS or with risk factors (*19*). The IAP policy, along with universal screening, was shown to decrease EOD incidence but did not decrease LOD incidence (*19*). However, 37% of low- to middle-income countries, including China, have not implemented the IAP program on a systematic basis, and China currently does not have specific recommendations or guidelines for preventing GBS. During 2015–2017, eighteen hospitals in this study reported only 13.2% of GBS case-mothers received GBS screening before delivery. Currently, not every hospital has a GBS screening program. Some hospitals offer optional GBS screening, but patients must cover the cost, and screening is not covered by any medical insurance. We also have seen previously that surveyed pregnant women thought GBS incidence was low and most chose not to have GBS screening (W. Ji, H. Liu, and Y. Fang, unpub. data).

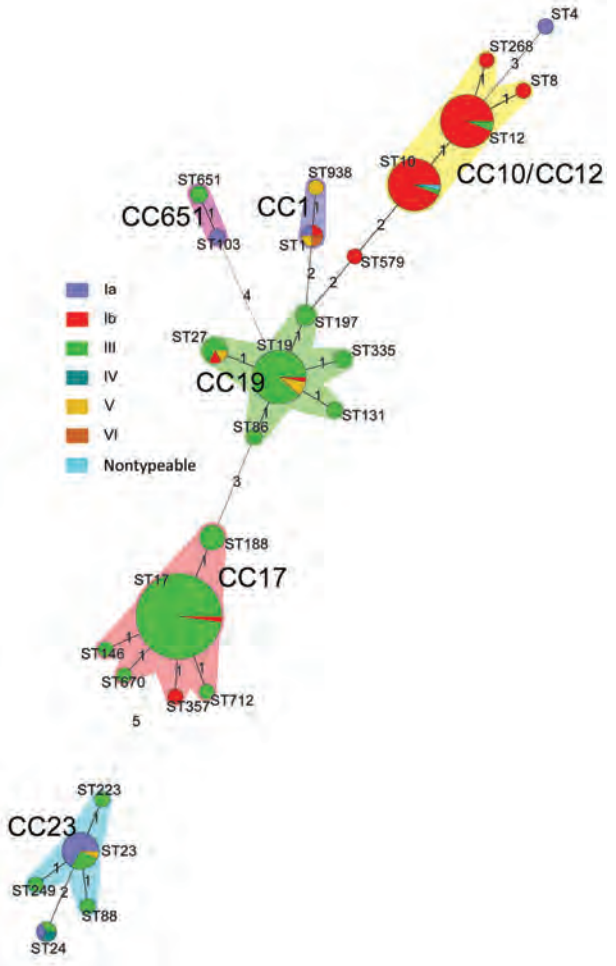

Figure 5. Minimum spanning tree of invasive group B *Streptococcus* (GBS) isolates by serotype showing the relationship between ST and clonal complex CC by serotype. Circles represent STs; size of each circle indicates the number of isolates within the specific type. The ST with the greatest number of single-locus variants is the founder ST. GBS isolate serotypes appear as different colors; shading denotes STs belonging to the same CC. CC, clonal complex; ST, sequence type.

Vaccination of pregnant women against GBS is a promising strategy to prevent invasive GBS disease in their infants (*20*). The World Health Organization proposed priority research and development pathways for GBS vaccines to facilitate and accelerate vaccine licensure (*21*). Our results indicated that serotype III was the most common type (61.2%). Other studies also have reported the comparable proportion of serotype III in China (*7*,*22*–*24*). In general, GBS serotype distributions were similar across regions of China, and were similar to findings for other countries, with serotype III dominating (61.5%) and serotypes Ia, Ib, II, III, and V causing 97% of invasive GBS disease (*3*). However, serotype Ib accounted for a higher proportion of disease in the northeast and north in China compared with other regions. The 244 isolates belonged to 27 STs and were clustered into 5 main CCs. ST17/CC17 and ST19/CC19 were found almost exclusively in serotype III, which is comparable with findings from other studies (*12*,*25*–*27*). The ST17 clone of serotype III is known to be a hypervirulent strain and a leading cause of meningitis in neonates (*28*,*29*). A pentavalent GBS vaccine including serotypes Ia, Ib, II, III, and V (*30*) or a hexavalent vaccine including serotypes Ia, Ib, II, III, IV, and V would cover all invasive GBS disease–causing serotypes in China.

Our study has several limitations. First, all participating hospitals were large urban tertiary hospitals. We did not include secondary hospitals and primary care institutions mainly because they lacked adequate laboratory facilities for GBS culture and identification of invasive GBS disease, which might have led to delayed confirmation of EOD cases. We found 56.2% of EOD cases were identified ≤24 hours after birth. Findings might differ in primary and secondary settings because of differences in populations and clinical practice, so these settings should be included in future studies. Second, the retrospective collection of data during January 2015–May 2016 did not include all isolates and only 46.3% were available for strain analysis. Last, rates of GBS could be underestimated because our study did not account for GBS in infants treated at nonstudy hospitals after discharge from study hospitals.

In summary, our large generalizable study on invasive GBS disease in infants in China informs the current clinical and molecular epidemiology of GBS. Despite the low rates of fatality and detectable neurologic sequelae at discharge, clinicians, laboratory technicians, and epidemiologists should be aware that GBS is prevalent, especially in the south and central regions of China. Future work and continuous surveillance are warranted to better understand

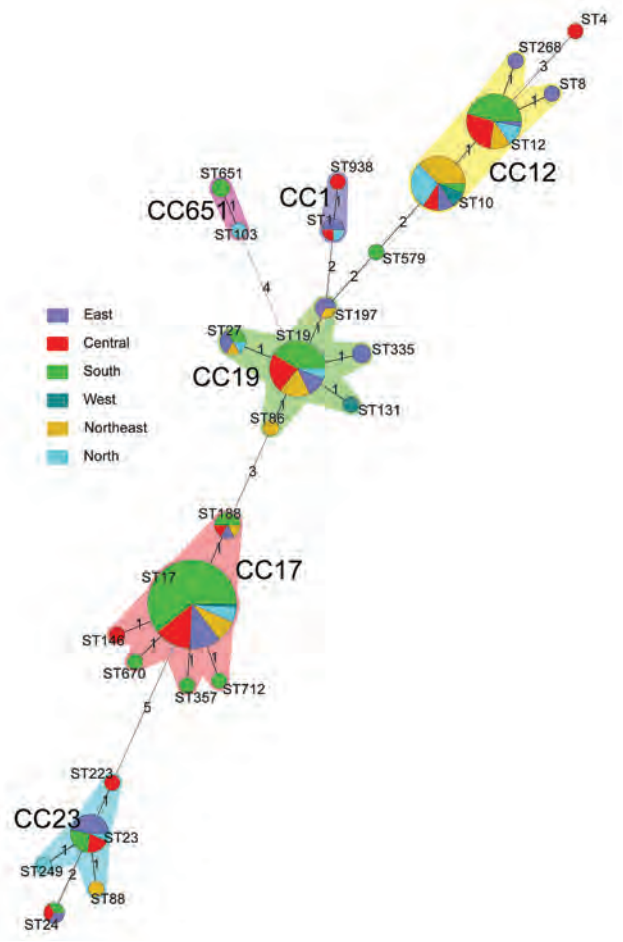

Figure 6. Minimum spanning tree analysis of invasive group B *Streptococcus* isolates showing the relationship between ST and CCs by region in China. Circles represent STs; size of each circle indicates the number of isolates within the specific type. The ST with the greatest number of single-locus variants is the founder ST. Regions appear as different colors; shading denotes STs belonging to the same CC. CC, clonal complex; ST, sequence type.

the heterogeneity of incidence across regions, identify risk factors, and conduct long-term follow-up to further assess the prevalence of invasive GBS disease on neurologic development in surviving infants.

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Conflicts of interest: M.C. is a permanent employee of and has shares in GlaxoSmithKline Plc. All other authors have no conflicts of interest to declare in relation to this study.

Authors' contributions: W.J., Y.F., and H.L. conceptualized the study and contacted study sites. W.J. finished data management and analysis and drafted the manuscript. Y.F., H.L., S.A.M., M.C., Z.Z., Z.D., and H.Z. provided valuable comments for revision. H.Z. completed minimum spanning tree analysis. H.L., Z.J., A.W., X.M., X.Q., C.G., Y.Z., X.F., J.L., S.S., L.J., J.L., S.Y., Z.L., G.L., and Q.L. contributed to the study oversight, management, coordination, implementation, and data collection. Q.D., K.G., and H.L. performed isolate analysis. All authors reviewed and approved the final manuscript.

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References

1. United Nations Children's Fund. Committing to child survival: a promise renewed, progress report 2015. New York: The Fund; 2015.
2. Seale AC, Bianchi-Jassir F, Russell NJ, Kohli-Lynch M, Tann CJ, Hall J, et al. Estimates of the burden of group B streptococcal disease worldwide for pregnant women, stillbirths, and children. Clin Infect Dis. 2017;65(suppl_2):S200–S19. https://doi.org/10.1093/cid/cix664
3. Madrid L, Seale AC, Kohli-Lynch M, Edmond KM, Lawn JE, Heath PT, et al. Infant group B streptococcal disease incidence and serotypes worldwide: systematic review and meta-analyses. Clin Infect Dis. 2017;65(suppl_2):S160–S72. https://doi.org/10.1093/cid/cix656
4. Dagnew AF, Cunnington MC, Dube Q, Edwards MS, French N, Heyderman RS, et al. Variation in reported neonatal group B streptococcal disease incidence in developing countries. Clin Infect Dis. 2012;55:91–102. https://doi.org/10.1093/cid/cis395
5. Iroh Tam PY, Delair SF, Obaro SK. Neonatal group B streptococcus disease in developing countries: are we ready to deploy a vaccine? Expert Rev Vaccines. 2015;14:1401–3. https://doi.org/10.1586/14760584.2015.1077121
6. Johri AK, Lata H, Yadav P, Dua M, Yang Y, Xu X, et al. Epidemiology of group B *Streptococcus* in developing countries. Vaccine. 2013;31(Suppl 4):D43–5. https://doi.org/10.1016/j.vaccine.2013.05.094
7. Guan X, Mu X, Ji W, Yuan C, He P, Zhang L, et al. Epidemiology of invasive group B streptococcal disease in infants from urban area of South China, 2011–2014. BMC Infect Dis. 2018;18:14. https://doi.org/10.1186/s12879-017-2811-0
8. Ji W, Liu H, Jin Z, Wang A, Mu X, Qin X, et al. Disease burden and antimicrobial resistance of invasive group B streptococcus

among infants in China: a protocol for a national prospective observational study. BMC Infect Dis. 2017;17:377. https://doi.org/10.1186/s12879-017-2475-9
9. Poyart C, Tazi A, Réglier-Poupet H, Billoët A, Tavares N, Raymond J, et al. Multiplex PCR assay for rapid and accurate capsular typing of group B streptococci. J Clin Microbiol. 2007;45:1985–8. https://doi.org/10.1128/JCM.00159-07
10. Yao K, Poulsen K, Maione D, Rinaudo CD, Baldassarri L, Telford JL, et al.; Members of the DEVANI Study Group. Capsular gene typing of *Streptococcus agalactiae* compared to serotyping by latex agglutination. J Clin Microbiol. 2013;51:503–7. https://doi.org/10.1128/JCM.02417-12
11. Jiang ZF, Shen KL, Shen Y. Zhu Futang Practice of Pediatrics. 8th ed. [in Chinese]. Beijing: People's Medical Publishing House; 2015.
12. Chu YW, Tse C, Tsang GKL, So DKS, Fung JTL, Lo JYC. Invasive group B *Streptococcus* isolates showing reduced susceptibility to penicillin in Hong Kong. J Antimicrob Chemother. 2007;60: 1407–9. https://doi.org/10.1093/jac/dkm390
13. Guo D, Cao X, Li S, Ou Q, Lin D, Yao Z, et al. Neonatal colonization of group B *Streptococcus* in China: prevalence, antimicrobial resistance, serotypes, and molecular characterization. Am J Infect Control. 2018;46:e19–24. https://doi.org/10.1016/j.ajic.2017.10.020
14. Ji W, Zhang L, Guo Z, Xie S, Yang W, Chen J, et al. Colonization prevalence and antibiotic susceptibility of group B *Streptococcus* in pregnant women over a 6-year period in Dongguan, China. PLoS One. 2017;12:e0183083. https://doi.org/10.1371/journal.pone.0183083
15. Lu B, Li D, Cui Y, Sui W, Huang L, Lu X. Epidemiology of group B *Streptococcus* isolated from pregnant women in Beijing, China. Clin Microbiol Infect. 2014;20:O370–3. https://doi.org/10.1111/1469-0691.12416
16. Liu H, Zeng H, Wang W, Deng Q, Margarit I, Rinaudo CD, et al. Estimating the burden of invasive group B streptococcal disease in young infants in southern mainland China: an observational study. Int J Clin Exp Med. 2015;8:13699–707.
17. Zeng Y, Hesketh T. The effects of China's universal two-child policy. Lancet. 2016;388:1930–8. https://doi.org/10.1016/S0140-6736(16)31405-2
18. Quan V, Verani JR, Cohen C, von Gottberg A, Meiring S, Cutland CL, et al. Invasive group B streptococcal disease in South Africa: importance of surveillance methodology. PLoS One. 2016;11:e0152524. https://doi.org/10.1371/journal.pone.0152524
19. Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease: revised guidelines from CDC, 2010. MMWR Morb Mortal Wkly Rep. MMWR Recomm Rep. 2010;59(RR-10): 1–36.
20. Heath PT. Status of vaccine research and development of vaccines for GBS. Vaccine. 2016;34:2876–9. https://doi.org/10.1016/j.vaccine.2015.12.072
21. Vekemans J, Moorthy V, Friede M, Alderson MR, Sobanjo-Ter Meulen A, Baker CJ, et al. Maternal immunization against group B *Streptococcus*: World Health Organization research and development technological roadmap and preferred product characteristics. Vaccine. 2018;S0264-410X(17)31359-2. https://doi.org/10.1016/j.vaccine.2017.09.087
22. Liu J, Xu R, Zhong H, Zhong Y, Xie Y, Li L, et al. Prevalence of GBS serotype III and identification of a ST 17–like genotype from neonates with invasive diseases in Guangzhou, China. Microb Pathog. 2018;120:213–8. https://doi.org/10.1016/j.micpath.2018.05.002
23. Li J, Ji W, Gao K, Zhou H, Zhang L, Mu X, et al. Molecular characteristics of group B *Streptococcus* isolates from infants in southern mainland China. BMC Infect Dis. 2019;19:812. https://doi.org/10.1186/s12879-019-4434-0
24. Nie S, Lu X, Jin Z, Gao J, Ma D, Deng J, et al. Characterization of group B *Streptococcus* isolated from sterile and non-sterile specimens in China. Diagn Microbiol Infect Dis. 2018;92:56–61. https://doi.org/10.1016/j.diagmicrobio.2018.04.014
25. Wang X, Cao X, Li S, Ou Q, Lin D, Yao Z, et al. Phenotypic and molecular characterization of *Streptococcus agalactiae* colonized in Chinese pregnant women: predominance of ST19/III and ST17/III. Res Microbiol. 2018;169:101–7. https://doi.org/10.1016/j.resmic.2017.12.004
26. Lo CW, Liu HC, Lee CC, Lin CL, Chen CL, Jeng MJ, et al. Serotype distribution and clinical correlation of *Streptococcus agalactiae* causing invasive disease in infants and children in Taiwan. J Microbiol Immunol Infect. 2017;S1684-1182(17)30228-1. https://doi.org/10.1016/j.jmii.2017.09.002
27. Bekker V, Bijlsma MW, van de Beek D, Kuijpers TW, van der Ende A. Incidence of invasive group B streptococcal disease and pathogen genotype distribution in newborn babies in the Netherlands over 25 years: a nationwide surveillance study. Lancet Infect Dis. 2014;14:1083–9. https://doi.org/10.1016/S1473-3099(14)70919-3
28. Lin FY, Whiting A, Adderson E, Takahashi S, Dunn DM, Weiss R, et al. Phylogenetic lineages of invasive and colonizing strains of serotype III group B *Streptococci* from neonates: a multicenter prospective study. J Clin Microbiol. 2006;44:1257–61. https://doi.org/10.1128/JCM.44.4.1257-1261.2006
29. Jones N, Oliver KA, Barry J, Harding RM, Bisharat N, Spratt BG, et al.; Oxford Group B Streptococcus Consortium. Enhanced invasiveness of bovine-derived neonatal sequence type 17 group B *Streptococcus* is independent of capsular serotype. Clin Infect Dis. 2006;42:915–24. https://doi.org/10.1086/500324
30. Kobayashi M, Schrag SJ, Alderson MR, Madhi SA, Baker CJ, Sobanjo-Ter Meulen A, et al. WHO consultation on group B *Streptococcus* vaccine development: report from a meeting held on 27–28 April 2016. Vaccine. 2016;S0264-410X(16)31236-1. https://doi.org/10.1016/j.vaccine.2016.12.029

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Seasonal Influenza and Avian Influenza A(H5N1) Virus Surveillance among Inpatients and Outpatients, East Jakarta, Indonesia, 2011–2014

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During October 2011–September 2014, we screened respiratory specimens for seasonal and avian influenza A(H5N1) virus infections among outpatients with influenza-like illness and inpatients with severe acute respiratory infection (SARI) in East Jakarta, an Indonesia district with high incidence of H5N1 virus infection among poultry. In total, 31% (1,875/6,008) of influenza-like illness case-patients and 15% (571/3,811) of SARI case-patients tested positive for influenza virus. Influenza A(H1N1)pdm09, influenza A(H3N2), and influenza B virus infections were detected in all 3 years, and the epidemic season extended from November through May. Although 28% (2,810/10,135) of case-patients reported exposure to poultry, only 1 SARI case-patient with an H5N1 virus infection was detected. Therefore, targeted screening among case-patients with high-risk poultry exposures (e.g., a recent visit to a live bird market or close proximity to sick or dead poultry) may be a more efficient routine surveillance strategy for H5N1 virus in these types of settings.

Seasonal influenza contributes substantially to acute respiratory disease in Indonesia and across the world. Influenza virus causes ≈3–5 million cases of severe illness (*1*) and ≈291,000–646,000 respiratory deaths each year globally, most occurring in lower-income countries (*2*). In Indonesia, published data suggest that influenza virus infection has a substantial effect on population health and the healthcare system, causing both inpatient and outpatient respiratory illness (*3*–*5*). Among the more densely populated western and central islands of Indonesia, influenza activity peaks in December and January, correlating with the rainy season. However, limited data from 1 district of Jakarta suggest that a longer peak in influenza activity occurs December–May, with multiple influenza viruses co-circulating (*4*).

In addition to seasonal influenza A and B epidemics, highly pathogenic avian influenza A(H5N1) virus also circulates among poultry in Indonesia (*6*). Jakarta Province is a hub for Indonesia's commercial poultry trade, and East Jakarta (1 of its 5 districts) is the main entry point for national poultry shipments (*7*). During 2005–2017, Indonesia detected and reported 200 H5N1 infections in humans, of which 168 (84%) were fatal (*8*). Although the number of infections in humans has decreased in Indonesia since 2015, this country still has the second highest number of reported cases (after Egypt) and the highest reported case-fatality proportion among all countries reporting H5N1 virus infections in humans. In East Jakarta, 12 of 13 H5N1 cases reported in humans during 2005–2015 were fatal (*9*).

Although influenza surveillance capacity in Indonesia has increased (*5*,*10*), national policies for influenza vaccination and antiviral use are limited. Influenza vaccination is recommended only for Hajj pilgrims and antivirals only for those with H5N1 virus infection (*11*,*12*). Thus, multiyear data are needed to explore trends in seasonal influenza and avian H5N1 virus infections among humans. Data are particularly needed in regions of Indonesia where highly

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pathogenic avian influenza A(H5N1) viruses are prevalent among poultry and the risk for poultry-to-human H5N1 virus transmission is elevated. Here, we describe the findings from a 3-year enhanced surveillance platform among inpatients and outpatients of clinics in East Jakarta for both seasonal influenza and avian influenza A(H5N1) viruses.

Methods

During October 2011–September 2014, we conducted enhanced influenza surveillance in East Jakarta to monitor influenza-like illness (ILI) among case-patients seeking treatment at 4 public primary health centers and severe acute respiratory infection (SARI) among inpatients at 6 hospitals (3 public and 3 private, all equipped with an intensive care unit [ICU]). We selected hospitals on the basis of their location within or bordering the district and the number of persons admitted for respiratory disease. We selected outpatient sites (among 10 subdistrict-level clinics present in East Jakarta) on the basis of proximity to live bird markets. ILI surveillance was conducted Monday–Friday, and surveillance staff used the case definition of recorded temperature ≥38°C with cough or sore throat to identify ILI case-patients. SARI surveillance was conducted every day in adult and pediatric wards (excluding surgery, obstetric, and gynecology) at all 6 hospitals. Hospital staff used the 2005 World Health Organization (WHO) Integrated Management of Childhood Illness case definition for pneumonia for case-patients <5 years of age (*13*) to identify SARI cases in this age group. To identify SARI in case-patients ≥5 years of age, staff used the 2011 WHO SARI case definition, which defines SARI as fever (measured temperature ≥38°C or subjective report of feverishness), disease onset within 7 days, and ≥1 of the following signs or symptoms: cough, sore throat, or shortness of breath (*14*). We defined elevated respiratory rate as >60 breaths/min for case-patients <2 months of age, ≥50 breaths/min for case-patients 2–11 months of age, ≥40 breaths/min for case-patients 1–4 years of age, ≥35 breaths/min for case-patients 5–7 years of age, ≥31 breaths/min for case-patients 8–11 years of age, ≥28 breaths/min for case-patients 12–14 years of age, and ≥25 breaths/min for case-patients ≥15 years of age (*15*,*16*). Staff obtained written consent from all enrolled ILI and SARI case-patients; surveillance staff completed case forms for and collected specimens from all enrolled case-patients. We collected data on demographic characteristics, clinical presentation, and exposure to poultry. Poultry exposure questions included questions on the exposures listed in the Indonesia case definition for suspected H5N1 virus infection (i.e., touching healthy, sick, or dead poultry or poultry products; slaughtering or cleaning poultry; or contact with chicken manure), as well as on poultry ownership, visiting live poultry markets, and neighborhood poultry die-offs (*4*). For SARI case-patients, we additionally collected data on risk factors for severe disease (chronic conditions, pregnancy, and smoking status), self-reported influenza vaccine use, and duration and outcome of hospitalization.

Surveillance staff collected nasal and throat swab samples from all enrolled ILI and SARI case-patients and sent them to 2 laboratories in Jakarta for influenza diagnosis (Provincial Health Laboratory of Daerah Khusus Ibukota [DKI] Jakarta and the Infectious Disease Hospital Sulianti Saroso Laboratory). Quality control testing was provided by the National Institute of Health Research and Development, a WHO-designated National Influenza Centre. These laboratories tested samples by real-time reverse transcription PCR (RT-PCR) following the US Centers for Disease Control and Prevention in-house real-time RT-PCR protocol for detection and characterization of influenza viruses. All specimens were tested for influenza A and B viruses, and influenza A virus–positive specimens were further subtyped for influenza A(H1N1)pdm09 and A(H3N2) viruses; specimens that were negative for these subtypes were then tested for H5N1 virus.

All surveillance and laboratory records were entered into a secured web-based electronic reporting system (Surveilans ILI-SARI DKI Jakarta) hosted by the DKI Jakarta Provincial Health Office (Jakarta, Indonesia). All eligible ILI and SARI case-patients were enumerated and reported through this electronic system on a weekly basis. The central project team trained site-level surveillance staff at the beginning of the project and conducted regular monitoring visits throughout the study to ensure thorough case ascertainment and data quality assurance at each site. Complete information on surveillance and data management methods were published previously (*4*).

We analyzed bivariate comparisons using χ^2 and Fisher exact tests. For time variables (e.g., time from illness onset to seeking care, duration of hospitalization), we determined median values and evaluated differences using the Mann-Whitney U test. We compared influenza virus detection across seasons qualitatively and identified time periods with elevated influenza virus circulation by determining the percentage of influenza virus–positive ILI or SARI episodes among all ILI or SARI episodes each week. We used poultry contact data to determine the percentage of case-patients who met the Indonesia case definition for suspected H5N1 virus infection (i.e., acute lower respiratory tract illness with fever and recent exposure to poultry or a human with a suspected H5N1 virus infection within 7 days of illness onset, based on WHO case definition) (*17*). We defined children as persons <18 years of age. We conducted all statistical analyses using SPSS version 21 (IBM, https://www.ibm.com).

This surveillance project was reviewed and considered to be routine public health surveillance by the Ministry of Health (Jakarta, Indonesia) and US Centers for

Disease Control and Prevention (Atlanta, Georgia, USA). Thus, our surveillance was exempted from institutional review board approval.

Results

During October 2011–September 2014, we enrolled 6,064 ILI case-patients (1% of 476,537 outpatient visits) (Table 1). Most (87%, 5,261/6,064) of these case-patients were <18 years of age. Because of case-patient refusal, we could not collect 56 respiratory specimens (from 1% of those enrolled). Of the 6,008 ILI specimens collected, 1,875 (31%) were positive for influenza virus. Median time from illness onset to outpatient visit among all ILI case-patients was 1 (interquartile range [IQR] 1–2) day (Table 2). Influenza virus–positive ILI case-patients (median age 7 [IQR 3–13] years) were older than influenza virus–negative ILI case-patients (median age 4 [IQR 1–8] years; $p<0.001$ by χ^2 test). Among ILI case-patients, influenza virus–negative case-patients (19%) were more likely than influenza virus–positive case-patients (12%; $p<0.001$) to have an elevated respiratory rate. More than one quarter (29%, 546/1,875) of influenza virus–positive ILI case-patients met the national case definition for suspected H5N1 virus infection.

We enrolled 4,071 SARI case-patients (2% of 184,576 hospitalizations) (Table 1). We could not collect 260 (6%) SARI specimens because of case-patient refusal or discharge before specimen collection; thus, we excluded these case-patients. Median time from illness onset to hospital admission was 3 days for children and adults, and median time from illness onset to specimen collection was 5 days (Table 3). Of the 3,811 SARI specimens collected, 571 (15%), including 1 positive for H5N1 virus, were positive for influenza virus (Table 1). Among all influenza virus–positive SARI case-patients, 97 (17%) met the national case definition for suspected H5N1 virus infection (Table 3).

The H5N1 virus–positive case-patient was a 33-year-old man (Table 4) with an onset of illness of June 1, 2014. On June 4, he was admitted into a hospital, where he received a diagnosis of pneumonia (chest radiograph data unavailable) and was enrolled into surveillance; his respiratory specimen was collected on June 6. On June 13, he was transferred to a designated referral hospital, where he began antiviral treatment and died on June 14. He was obese but had no other concurrent medical condition; exposure history included visiting a live bird market, where he purchased live poultry within 7 days before hospital admission. This case was not linked to any documented bird outbreaks. No animal or environmental specimen collection occurred because of the length of time that elapsed before linkage to a live poultry market and because of rapid turnover of poultry through these markets.

SARI case-patients were 0–90 years of age. A higher proportion of SARI cases among children 5–17 years of age

Table 1. Characteristics of ILI and SARI case-patients in study of incidence of seasonal and avian influenza A(H5N1) virus infections, East Jakarta, Indonesia, October 2011–September 2014*

Characteristic	ILI, n = 6,064	SARI, n = 4,071
Age, y		
<5	2,715 (44.8)	1,414 (35)
5–17	2,546 (42.0)	650 (16)
18–49	705 (11.6)	1,298 (32)
50–64	85 (1.4)	463 (11)
≥65	13 (0.2)	246 (6)
Age, y, median (IQR)	5 (2–9)	17 (2–40)
Sex		
M	2,930 (48)	2,205 (54)
F	3,134 (52)	1,866 (46)
Specimens collected and tested for influenza	6,008 (99)	3,811 (94)
Influenza virus positive		
Any	1,875/6,008 (31)	571/3,811 (15)
A	1,057/1,875 (56)	330/571 (58)
A(H3N2)	605/1,057 (57)	177/330 (53.6)
A(H1N1)pdm09	452/1,057 (43)	152/330 (46.1)
A(H5N1)	0	1/330 (0.3)
B	827/1,875 (44)	241/571 (42)
Met Indonesia case definition for suspected avian H5N1 virus infection†	1,943 (32)	867 (21)

*Values are no. (%), except where indicated otherwise. ILI and SARI were defined as described in the text. ILI, influenza-like illness; IQR, interquartile range; SARI, severe acute respiratory infection.
†Acute lower respiratory tract illness with fever and recent exposure to poultry (i.e., touching healthy, sick, or dead poultry or poultry products; slaughtering or cleaning poultry; or contact with chicken manure) or a human with a suspected H5N1 virus infection within 7 days of illness onset.

Table 2. Characteristics of ILI case-patients by influenza virus positivity, East Jakarta, Indonesia, October 2011–September 2014*

	Influenza virus positive		
Characteristic	Yes, n = 1,875	No, n = 4,133	p value
Sex			
M	900 (48)	2,007 (49)	0.687
F	975 (52)	2,126 (51)	
Age, y†			
<5	562 (30.0)	2,150 (52.0)	
5–17	915 (48.8)	1,592 (38.5)	
18–49	350 (18.7)	341 (8.3)	
50–64	41 (2.2)	44 (1.1)	
≥65	7 (0.4)	6 (0.1)	
Age, y, median (IQR)	7 (3–13)	4 (1–8)	<0.001
Time from illness onset to outpatient visit, d, median (IQR)	1 (1–2)	1 (1–2)	0.526
Elevated respiratory rate‡	229 (12)	796 (19)	<0.001
Met Indonesia case definition for suspected avian influenza A(H5N1) virus infection§	546 (29)	1,387 (34)	<0.001

*Values are no. (%), except where indicated otherwise. ILI was defined as stated in the text. ILI, influenza-like illness; IQR, interquartile range.
†Percentages in group do not always add up to 100% because of rounding.
‡Defined as described in the text.
§Acute lower respiratory tract illness with fever and recent exposure to poultry (i.e., touching healthy, sick, or dead poultry or poultry products; slaughtering or cleaning poultry; or contact with chicken manure) or a human with a suspected H5N1 virus infection within 7 days of illness onset.

Table 3. Characteristics of case-patients with SARI, by influenza virus positivity, East Jakarta, Indonesia, October 2011–September 2014*

Characteristic	Influenza virus positive		p value
	Yes, n = 571	No, n = 3,240	
Sex			0.816
M	310 (54)	1,742 (54)	
F	261 (46)	1,498 (46)	
Age, y†			
<5	164 (29)	1,109 (34)	
5–17	111 (19)	503 (16)	
18–49	186 (33)	1,065 (33)	
50–64	78 (14)	364 (11)	
≥65	32 (6)	199 (6)	
Age, y, median (IQR)	21 (4–43)	18 (2–40)	0.019
Time from illness onset to admission, d, median (IQR)	3 (2–4)	3 (2–5)	0.020
Time from illness onset to specimen collection, d, median (IQR)	5 (4–6)	5 (4–7)	0.012
Concurrent medical conditions			
≥1	123 (22)	779 (24)	0.195
Asthma	55 (10)	253 (8)	0.294
Tuberculosis	43 (8)	345 (11)	0.045
Diabetes	27 (5)	141 (4)	0.007
Influenza vaccination in past year	2 (0)	36 (1)	0.066
Pregnancy‡	5/93 (5)	22/568 (4)	0.497
Current smoker	75 (13)	466 (14)	0.7
Elevated respiratory rate§	139 (24)	1,061 (33)	<0.001
Chest radiograph–confirmed pneumonia¶	177/481 (37)	1,153/2,799 (41)	0.07
Met Indonesia case definition for suspected influenza A(H5N1) virus infection#	97 (17)	721 (22)	0.005
Sought care before admission**			0.007
Yes	180/276 (65)	1,112/1,514 (73)	
No	96/276 (35)	402/1,514 (27)	
Primary discharge diagnosis available	517 (91)	2,912 (90)	0.625
Pneumonia	132/517 (26)	996/2,912 (34)	<0.001
Typhoid	103/517 (20)	303/2,912 (10)	
Unspecified febrile Illness	57/517 (11)	189/2,912 (6)	
Upper respiratory tract infection	43/517 (8)	235/2,912 (8)	
Tuberculosis	32/517 (6)	311/2,912 (11)	
Discharge form completed	568 (99)	3,211 (99)	0.465
Medication during hospitalization			
Antibiotics	554/568 (98)	3,093/3,211 (96)	0.14
Oseltamivir	14/568 (2)	22/3,211 (1)	<0.001
Corticosteroid	10/568 (2)	116/3,211 (4)	0.064
Intensive care unit	6/568 (1)	101/3,211 (3)	0.006
Mechanical ventilation	6/568 (1)	76/3,211 (2)	0.049
Length of hospitalization, d, median (IQR)	4 (3–6)	5 (3–8)	<0.001
Outcome			0.075
Recovered	550/568 (97)	3,022/3,211 (94)	
Died	10/568 (2)	103/3,211 (3)	
Other††	8/568 (1)	86/3,211 (3)	

*Values are no. (%), except where indicated. SARI was defined as stated in the text. IQR, interquartile range; SARI, severe acute respiratory infection.
†Percentages in group do not always add up to 100% because of rounding.
‡Among female case-patients 15–49 years of age.
§Defined as described in the text.
¶Among those with a chest radiograph performed.
#Acute lower respiratory tract illness with fever and recent exposure to poultry (i.e., touching healthy, sick, or dead poultry or poultry products; slaughtering or cleaning poultry; or contact with chicken manure) or a human with a suspected H5N1 virus infection within 7 days of illness onset.
**Among those for which prehospitalization care-seeking behaviors were known.
††Includes forced discharge and referrals.

and adults 50–64 years of age were influenza virus–positive compared with other age groups (p = 0.016 by χ^2 test) (Table 3). Adults ≥65 years of age comprised 6% of all influenza virus–positive and influenza virus–negative SARI cases. Concurrent medical conditions, such as tuberculosis (10%, 388/3,811), asthma (8%, 308/3,811), and diabetes (4%, 168/3,811), were reported among 24% (902/3,811) of SARI case-patients; of these conditions, only diabetes was more commonly associated with influenza virus infection (p = 0.007). Data on healthcare seeking before admission were available for about half (46%, 1,790/3,811) of SARI case-patients. Influenza virus–negative SARI case-patients (73%, 1,112/1,514) were more likely than influenza virus–positive case-patients (65%, 180/276) to have sought care before hospitalization (p = 0.007). Overall, 38 (1%) SARI case-patients reported receiving the influenza vaccine in the previous 12 months, of whom 36 (95%) had a negative influenza virus test finding.

Table 4. Characteristics of SARI case-patients with severe influenza, East Jakarta, Indonesia, October 2011–September 2014*

Case-patient no.	Age, y/sex	Influenza virus detected	Diagnosis	Influenza vaccine in previous year	Underlying conditions	ICU or HCU admission	Outcome
1	50/M	A(H3N2)	Suspected A(H5N1) infection	No	Smoker, diabetes, heart disease	Yes	Recovered
2	33/M	A(H5N1)	Pneumonia	No	Obesity	Unknown	Died
3	83/M	B	Pneumonia	No	Smoker, diabetes, heart disease, asthma	No	Died
4	70/M	A(H3N2)	Pneumonia	No	Smoker	No	Died
5	66/M	A(H1N1)pdm09	Pneumonia	Unknown	Unknown	Yes	Died
6	0.5/F	B	Pneumonia	No	None recorded	Yes	Referred
7	1/M	A(H3N2)	Pneumonia	No	None recorded	Yes	Recovered
8	2/F	A(H1N1)pdm09	Febrile illness	No	None recorded	No	Died
9	64/F	A(H3N2)	Febrile illness	No	Diabetes, heart disease, asthma	No	Died
10	59/M	A(H3N2)	Chronic obstructive pulmonary disease	No	Smoker, kidney disease, asthma	No	Died
11	62/M	A(H3N2)	Congestive heart failure exacerbation	No	Smoker, heart disease, chronic lung disease	No	Died
12	39/M	A(H1N1)pdm09	Suspected A(H5N1) infection	No	Smoker	Yes	Died
13	63/F	A(H1N1)pdm09	Tuberculosis	No	Tuberculosis, asthma	No	Died
14	1/M	A(H1N1)pdm09	Anemia, malnutrition	No	None recorded	Yes	Recovered

*SARI was defined as stated in the text. SARI case-patients with severe influenza were those who were admitted into the HCU or ICU, died, or both as a result of influenza virus infection. HCU, high care unit; ICU, intensive care unit; SARI, severe acute respiratory infection.

Chest radiograph–confirmed pneumonia was documented for 35% (1,330/3,811) of SARI case-patients (Table 3). Of case-patients with known primary discharge diagnoses, pneumonia (33%, 1,128/3,429) was the most common diagnosis, followed by typhoid (12%, 406/3,429). Almost all (96%, 3,647/3,811) SARI case-patients received antibiotics during hospitalization. Only 2% (14/568) of influenza virus–positive SARI case-patients and 1% (22/3,211) of influenza virus–negative case-patients with data on medication use were treated with oseltamivir. Median duration of hospital stay was slightly longer for adults (5 days) than children (4 days; $p<0.001$) and influenza virus–negative (5 days) than influenza virus–positive (4 days; $p<0.001$) case-patients. Overall, 3% (107/3,779) of case-patients with a completed discharge form had been admitted to the ICU; ICU admission was less common among influenza virus–positive case-patients (1%, 6/568) than influenza virus–negative case-patients (3%, 101/3,211; $p = 0.006$). Of the 6 case-patients with influenza who were admitted to an ICU, 3 were children and 3 were adults (Table 4). One of these adult case-patients had concurrent medical conditions, and 2 were smokers. At the time of discharge, 95% (3,572/3,779) of SARI case-patients had recovered (Table 3). In total, 113 SARI case-patients died; 10 were positive for influenza virus. Of those 10 deaths, 9 were associated with seasonal influenza viruses and 1 with H5N1 virus. Two SARI case-patients with severe influenza had a discharge diagnosis of suspected H5N1; both had seasonal influenza virus infection.

Collectively among ILI and SARI case-patients, influenza A(H1N1)pdm09, influenza A(H3N2), and influenza B viruses were detected in all 3 years (Figure). The percentage of ILI case-patients who were influenza virus positive during year 1 (35%, 1,131/3,278) was slightly higher than the percentages positive during years 2 (27%, 427/1,562) and 3 (27%, 317/1,168). The percentage of SARI case-patients who were influenza virus positive was consistent across all 3 years: 15% (276/1,787) in year 1, 14% (153/1,122) in year 2, and 16% (142/902) in year 3. Each year, the percentage of samples positive for influenza virus per week started increasing in November, peaked in January or February, and declined in March but persisted through May. Influenza B virus infections were typically identified later in each season. Each year, the rate of influenza virus–positive samples per week peaked around 72%–85% for ILI and 38%–50% for SARI.

Discussion

During this 3-year period of enhanced surveillance, influenza viruses were commonly detected among ILI and SARI case-patients in East Jakarta, representing 31% of ILI visits and 15% of SARI hospitalizations, many of which were chest radiograph–confirmed pneumonia. Although many persons met the Indonesia case definition for suspected avian H5N1 virus infection because of their interaction with poultry, only 1 case of H5N1 virus infection was identified. Within 7 days before illness onset, this H5N1 case-patient had visited a live bird market, a type of poultry exposure reported among other persons with H5N1 virus infection in Indonesia; this type of exposure was reported among only 4% of SARI case-patients in this surveillance.

One of the primary objectives of this project was to systematically test both inpatients and outpatients for H5N1 virus infection. Although the case-fatality proportion reported for humans with H5N1 virus infection in Indonesia is high, underdetection in less severely ill patients was still

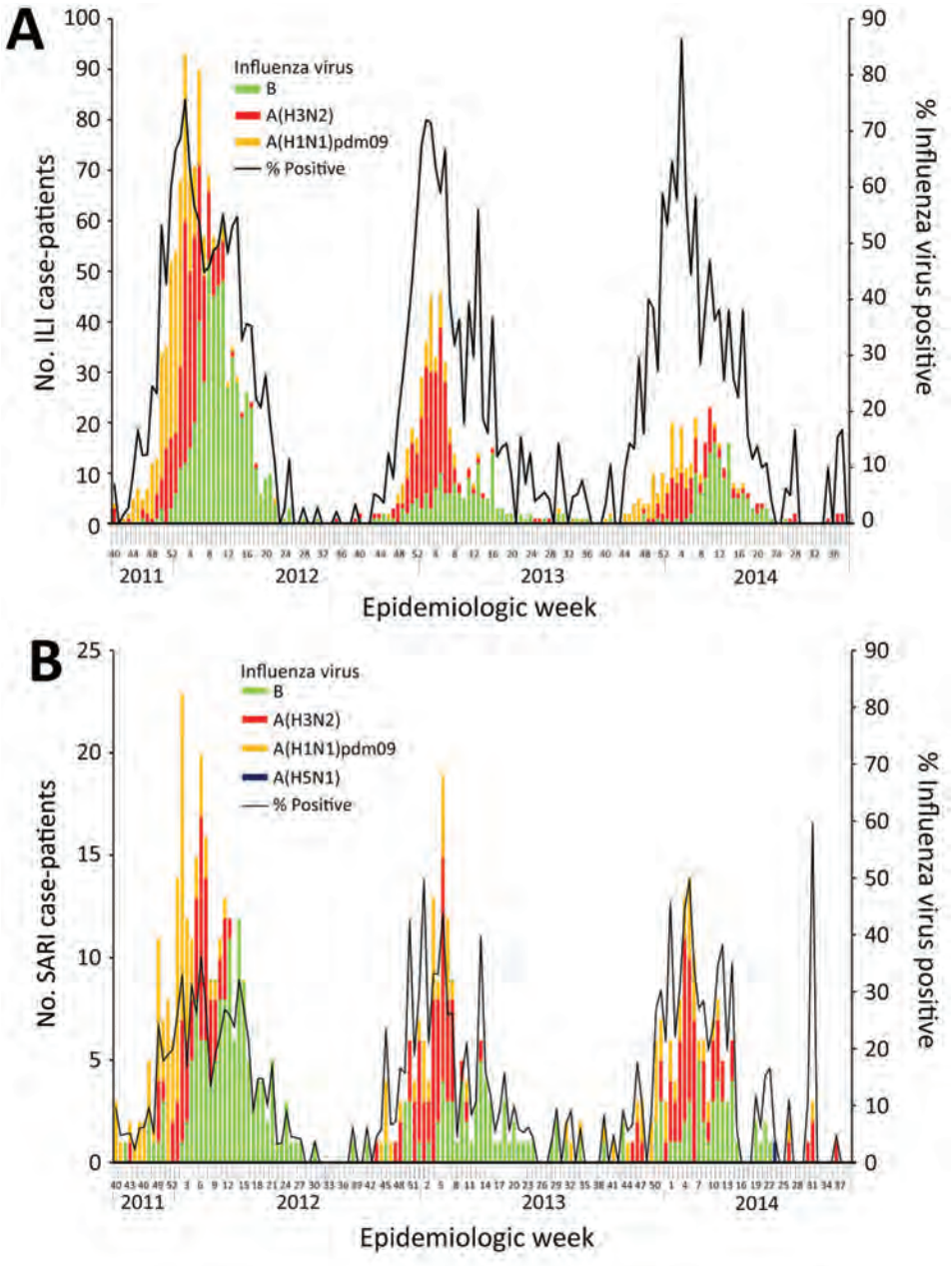

Figure. Seasonal and avian influenza A(H5N1) virus–positive ILI (A) and SARI (B) case-patients by clinical presentation and epidemiologic week, East Jakarta, Indonesia, October 2011–September 2014. ILI and SARI were defined as stated in the text. ILI, influenza-like illness; SARI, severe acute respiratory infection.

a possibility. Given that fatal outcomes are relatively rare, the ubiquity of exposure to poultry in parts of this country prohibits rigorous screening of all respiratory patients for H5N1 virus infection by sophisticated laboratory diagnostic methods such as RT-PCR. Our detection of only 1 case-patient with H5N1 virus infection in this high-risk district with endemic H5N1 virus circulation among poultry provides evidence to suggest that the likelihood of underascertainment in high-risk areas of Indonesia is low. Routine influenza surveillance in Indonesia involves inpatient and outpatient facilities, identification of SARI and ILI cases, and RT-PCR diagnostic testing; however, this surveillance is not specifically targeted to areas with endemic H5N1 virus circulation, and the volume of samples tested is much lower than the volume tested in this project.

Contrary to the infrequent detection of avian H5N1 virus, seasonal influenza A and B viruses were major contributors to medically treated respiratory illnesses in this population. Influenza A(H3N2), influenza A(H1N1)pdm09, and influenza B viruses were all identified, and no single virus type or influenza A virus subtype predominated in any year. In this region, influenza A and B viruses were most frequently detected during the beginning of each calendar year during the rainy season. Months of peak seasonal influenza virus circulation were consistent across all 3 years and covered at least half of each year. These long seasons

comprised consecutive peaks of influenza A and B viruses, similar to the pattern seen in US surveillance systems (*18*). The correspondence of influenza virus circulation with the rainy season is also consistent with evidence from other tropical countries of southern and southeastern Asia, including India and Thailand (*19*), as well as with previous outpatient influenza surveillance data from other parts of Indonesia (*3*).

In the study population, ILI case-patients were typically young children, and SARI case-patients included both children and adults. This observation is consistent with the age distribution reported by influenza surveillance platforms in other countries of Asia, such as the Philippines and Mongolia (*20*,*21*). Although older adults are more frequently identified in inpatient surveillance platforms in high-income settings (*22*), adults ≥65 years of age comprised only 6% of SARI case-patients in our platform. Several factors should be considered when interpreting this finding. First, this finding is consistent with the relatively young age structure of the Jakarta population, where only 5% of persons are ≥60 years of age (*23*). In addition, we conducted routine monitoring and validation activities to ensure enrollment of all eligible SARI case-patients, so the low number of case-patients enrolled in this age group is unlikely to be attributable to underenrollment. Instead, this trend might reflect differences in clinical presentation of older adults (who might not always meet the SARI case definition), decreased healthcare seeking by older adults, or a high threshold for admission by physicians. In this community, we previously conducted a survey on healthcare-seeking practices and found reported hospitalizations to be higher in older adults than younger adults and children, although the older adult population comprised a small proportion of the surveyed population (*24*).

In addition to age distribution differences between inpatients and outpatients, influenza virus detection status also differed by age. The youngest children with ILI and SARI were less likely to be influenza virus positive; influenza virus detection was more frequent among older children and adults. This trend is consistent with findings from a global meta-analysis on pediatric influenza hospitalizations (*25*) and is likely driven by the high prevalence of respiratory syncytial virus among children <1 year of age; this virus causes a large proportion of hospitalizations among children <5 years of age. Although we did not test respiratory specimens for respiratory syncytial virus, this pathogen has been shown to differentially infect young children who are also at high risk for SARI.

Less than one quarter of influenza virus–positive SARI case-patients reported underlying conditions, such as asthma, tuberculosis, or diabetes, and a small number of SARI case-patients were pregnant women. These findings might be a reflection of care-seeking patterns, particularly of pregnant women, who might prefer to receive care in private maternity hospitals. Our observation of a low percentage of pregnant women with influenza is in contrast with other published findings, such as those from routine SARI surveillance in South Africa, where pregnancy was identified as a risk factor for influenza-associated hospitalization (*26*). Concurrent medical conditions were also more prevalent among influenza virus–positive SARI case-patients in South Africa (35%), although surveillance in other locations, such as Damanhour, Egypt (*27*), revealed lower prevalences of concurrent conditions (19%) than that found in our study (22%). These differences might reflect baseline population variability in prevalence of diseases or variability in the types of persons seeking care at the facilities participating in surveillance.

Although use of antibiotics was common among patients in this study, only 2% of influenza virus–positive and 1% of influenza virus–negative SARI case-patients received antiviral treatment with oseltamivir. Oseltamivir is typically prescribed by physicians who suspect their patient has an H5N1 virus infection, per the Indonesia national policy for antiviral use (*12*). However, treating hospitalized influenza patients with oseltamivir is clinically beneficial regardless of whether the infection is seasonal or pandemic influenza virus, and recommendations outside of Indonesia include initiation of antiviral treatment as soon as possible for hospitalized patients with seasonal influenza (*28*,*29*). Likewise, the report of influenza vaccination among case-patients was low, probably because of the lack of a national influenza vaccination program in Indonesia, aside from the recommendation for vaccination for Hajj travelers.

The surveillance platform in this study, involving an urban district within the capital province of Jakarta, was specifically selected for its high prevalence of H5N1 viruses in poultry (*30*); therefore, our findings cannot be generalized to all of Indonesia. Although routine surveillance in other districts might potentially detect even lower percentages of patients with H5N1 virus infection, likelihood of detection through a surveillance system varies by location and over time, and the true risk for infection cannot be known. Confirmed H5N1 virus infections in humans decreased in Indonesia in the 2010s; infections peaked in 2006 with 55 cases, and only 3 cases were identified during 2015–2018. This decrease in H5N1 virus infections in humans has also been observed in other countries where the virus is endemic in poultry. In addition, only a fraction of patients with seasonal influenza or avian H5N1 virus infection will seek care, and those who do seek care might not meet the typical surveillance case definition or might no longer be shedding detectable influenza virus in the upper respiratory tract by the time they provide a respiratory specimen. Avian H5N1 viruses, in particular, preferentially bind receptors in the lower respiratory tract and can be missed in upper respiratory tract

specimen collection. Last, our results are reliant on self-report of risk factors, such as poultry exposure and chronic medical conditions, meaning we could be underestimating the frequency of these risk factors, although recall is unlikely to differ between those with and without influenza virus infection. Despite these limitations, we believe our findings provide valuable insight into patterns of seasonal influenza virus circulation and severe respiratory disease in this urban setting in Indonesia.

In conclusion, seasonal influenza was found to be a key contributor to outpatient and inpatient respiratory disease in this urban setting of Indonesia, where avian H5N1 viruses are frequently detected among poultry. In contrast, avian H5N1 virus infection was only detected in 1 SARI case-patient, despite rigorous and systematic screening of both inpatients with SARI and outpatients with ILI. In settings like East Jakarta, where poultry exposure is common, our findings support restricting RT-PCR testing for H5N1 viruses to sick patients with unsubtypeable influenza A virus infection and those with high-risk poultry exposures, such as a recent visit to a live bird market or close proximity to sick or dead poultry. Knowledge about the prevalence of seasonal influenza and timing of the local influenza season could also be leveraged by policy makers, public health officials, and healthcare providers to improve risk communication and develop appropriate prevention and control measures, such as early empiric antiviral use and optimally timed influenza vaccination activities.

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References

1. World Health Organization. Fact sheet: influenza (seasonal). 2018 [cited 2018 Nov 30]. https://www.who.int/news-room/fact-sheets/detail/influenza-(seasonal)
2. Iuliano AD, Roguski KM, Chang HH, Muscatello DJ, Palekar R, Tempia S, et al.; Global Seasonal Influenza-associated Mortality Collaborator Network. Estimates of global seasonal influenza-associated respiratory mortality: a modelling study. Lancet. 2018;391:1285–300. http://dx.doi.org/10.1016/S0140-6736(17)33293-2
3 Kosasih H, Roselinda, Nurhayati, Klimov A, Xiyan X, Lindstrom S, et al. Surveillance of influenza in Indonesia, 2003–2007. Influenza Other Respir Viruses. 2013;7:312–20. https://doi.org/10.1111/j.1750-2659.2012.00403.x
4. Storms AD, Kusriastuti R, Misriyah S, Praptiningsih CY, Amalya M, Lafond KE, et al. The East Jakarta Project: surveillance for highly pathogenic avian influenza A(H5N1) and seasonal influenza viruses in patients seeking care for respiratory disease, Jakarta, Indonesia, October 2011–September 2012. Epidemiol Infect. 2015;143:3394–404. https://doi.org/10.1017/S0950268815000771
5. Susilarini NK, Haryanto E, Praptiningsih CY, Mangiri A, Kipuw N, Tarya I, et al. Estimated incidence of influenza-associated severe acute respiratory infections in Indonesia, 2013-2016. Influenza Other Respir Viruses. 2018;12:81–7. https://doi.org/10.1111/irv.12496
6. Daniels P, Wiyono A, Sawitri E, Poermadjaja B, Sims LD. H5N1 highly pathogenic avian influenza in Indonesia: retrospective considerations. Curr Top Microbiol Immunol. 2013;365:171–84. https://doi.org/10.1007/82_2012_265
7. Food and Agriculture Organization. Approaches to controlling, preventing and eliminating H5N1 highly pathogenic avian influenza in endemic countries. 2011 [cited 2018 Nov 30]. http://www.fao.org/3/i2150e/i2150e00.htm
8. World Health Organization. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO, 2003-2018. 2018 Mar 2 [cited 2018 Nov 30]. http://www.who.int/entity/influenza/human_animal_interface/2018_03_02_tableH5N1.pdf?ua=1
9. DKI Jakarta Provincial Health Office. Policy of harmonization of epidemiology and virology (H5N1 and seasonal influenza) surveillance based on sentinel in East Jakarta. Presented at: East Jakarta Project stakeholder meeting; 2015 Sep 30; Jakarta, Indonesia.
10. Hanafusa S, Muhadir A, Santoso H, Tanaka K, Anwar M, Sulistyo ET, et al. A surveillance model for human avian influenza with a comprehensive surveillance system for local-priority communicable diseases in South Sulawesi, Indonesia. Trop Med Health. 2012;40:141–7. https://doi.org/10.2149/tmh.2012-10
11. Pane M, Imari S, Alwi Q, Nyoman Kandun I, Cook AR, Samaan G. Causes of mortality for Indonesian Hajj pilgrims: comparison between routine death certificate and verbal autopsy findings. PLoS One. 2013;8:e73243. https://doi.org/10.1371/journal.pone.0073243

12. Kosasih H, Bratasena A, Pangesti K, Laras K, Samaan G. Managing seasonal influenza: oseltamivir treatment policy in Indonesia? Acta Med Indones. 2014;46:58–65.
13. World Health Organization Department of HIV/AIDS, World Health Organization Department of Child and Adolescent Health and Development. WHO recommendations on the management of diarrhoea and pneumonia in HIV-infected infants and children: integrated management of childhood illness. Geneva: The Organization; 2010 [cited 2018 Nov 30]. https://www.who.int/hiv/pub/children/9789241548083
14. World Health Organization. WHO global technical consultation: global standards and tools for influenza surveillance. 2011 Mar 8–10 [cited 2018 Nov 30]. https://www.who.int/influenza/resources/documents/technical_consultation
15. Bonafide CP, Brady PW, Keren R, Conway PH, Marsolo K, Daymont C. Development of heart and respiratory rate percentile curves for hospitalized children. Pediatrics. 2013;131:e1150–7. https://doi.org/10.1542/peds.2012-2443
16. Cretikos MA, Bellomo R, Hillman K, Chen J, Finfer S, Flabouris A. Respiratory rate: the neglected vital sign. Med J Aust. 2008;188:657–9.
17. World Health Organization. WHO case definitions for human infections with influenza A(H5N1) virus. 2006 Aug 29 [cited 2018 Nov 30]. http://www.who.int/influenza/resources/documents/case_definition2006_08_29
18. World Health Organization. Influenza. FluNet - CHARTS. Influenza virus detections. 2019 [cited 2018 Nov 30]. https://www.who.int/influenza/gisrs_laboratory/flunet/charts
19. Saha S, Chadha M, Al Mamun A, Rahman M, Sturm-Ramirez K, Chittaganpitch M, et al. Influenza seasonality and vaccination timing in tropical and subtropical areas of southern and south-eastern Asia. Bull World Health Organ. 2014;92:318–30. https://doi.org/10.2471/BLT.13.124412
20. Burmaa A, Kamigaki T, Darmaa B, Nymadawa P, Oshitani H. Epidemiology and impact of influenza in Mongolia, 2007–2012. Influenza Other Respir Viruses. 2014;8:530–7. https://doi.org/10.1111/irv.12268
21. Tallo VL, Kamigaki T, Tan AG, Pamaran RR, Alday PP, Mercado ES, et al. Estimating influenza outpatients' and inpatients' incidences from 2009 to 2011 in a tropical urban setting in the Philippines. Influenza Other Respir Viruses. 2014;8:159–68. https://doi.org/10.1111/irv.12223
22. Jain S, Self WH, Wunderink RG, Fakhran S, Balk R, Bramley AM, et al.; Centers for Disease Control and Prevention EPIC Study Team. Community-acquired pneumonia requiring hospitalization among U.S. Adults. N Engl J Med. 2015;373:415–27. https://doi.org/10.1056/NEJMoa1500245
23. Badan Pusat Statistik. Population by age group, urban/rural area, and sex, DKI Jakarta Province [in Indonesian]. 2010 [cited 2018 Nov 30]. http://sp2010.bps.go.id/index.php/site/tabel?tid=263&wid=3100000000
24. Praptiningsih CY, Lafond KE, Wahyuningrum Y, Storms AD, Mangiri A, Iuliano AD, et al. Healthcare-seeking behaviors for acute respiratory illness in two communities of Java, Indonesia: a cross-sectional survey. J Epidemiol Glob Health. 2016;6:77–86. https://doi.org/10.1016/j.jegh.2016.01.002
25. Lafond KE, Nair H, Rasooly MH, Valente F, Booy R, Rahman M, et al.; Global Respiratory Hospitalizations—Influenza Proportion Positive (GRIPP) Working Group. Global role and burden of influenza in pediatric respiratory hospitalizations, 1982–2012: a systematic analysis. PLoS Med. 2016;13:e1001977. https://doi.org/10.1371/journal.pmed.1001977
26. Tempia S, Walaza S, Moyes J, Cohen AL, von Mollendorf C, Treurnicht FK, et al. Risk factors for influenza-associated severe acute respiratory illness hospitalization in South Africa, 2012–2015. Open Forum Infect Dis. 2017;4:ofw262. https://doi.org/10.1093/ofid/ofw262
27. Rowlinson E, Dueger E, Mansour A, Azzazy N, Mansour H, Peters L, et al. Incidence and etiology of hospitalized acute respiratory infections in the Egyptian Delta. Influenza Other Respir Viruses. 2017;11:23–32. https://doi.org/10.1111/irv.12409
28. Muthuri SG, Venkatesan S, Myles PR, Leonardi-Bee J, A l Khuwaitir TS, Al Mamun A, et al.; PRIDE Consortium Investigators. Effectiveness of neuraminidase inhibitors in reducing mortality in patients admitted to hospital with influenza A H1N1pdm09 virus infection: a meta-analysis of individual participant data. Lancet Respir Med. 2014;2:395–404. https://doi.org/10.1016/S2213-2600(14)70041-4
29. Uyeki TM, Bernstein HH, Bradley JS, Englund JA, File TM Jr, Fry AM, et al. Clinical practice guidelines by the Infectious Diseases Society of America: 2018 update on diagnosis, treatment, chemoprophylaxis, and institutional outbreak management of seasonal influenza. Clin Infect Dis. 2019;68:e1–47. https://doi.org/10.1093/cid/ciy866
30. Indriani R, Samaan G, Gultom A, Loth L, Irianti S, Adjid R, et al. Environmental sampling for avian influenza virus A (H5N1) in live-bird markets, Indonesia. Emerg Infect Dis. 2010;16:1889–95. https://doi.org/10.3201/eid1612.100402

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Nasopharyngeal Pneumococcal Density during Asymptomatic Respiratory Virus Infection and Risk for Subsequent Acute Respiratory Illness

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Increased nasopharyngeal pneumococcal (*Streptococcus pneumoniae*) colonization density has been associated with invasive pneumococcal disease, but factors that increase pneumococcal density are poorly understood. We evaluated pneumococcal densities in nasopharyngeal samples from asymptomatic young children from Peru and their association with subsequent acute respiratory illness (ARI). Total pneumococcal densities (encompassing all present serotypes) during asymptomatic periods were significantly higher when a respiratory virus was detected versus when no virus was detected (p<0.001). In adjusted analyses, increased pneumococcal density was significantly associated with the risk for a subsequent ARI (p<0.001), whereas asymptomatic viral detection alone was associated with lower risk for subsequent ARI. These findings suggest that interactions between viruses and pneumococci in the nasopharynx during asymptomatic periods might have a role in onset of subsequent ARI. The mechanisms for these interactions, along with other potentially associated host and environmental factors, and their role in ARI pathogenesis and pneumococcal transmission require further elucidation.

Streptococcus pneumoniae (pneumococcus) is one of the most important bacterial causes of pneumonia among children and adults worldwide (*1*–*3*). Nasopharyngeal pneumococcal colonization is common in young children and represents a critical initial step in the progression to invasive disease (*4*,*5*). Increases in the density of pneumococci in the nasopharynx have been associated with the onset of respiratory illness (*6*,*7*) and might also play a role in transmission of bacteria to others (*8*). Several studies have found higher nasopharyngeal pneumococcal densities in patients with pneumonia than in healthy controls. However, no specific level of pneumococcal density has been identified that can establish the diagnosis of pneumococcal pneumonia (*9*–*11*).

The specific factors that drive increases in nasopharyngeal pneumococcal density that might lead to pneumonia or other invasive disease are not well characterized. Although an increase in density might represent expansion of a single preexisting serotype, it could also represent acquisition of a new colonizing pneumococcal serotype. New acquisition has been temporally associated with the acute onset of invasive disease (*12*,*13*). Co-detection of respiratory viruses has also been associated with increases in pneumococcal density during periods of acute respiratory illness (ARI), pneumonia, or both (*6*,*7*,*14*,*15*), suggesting that respiratory viruses and pneumococci might work synergistically in pneumonia and invasive disease pathogenesis (*16*–*20*). In addition, younger children have been shown to have higher pneumococcal densities than older children, whereas children who had received pneumococcal conjugate vaccines or lived in vaccinated communities were reported to have lower pneumococcal densities (*21*,*22*).

Respiratory viruses are frequently detected in the nasopharynx of young children during asymptomatic periods (*23*–*26*). A recent cross-sectional study of children 4–7 years of age indicated similar pneumococcal densities in asymptomatic children who had respiratory viruses detected and in children with viral upper respiratory illness (*27*). However, whether viral detection or duration of viral carriage during asymptomatic periods is associated with increases in nasopharyngeal pneumococcal density or

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subsequent ARI risk is unclear. This assessment requires longitudinal follow-up of individual young children. Our goal was to assess the association between viral detection and pneumococcal density during asymptomatic periods and determine whether viral–pneumococcal interactions during these periods predisposed to subsequent symptomatic ARI among young children.

Methods

Study Design and Setting

The Respiratory Infections in Andean Peruvian Children (RESPIRA-Peru) study is a prospective cohort study conducted in the Province of San Marcos, Department of Cajamarca, located in the northern highlands of Peru. Malaria is not endemic in this high-altitude study community (median altitude of study households 2,726 m). The population is primarily rural with low income and limited access to healthcare services, as previously described (*6*,*7*,*16*,*23*,*28*–*34*). Nearly all residents of San Marcos descended from the same ethnic group of Spanish people who mixed with the local Quechua population. During May 2009–September 2011, enrolled children <3 years of age residing in the study area were prospectively assessed for ARI symptoms during weekly household visits. An ARI episode was defined as the presence of either cough or fever. This definition of ARI has been applied in many large surveillance studies (*35*,*36*). Although fever alone is not highly specific for the diagnosis of ARI, it is well established that fever is often the only sign of ARI, especially in young children (*35*). In addition to cough and fever, we collected data on other respiratory signs and symptoms, including rhinorrhea, ear pain, malaise, tachypnea, nasal flaring, stridor, wheezing, and accessory muscle retractions. We considered a child to be asymptomatic if the child had rhinorrhea alone or no ARI symptoms.

The 7-valent pneumococcal conjugate vaccine (PCV7) was introduced in the study region in late 2009 in a 2+1 schedule (2 primary doses at 3 and 5 months of age and a booster dose at 12 months). Very few children enrolled in 2009 had received any PCV7 doses at the time of enrollment. By the end of the study (September 2011), 62% of the study cohort had received ≥1 PCV7 dose (*30*). This study was approved by the Institutional Review Boards of Vanderbilt University (Nashville, Tennessee, USA) and the Instituto de Investigacion Nutricional (Lima, Peru).

Respiratory Sample Collection and Testing

We collected nasopharyngeal swabs from each child monthly, whether or not ARI symptoms were present, and tested the swabs at Emory University (Atlanta, GA, USA) by bacterial culture for pneumococcal identification and quantitative PCR (qPCR) for pneumococcal density determinations (*6*,*7*,*16*,*29*,*31*). We also randomly selected a subset of nasopharyngeal samples collected during asymptomatic periods, ≥8 days apart from an ARI episode, to undergo real-time reverse transcription PCR (rRT-PCR) viral testing at Vanderbilt University for influenza virus (types A, B, and C), respiratory syncytial virus (RSV), human metapneumovirus (MPV), rhinovirus (HRV), adenovirus (AdV), and parainfluenza virus (PIV) types 1–3 (*23*,*32*). Because detections of respiratory viruses other than HRV and AdV were infrequent in nasopharyngeal samples collected during those asymptomatic periods (*23*), we classified viral detections into distinct groups: HRV only; AdV only; sole detection of other virus (influenza, RSV, MPV, or PIV); co-detection of >1 respiratory virus; and no virus detected.

Statistical Analysis

We transformed pneumococcal density values to stabilize their variance, as previously described (*6*,*7*), by applying $\log_{10}(x + 1)$ transformation to colonization density values, where x represents the measured density. We performed unadjusted group comparisons by using the Wilcoxon rank-sum test, Kruskal-Wallis test, or median test with Bonferroni adjustments, as appropriate. We used multivariable linear quantile mixed effects regression to model the median of log-transformed pneumococcal density against viral detection group, with random effects to account for correlation among multiple measurements collected from the same child. Other covariates included age at the time of sample collection, sex, recent exposure to antibiotics (i.e., aminopenicillins, cephalosporins, co-trimoxazole, chloramphenicol, or furazolidone within the previous 7 days), pneumococcal vaccination status (receipt of ≥1 dose of PCV7), and calendar month. Because viral infections could lead to rhinorrhea, we did not directly include this variable in our main regression model, and rhinorrhea was considered as a potential variable in the causal pathway between viral infections and pneumococcal density changes. To examine the association of pneumococcal densities during asymptomatic periods with the risk for subsequent ARI, we conducted a survival analysis by using a frailty model and accounted for repeated measurements from individual children. Because severe ARI occurred uncommonly in this household surveillance study, we did not stratify the outcome variable (ARI) by severity (*28*). For this assessment, follow-up extended from the date of an asymptomatic nasopharyngeal sample collection (≥8 days after the last day of ARI symptoms) through the earliest of the following: ARI, the collection of another nasopharyngeal sample, or 60 days after collection of the initial asymptomatic nasopharyngeal sample. These censoring criteria were implemented on the basis of our monthly nasopharyngeal collection strategy and the anticipated transient duration of colonization with an individual serotype in young children,

typically 6–8 weeks or less (*37–40*). Because viral infection or its manifestation as rhinorrhea could modify the risk for subsequent ARI (*41*), alternate models accounted for either viral infections or rhinorrhea in addition to the aforementioned covariates. We applied restricted cubic splines (RCS) to log-transformed pneumococcal densities in our frailty model to relax the assumption of linearity in our models (*42*,*43*). The estimated effects of RCS-transformed densities are presented in figures. The proportional hazards assumption of the frailty model was examined and satisfied by using a Schoenfeld's global test. Statistical analyses were done in Stata version 14.2 (https://www.stata.com) and R version 3.5.0 (https://www.r-project.org), including lqmm and survival packages.

Results

Pneumococcal Colonization and Viral Detections

In total, 849 nasopharyngeal samples collected during asymptomatic periods from 480 children underwent both viral testing and pneumococcal density determinations (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/25/11/19-0157-App1.pdf). Relevant demographic characteristics of study children are shown in the Table. Pneumococcus was detected in 566/849 (67%) nasopharyngeal samples from asymptomatic children. At least 1 respiratory virus was detected in 357/849 (42%) samples from asymptomatic children, most commonly HRV (31%) and AdV (11%), whereas detections of influenza, MPV, PIV, and RSV in asymptomatic children were uncommon (≤3%), as previously reported (*23*).

Co-detection of >1 respiratory virus occurred in 48/849 (6%) samples. HRV was present in 40/48 (83%) co-detections, most commonly in combination with AdV, with or without an additional virus (36/40 [90%]). Colonization with pneumococcus was more common in samples in which ≥1 respiratory virus was detected (280/357 [78%]) than in those in which a respiratory virus was not detected (286/492 [58%]) (p<0.001). Rhinorrhea was reported in 219/357 (61%) children in whom a virus was detected, compared with 227/492 (46%) in whom a virus was not detected (p<0.001). At least 1 respiratory virus was detected in 219/446 (49%) children with rhinorrhea, compared to 138/403 (34%) without rhinorrhea. Antibiotic exposure preceding collection of each asymptomatic nasopharyngeal sample was uncommon, occurring in only 12/849 (1%) samples (antibiotic exposure was unknown in 9/849 samples [1%]).

Table. Selected demographic characteristics of 480 children from whom nasopharyngeal samples were obtained during asymptomatic periods, Respiratory Infections in Andean Peruvian Children study, May 2009–September 2011*

Characteristic	Value
Sex	
M	253 (53)
F	227 (47)
Median age at enrollment (IQR), mo	7.7 (1.0–18.8)
No. samples per child (IQR)	2 (1–2)
No. persons per household (IQR), n = 476	5 (4–6)
No. children age <5 y in household, n = 476	
1	323 (68)
2	140 (29)
≥3	13 (3)
Shares a bed, n = 473	461 (97)
Received >1 PCV7 dose, n = 468	262 (56)

*Values are no. (%) children unless indicated otherwise. IQR, interquartile range; PCV7, 7-valent pneumococcal conjugate vaccine.

Pneumococcal Densities and Associated Factors

In unadjusted comparisons, $\log_{10}$-transformed pneumococcal densities were higher during asymptomatic periods when ≥1 respiratory virus was detected (median 4.95 [interquartile range (IQR) 3.11–6.35]) than when no respiratory viruses were detected (median 3.35 [IQR 0–4.95]; p<0.001). When densities were examined according to detection of specific viruses, densities differed significantly by viral group (p<0.001); the highest densities were observed in the HRV-only group. In Bonferroni-adjusted pairwise median comparisons, pneumococcal densities were significantly higher when HRV only (median 5.18 [IQR 3.40–6.43]; p<0.001) or viral co-detections (median 4.71 [IQR 3.46–6.35]; p = 0.003) were present compared with samples that were virus-negative (3.35 [IQR 0–4.95]). In Bonferroni-adjusted median comparisons, pneumococcal density from samples with AdV-only infection (median 4.39 [IQR 3.15–6.08]) was not significantly different from that from samples with no respiratory viruses detected (p = 0.087).

In the multivariable mixed effects quantile regression model, pneumococcal densities during asymptomatic periods were not associated with age, sex, antibiotic exposure, or history of pneumococcal vaccination. However, detection of any (≥1) virus was significantly associated with higher $\log_{10}$-transformed pneumococcal densities (Appendix Table 1). The addition of rhinorrhea, a common manifestation of viral infections, to the model attenuated the observed association between viral infections and pneumococcal density, but the association remained statistically significant.

We compiled violin plots of predicted pneumococcal densities according to detection of any respiratory virus as estimated from the multivariable linear quantile mixed effects model (Figure 1). When specific viruses were examined in multivariable analyses, detection of HRV only, detection of AdV only, or co-detection of >1 virus were significantly associated with increased pneumococcal densities compared with densities in samples that were virus-negative. We observed no significant association between detection of other less frequently detected viruses (RSV,

PIV, influenza, MPV) and pneumococcal densities (Appendix Table 2). As in the main analysis, the addition of rhinorrhea to the model slightly attenuated, but did not eliminate, the observed associations. We compiled predicted densities associated with specific viral infections as estimated from the multivariable linear quantile mixed effects model (Figure 2).

Exploration of Asymptomatic Pneumococcal Densities and Risk for Subsequent ARI

We explored the association of pneumococcal density during asymptomatic periods and the time to next ARI by using log-transformed pneumococcal density as the exposure of interest and ARI as the outcome in a frailty model. Compared with the lowest pneumococcal density, increases in pneumococcal density were significantly associated with increased incidence of subsequent ARI (Figure 3; Appendix Table 3) ($p<0.001$). Younger age, receipt of ≥1 PCV7 dose, and detection of ≥1 respiratory virus during asymptomatic periods were significantly associated with lower incidence of ARI. In addition, sample collection during the middle months of spring (October and November) was associated with lower incidence of subsequent ARI. Similar findings were observed when rhinorrhea was included in the model instead of viral detection (Appendix Table 4, Figure 2), suggesting that the association between viral detection and ARI might be at least partially mediated by rhinorrhea.

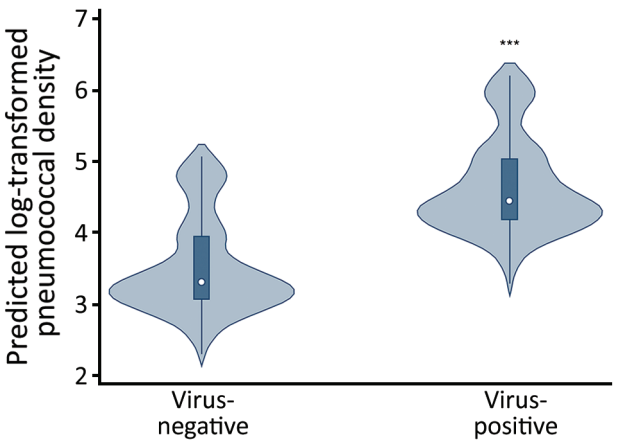

Figure 1. Violin plots of predicted $\log_{10}$-transformed pneumococcal colonization densities by any viral detection among children <3 years of age, Respiratory Infections in Andean Peruvian Children study, May 2009–September 2011. Predicted densities were estimated from the final multivariable linear quantile mixed effects model. Circles indicate median densities, boxes represent interquartile range, lines extend through the upper and lower adjacent values, and the density plot width indicates the predicted frequency of observations. ***p<0.001.

Discussion

Our findings demonstrate that viral detections during asymptomatic periods are associated with increases in nasopharyngeal pneumococcal colonization density, and further, that higher pneumococcal density during asymptomatic periods is associated with subsequent onset of an ARI. These findings expand upon earlier observations from our group and others that increases in nasopharyngeal pneumococcal colonization density are associated with symptomatic respiratory illness and pneumonia and with the detection of respiratory viruses during ARI periods (*6*,*7*,*14*,*15*). We now report that those associations are not restricted to periods of symptomatic disease and demonstrate an increased risk for ARI with increasing pneumococcal density during asymptomatic periods as well. Of interest, increased pneumococcal density was associated with the risk for subsequent ARI in a nonlinear manner and appeared particularly evident at higher pneumococcal densities ($>10^4$). These findings suggest that asymptomatic viral infection during asymptomatic periods might present colonizing pneumococci with an opportunity to expand.

Most ARI episodes in our study were mild, self-limited without antibiotic use, and associated with respiratory virus detection (*28*). Our finding that increases in pneumococcal density during asymptomatic episodes were associated with increased likelihood of subsequent onset of these mostly mild, viral ARI might suggest a contributing role of colonizing pneumococci in these illnesses. Of note, this association only became significant at the highest levels of pneumococcal density. A previous study of young children with nasopharyngeal pneumococcal colonization indicated that higher colonization density was associated with high levels of markers of nasopharyngeal inflammation compared with children with lower-density colonization (*44*). We postulate that increased nasopharyngeal inflammation from increased pneumococcal density might facilitate viral infection of an exposed susceptible person. The hypothesis that nasopharyngeal pneumococcal colonization patterns and density might influence subsequent onset of nonpneumococcal ARI is also consistent with our observation that children who had received ≥1 PCV vaccination had a reduced risk for ARI following asymptomatic viral detection compared with unvaccinated children.

A recent study by DeMuri et al. (*27*) examined colonization density with pneumococci and other respiratory bacteria during asymptomatic periods in children 4–7 years of age. They found that the densities of pneumococcus, *Moraxella catarrhalis*, and *Haemophilus influenzae* all increased when respiratory viruses were detected, although the study design did not allow determination of subsequent ARI risk (*27*). In that study, the association of viral detection and nasopharyngeal bacterial density was strongest with pneumococcus; pneumococcal densities approached those observed during periods of viral upper respiratory

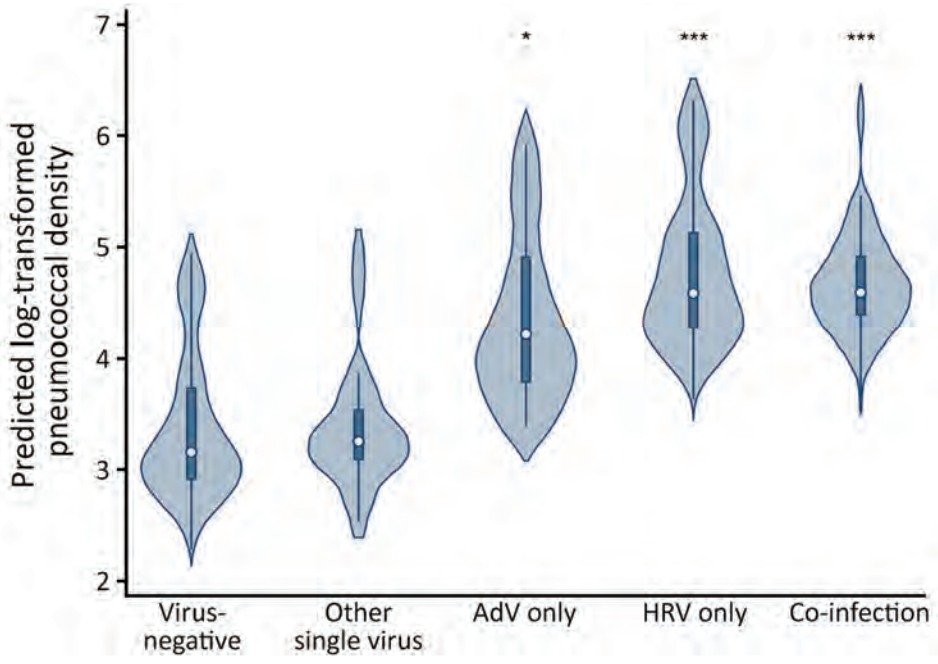

Figure 2. Violin plots of predicted $\log_{10}$-transformed pneumococcal colonization densities by detection of specific viruses among children <3 years of age, Respiratory Infections in Andean Peruvian Children study, May 2009–September 2011. Predicted densities were estimated from the final multivariable linear quantile mixed effects model. Circles indicate median densities, boxes represent interquartile range, lines extend through the upper and lower adjacent values, and the density plot width indicates the predicted frequency of observations. *p<0.05; ***p<0.001. AdV, adenovirus; HRV, rhinovirus.

illness. HRV, coronaviruses, and viral co-detections (≥1 virus present) were also significantly associated with higher densities than when no virus was detected. Detection of AdV in their patients was not significantly associated with increased pneumococcal densities during asymptomatic periods, but those observations were limited to only 4 AdV-positive samples.

Although both viral detection and rhinorrhea were associated with increased pneumococcal density, both were also associated with a lower incidence of subsequent ARI. Specifically, children with viral infection or rhinorrhea had a lower incidence of subsequent ARI than children without viral infection or rhinorrhea. The mechanism of this association is unclear but might involve the phenomenon of viral interference, in which infection with 1 virus prevents or mitigates infection with a different virus, perhaps through the induction of proinflammatory cytokines or other immune mediators (*41*), because the presence of rhinorrhea might indicate a viral infection with a virus detected or not detected in our study. Rhinitis has also been shown to occur after intranasal inoculation of pneumococcus in healthy adults and in mouse models (*45*), and nasal discharge has been associated with pneumococcal detection even in the absence of respiratory viral infection (*46*), suggesting that an inflammatory response might be an important component of early pneumococcal colonization, which might have important host consequences. Furthermore, several studies have also demonstrated the role of normal airway mucus and nasal secretions in lining epithelial surfaces and trapping and removing pathogens from the airway through mucociliary clearance (*47*,*48*), potentially providing an effective barrier to acquisition of new respiratory viruses, bacteria, or both.

In spite of the strengths of our longitudinal design, our study has several limitations. Because detection of viruses other than HRV and AdV during asymptomatic periods was uncommon in our cohort, the power to detect associations with these other specific viruses and pneumococcal densities was low. HRV was detected in >80% of samples in which >1 virus was detected, limiting inferences about co-detections with other viruses. Because of enhanced sensitivity of rRT-PCR for detection of respiratory viruses relative to culture, it might be difficult to interpret the clinical significance of detection of HRV and AdV by PCR in asymptomatic children (i.e., whether these detections might represent prolonged shedding from a prior illness or a recent asymptomatic infection). The study was conducted in a rural region in which the incidence of pneumonia is higher than national rates (*28*,*49*). However, incidence of other common respiratory diseases, such as otitis media, sinusitis, and other types of respiratory illnesses, was not available. Highest densities were observed during the 2009 mid-spring season, but examination of several consecutive seasons would be useful to clarify the role of seasonality in our observations. In our assessment of the role of colonization density during asymptomatic periods on the risk for subsequent ARI, we did not systematically assess for pneumococcal colonization or density during the subsequent ARI, so direct density comparisons were not possible between the asymptomatic and subsequent ARI periods. Furthermore, we must

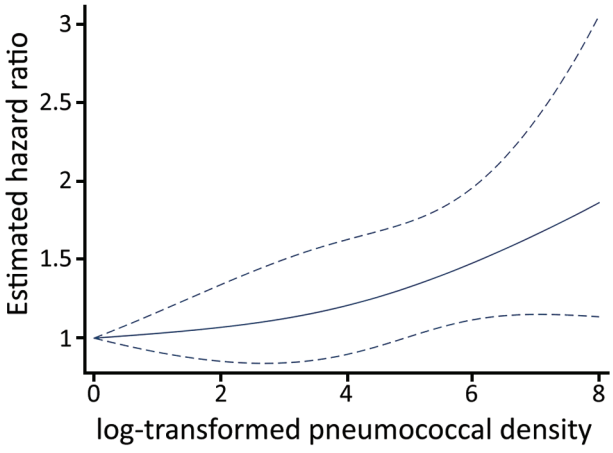

Figure 3. Association between asymptomatic pneumococcal densities and risk of subsequent acute respiratory illness among children <3 years of age, Respiratory Infections in Andean Peruvian Children study, May 2009–September 2011. Estimated hazard ratios correspond to comparisons of increasing $\log_{10}$-transformed pneumococcal density relative to the lowest detectable densities (p = 0.013). Solid lines represent the point estimates for the hazard ratio by log-transformed pneumococcal density; dashed lines represent 95% CIs. Estimates were obtained from a frailty model that adjusted for age, sex, month, prior antibiotic exposure, viral detection, and pneumococcal conjugate vaccination status. Pneumococcal densities were modeled by using restricted cubic splines to allow examination of nonlinear associations.

note that several factors might play a role in increases in pneumococcal colonization density. A recent study from Kenya found that increases in density associated with viral detection were modest relative to the baseline variation in density in individual patients (*50*) and postulated that other factors, such as the host immune response to previous viral infections (*6*,*7*,*27*), also play an important role in the association between high levels of pneumococcal density and risk for subsequent ARI. However, the potential influence of immune, genetic, or other environmental factors on subsequent ARI risk was not measured in our study. Also, the associations observed with pneumococcal density might be serotype-specific, but serotype information was not available for those samples from patients with subsequent ARI. Furthermore, interactions between pneumococcus and other colonizing nasopharyngeal bacteria might play an important role in the host response to pneumococcal acquisition as well as the dynamics of pneumococcal density and pathogenesis (*29*), but associated changes in the nasopharyngeal microbiome were not assessed in this study. The presence of an asymptomatic viral infection was associated with a lower risk for subsequent symptomatic ARI in this study. Although we postulate that such a relationship might have a time-limited impact, our study was set up with a short follow-up time and was not designed to determine the duration of this association. Additional studies that have a longer follow-up time and are specifically designed to determine the temporality of the observed association would be useful. Future studies will assess the relationship between viral detection and serotype and density patterns over time in individual patients, along with associated changes in the respiratory microbiome during both asymptomatic and ARI periods. Finally, our study did not assess transmission patterns of colonizing pneumococci. Whether the increase in densities associated with viral detection have an impact on transmission is unclear.

In summary, we found that viral detections during asymptomatic periods are associated with increases in nasopharyngeal pneumococcal colonization density. Furthermore, we found that pneumococcal density, especially at high levels, is associated with subsequent development of ARI in young children in Peru. These findings suggest that interactions between viruses and pneumococci in the nasopharynx during asymptomatic periods might have a role in onset of subsequent ARI.

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Conflicts of interest: L.M.H. receives grant support from Pfizer. C.G.G. has served as a consultant to Pfizer, Merck, and Sanofi-Pasteur for unrelated work and received research support from Sanofi-Pasteur. M.R.G. receives grant funding from MedImmune. K.M.E. receives grant funding from Novartis in unrelated work. J.V.W. serves on a scientific advisory board for Quidel and an independent data monitoring committee for GlaxoSmithKline, neither related to the present work. C.F.L. serves as a scientific advisor to Takeda in subjects not related to the present work.

About the Author

Dr. Howard is an assistant professor of pediatric infectious diseases at Vanderbilt University Medical Center. Her primary research interests are respiratory viral and bacterial epidemiology, with an emphasis on pneumococcal infections, antimicrobial resistance patterns among pneumococci, and the impact of pneumococcal conjugate vaccines on pneumococcal colonization dynamics in children.

References

1. Cevey-Macherel M, Galetto-Lacour A, Gervaix A, Siegrist CA, Bille J, Bescher-Ninet B, et al. Etiology of community-acquired pneumonia in hospitalized children based on WHO clinical guidelines. Eur J Pediatr. 2009;168:1429–36. https://doi.org/10.1007/s00431-009-0943-y
2. Griffin MR, Zhu Y, Moore MR, Whitney CG, Grijalva CGUS. U.S. hospitalizations for pneumonia after a decade of pneumococcal vaccination. N Engl J Med. 2013;369:155–63. https://doi.org/10.1056/NEJMoa1209165
3. Izadnegahdar R, Cohen AL, Klugman KP, Qazi SA. Childhood pneumonia in developing countries. Lancet Respir Med. 2013;1:574–84. https://doi.org/10.1016/S2213-2600(13)70075-4
4. Simell B, Auranen K, Käyhty H, Goldblatt D, Dagan R, O'Brien KL; Pneumococcal Carriage Group. The fundamental link between pneumococcal carriage and disease. Expert Rev Vaccines. 2012;11:841–55. https://doi.org/10.1586/erv.12.53
5. Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. Lancet Infect Dis. 2004;4:144–54. https://doi.org/10.1016/S1473-3099(04)00938-7
6. Fan RR, Howard LM, Griffin MR, Edwards KM, Zhu Y, Williams JV, et al. Nasopharyngeal pneumococcal density and evolution of acute respiratory illnesses in young children, Peru, 2009–2011. Emerg Infect Dis. 2016;22:1996–9. https://doi.org/10.3201/eid2211.160902
7. Howard LM, Fan R, Zhu Y, Griffin MR, Edwards KM, Hartinger S, et al. Nasopharyngeal pneumococcal density is associated with viral activity but not with use of improved stoves among young andean children. Open Forum Infect Dis. 2017;4:ofx161. https://doi.org/10.1093/ofid/ofx161
8. Diavatopoulos DA, Short KR, Price JT, Wilksch JJ, Brown LE, Briles DE, et al. Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. FASEB J. 2010;24:1789–98. https://doi.org/10.1096/fj.09-146779
9. Albrich WC, Madhi SA, Adrian PV, van Niekerk N, Mareletsi T, Cutland C, et al. Use of a rapid test of pneumococcal colonization density to diagnose pneumococcal pneumonia. Clin Infect Dis. 2012;54:601–9. https://doi.org/10.1093/cid/cir859
10. Brotons P, Bassat Q, Lanaspa M, Henares D, Perez-Arguello A, Madrid L, et al. Nasopharyngeal bacterial load as a marker for rapid and easy diagnosis of invasive pneumococcal disease in children from Mozambique. PLoS One. 2017;12:e0184762. https://doi.org/10.1371/journal.pone.0184762
11. Baggett HC, Watson NL, Deloria Knoll M, Brooks WA, Feikin DR, Hammitt LL, et al. Density of upper respiratory colonization with *Streptococcus pneumoniae* and its role in the diagnosis of pneumococcal pneumonia among children aged <5 years in the PERCH Study. Clin Infect Dis. 2017;64(Suppl_3):S317–S27.
12. Auranen K, Syrjänen R, Leino T, Kilpi T. New pneumococcal carriage acquired in association with acute respiratory infection is prone to cause otitis media. PLoS One. 2016;11:e0156343. https://doi.org/10.1371/journal.pone.0156343
13. Käyhty H, Auranen K, Nohynek H, Dagan R, Mäkelä H. Nasopharyngeal colonization: a target for pneumococcal vaccination. Expert Rev Vaccines. 2006;5:651–67. https://doi.org/10.1586/14760584.5.5.651
14. Wolter N, Tempia S, Cohen C, Madhi SA, Venter M, Moyes J, et al. High nasopharyngeal pneumococcal density, increased by viral coinfection, is associated with invasive pneumococcal pneumonia. J Infect Dis. 2014;210:1649–57. https://doi.org/10.1093/infdis/jiu326
15. Vu HT, Yoshida LM, Suzuki M, Nguyen HA, Nguyen CD, Nguyen AT, et al. Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. Pediatr Infect Dis J. 2011;30:11–8. https://doi.org/10.1097/INF.0b013e3181f111a2
16. Grijalva CG, Griffin MR, Edwards KM, Williams JV, Gil AI, Verastegui H, et al. The role of influenza and parainfluenza infections in nasopharyngeal pneumococcal acquisition among young children. Clin Infect Dis. 2014;58:1369–76. https://doi.org/10.1093/cid/ciu148
17. Weinberger DM, Grant LR, Steiner CA, Weatherholtz R, Santosham M, Viboud C, et al. Seasonal drivers of pneumococcal disease incidence: impact of bacterial carriage and viral activity. Clin Infect Dis. 2014;58:188–94. https://doi.org/10.1093/cid/cit721
18. Ampofo K, Bender J, Sheng X, Korgenski K, Daly J, Pavia AT, et al. Seasonal invasive pneumococcal disease in children: role of preceding respiratory viral infection. Pediatrics. 2008;122:229–37. https://doi.org/10.1542/peds.2007-3192
19. Talbot TR, Poehling KA, Hartert TV, Arbogast PG, Halasa NB, Edwards KM, et al. Seasonality of invasive pneumococcal disease: temporal relation to documented influenza and respiratory syncytial viral circulation. Am J Med. 2005;118:285–91. https://doi.org/10.1016/j.amjmed.2004.09.016
20. Shrestha S, Foxman B, Weinberger DM, Steiner C, Viboud C, Rohani P. Identifying the interaction between influenza and pneumococcal pneumonia using incidence data. Sci Transl Med. 2013;5:191ra84. https://doi.org/10.1126/scitranslmed.3005982
21. Fadlyana E, Dunne EM, Rusmil K, Tarigan R, Sudigdoadi S, Murad C, et al. Risk factors associated with nasopharyngeal carriage and density of *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Staphylococcus aureus* in young children living in Indonesia. Pneumonia (Nathan). 2018;10:14. https://doi.org/10.1186/s41479-018-0058-1
22. Roca A, Bottomley C, Hill PC, Bojang A, Egere U, Antonio M, et al. Effect of age and vaccination with a pneumococcal conjugate vaccine on the density of pneumococcal nasopharyngeal carriage. Clin Infect Dis. 2012;55:816–24. https://doi.org/10.1093/cid/cis554
23. Howard LM, Johnson M, Williams JV, Zhu Y, Gil AI, Edwards KM, et al.; RESPIRA-PERU Group. Respiratory viral detections during symptomatic and asymptomatic periods in young Andean children. Pediatr Infect Dis J. 2015;34:1074–80. https://doi.org/10.1097/INF.0000000000000812
24. Self WH, Williams DJ, Zhu Y, Ampofo K, Pavia AT, Chappell JD, et al. Respiratory viral detection in children and adults: comparing asymptomatic controls and patients with community-acquired pneumonia. J Infect Dis. 2015.
25. Jartti T, Jartti L, Peltola V, Waris M, Ruuskanen O. Identification of respiratory viruses in asymptomatic subjects: asymptomatic respiratory viral infections. Pediatr Infect Dis J. 2008;27:1103–7. https://doi.org/10.1097/INF.0b013e31817e695d
26. Chonmaitree T, Alvarez-Fernandez P, Jennings K, Trujillo R, Marom T, Loeffelholz MJ, et al. Symptomatic and asymptomatic respiratory viral infections in the first year of life: association with acute otitis media development. Clin Infect Dis. 2015;60:1–9. https://doi.org/10.1093/cid/ciu714
27. DeMuri GP, Gern JE, Eickhoff JC, Lynch SV, Wald ER. Dynamics of bacterial colonization With *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* during symptomatic and asymptomatic viral upper respiratory tract

infection. Clin Infect Dis. 2018;66:1045–53. https://doi.org/10.1093/cid/cix941
28. Budge PJ, Griffin MR, Edwards KM, Williams JV, Verastegui H, Hartinger SM, et al.; RESPIRA-PERU Group. A household-based study of acute viral respiratory illnesses in Andean children. Pediatr Infect Dis J. 2014;33:443–7. https://doi.org/10.1097/INF.0000000000000135
29. Chien YW, Vidal JE, Grijalva CG, Bozio C, Edwards KM, Williams JV, et al. Density interactions among *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* in the nasopharynx of young Peruvian children. Pediatr Infect Dis J. 2013;32:72–7. https://doi.org/10.1097/INF.0b013e318270d850
30. Grijalva CG, Griffin MR, Edwards KM, Williams JV, Gil AI, Verastegui H, et al. Cohort profile: the study of respiratory pathogens in Andean children. Int J Epidemiol. 2014;43:1021–30. https://doi.org/10.1093/ije/dyt065
31. Hanke CR, Grijalva CG, Chochua S, Pletz MW, Hornberg C, Edwards KM, et al. Bacterial density, serotype distribution and antibiotic resistance of pneumococcal strains from the nasopharynx of Peruvian children before and after pneumococcal conjugate vaccine 7. Pediatr Infect Dis J. 2016;35:432–9. https://doi.org/10.1097/INF.0000000000001030
32. Howard LM, Johnson M, Gil AI, Griffin MR, Edwards KM, Lanata CF, et al. Molecular epidemiology of rhinovirus detections in young children. Open Forum Infect Dis. 2016;3:ofw001. https://doi.org/10.1093/ofid/ofw001
33. Howard LM, Johnson M, Gil AI, Pekosz A, Griffin MR, Edwards KM, et al.; RESPIRA-PERU Group. A novel real-time RT-PCR assay for influenza C tested in Peruvian children. J Clin Virol. 2017;96:12–6. https://doi.org/10.1016/j.jcv.2017.08.014
34. Wu A, Budge PJ, Williams J, Griffin MR, Edwards KM, Johnson M, et al. Incidence and risk factors for respiratory syncytial virus and human metapneumovirus infections among children in the remote highlands of Peru. PLoS One. 2015;10:e0130233. https://doi.org/10.1371/journal.pone.0130233
35. Poehling KA, Edwards KM, Weinberg GA, Szilagyi P, Staat MA, Iwane MK, et al.; New Vaccine Surveillance Network. The underrecognized burden of influenza in young children. N Engl J Med. 2006;355:31–40. https://doi.org/10.1056/NEJMoa054869
36. Lanata CF, Rudan I, Boschi-Pinto C, Tomaskovic L, Cherian T, Weber M, et al. Methodological and quality issues in epidemiological studies of acute lower respiratory infections in children in developing countries. Int J Epidemiol. 2004;33:1362–72. https://doi.org/10.1093/ije/dyh229
37. Turner P, Turner C, Jankhot A, Helen N, Lee SJ, Day NP, et al. A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand–Myanmar border. PLoS One. 2012;7:e38271. https://doi.org/10.1371/journal.pone.0038271
38. Gritzfeld JF, Cremers AJ, Ferwerda G, Ferreira DM, Kadioglu A, Hermans PW, et al. Density and duration of experimental human pneumococcal carriage. Clin Microbiol Infect. 2014;20:O1145–51. https://doi.org/10.1111/1469-0691.12752
39. Darboe MK, Fulford AJ, Secka O, Prentice AM. The dynamics of nasopharyngeal *Streptococcus pneumoniae* carriage among rural Gambian mother-infant pairs. BMC Infect Dis. 2010;10:195. https://doi.org/10.1186/1471-2334-10-195
40. Högberg L, Geli P, Ringberg H, Melander E, Lipsitch M, Ekdahl K. Age- and serogroup-related differences in observed durations of nasopharyngeal carriage of penicillin-resistant pneumococci. J Clin Microbiol. 2007;45:948–52. https://doi.org/10.1128/JCM.01913-06
41. Chan KF, Carolan LA, Korenkov D, Druce J, McCaw J, Reading PC, et al. Investigating viral interference between influenza A virus and human respiratory syncytial virus in a ferret model of infection. J Infect Dis. 2018;218:406–17. https://doi.org/10.1093/infdis/jiy184
42. Harrell FE. Regression modeling strategies: with applications to linear models, logistic regression, and survival analysis. New York: Springer; 2001.
43. Marrie RA, Dawson NV, Garland A. Quantile regression and restricted cubic splines are useful for exploring relationships between continuous variables. J Clin Epidemiol. 2009;62:511–7.e1. https://doi.org/10.1016/j.jclinepi.2008.05.015
44. Vissers M, Ahout IM, van den Kieboom CH, van der Gaast-de Jongh CE, Groh L, Cremers AJ, et al. High pneumococcal density correlates with more mucosal inflammation and reduced respiratory syncytial virus disease severity in infants. BMC Infect Dis. 2016;16:129. https://doi.org/10.1186/s12879-016-1454-x
45. Weiser JN. The pneumococcus: why a commensal misbehaves. J Mol Med (Berl). 2010;88:97–102. https://doi.org/10.1007/s00109-009-0557-x
46. Collins DA, Hoskins A, Snelling T, Senasinghe K, Bowman J, Stemberger NA, et al. Predictors of pneumococcal carriage and the effect of the 13-valent pneumococcal conjugate vaccination in the Western Australian Aboriginal population. Pneumonia (Nathan). 2017;9:14. https://doi.org/10.1186/s41479-017-0038-x
47. Voynow JA, Rubin BK. Mucins, mucus, and sputum. Chest. 2009;135:505–12. https://doi.org/10.1378/chest.08-0412
48. Fahy JV, Dickey BF. Airway mucus function and dysfunction. N Engl J Med. 2010;363:2233–47. https://doi.org/10.1056/NEJMra0910061
49. Rudan I, Boschi-Pinto C, Biloglav Z, Mulholland K, Campbell H. Epidemiology and etiology of childhood pneumonia. Bull World Health Organ. 2008;86:408–16. https://doi.org/10.2471/BLT.07.048769
50. Morpeth SC, Munywoki P, Hammitt LL, Bett A, Bottomley C, Onyango CO, et al. Impact of viral upper respiratory tract infection on the concentration of nasopharyngeal pneumococcal carriage among Kenyan children. Sci Rep. 2018;8:11030. https://doi.org/10.1038/s41598-018-29119-w

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Rare Detection of *Bordetella pertussis* Pertactin-Deficient Strains in Argentina

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Pertussis resurgence had been attributed to waning vaccine immunity and *Bordetella pertussis* adaptation to escape vaccine-induced immunity. Circulating bacteria differ genotypically from strains used in production of pertussis vaccine. Pertactin-deficient strains are highly prevalent in countries that use acellular vaccine (aP), suggesting strong aP-imposed selection of circulating bacteria. To corroborate this hypothesis, systematic studies on pertactin prevalence of infection in countries using whole-cell vaccine are needed. We provide pertussis epidemiologic data and molecular characterization of *B. pertussis* isolates from Buenos Aires, Argentina, during 2000–2017. This area used primary vaccination with whole-cell vaccine. Since 2002, pertussis case incidences increased at regular 4-year outbreaks; most cases were in infants <1 year of age. Of the *B. pertussis* isolates analyzed, 90.6% (317/350) contained the *ptx*P3-*ptx*A1-*prn*2-*fim*3-2 allelic profile. Immunoblotting and sequencing techniques detected only the 2 pertactin-deficient isolates. The low prevalence of pertactin-deficient strains in Argentina suggests that loss of pertactin gene expression might be driven by aP vaccine.

Vaccination against pertussis is mandatory worldwide. Two types of vaccines are currently in use: whole-cell vaccine (wP), which was the first vaccine developed, and acellular vaccine (aP), which contains purified components of *Bordetella pertussis* and was formulated because of adverse reactions associated with wP (*1*). Many countries continue to use wP for the primary vaccination series and for boosters recommended for children <7 years of age. Industrialized countries have switched to vaccination with aP. However, in the past 2 decades, the number of pertussis cases detected increased to ≈24.1 million/year; ≈161,000 deaths occurred during 2014 (*2*,*3*). Although most cases occur in developing countries, industrialized countries have also had large-scale outbreaks, even nations with high vaccination rates (*2*,*4*–*6*).

The main causes proposed for this changing pertussis epidemiology are vaccination coverage rates lower than the 90% recommended by the World Health Organization, waning of vaccine-induced immunity (*7*,*8*) (which occurs faster in the aP-vaccinated population), and evolution of circulating bacteria to vaccine immunity–evasive phenotypes (*9*,*10*). The first reports on bacterial evolution documented genetic polymorphisms encoding proteins included in the vaccines (e.g., pertactin [PRN], pertussis toxin, and the pertussis toxin promoter [*ptx*P]) (*11*,*12*). More recently, a major increase in the isolation of *B. pertussis* bacteria that do not express certain vaccine antigens was reported (*10*,*13*,*14*). In countries using PRN-containing aP vaccines, such as the United States, Canada, and Australia, the PRN-deficient isolates have increased substantially in the past 4 years (*10*,*15*,*16*). The expansion of strains deficient in PRN in populations vaccinated with PRN-containing aP vaccines indicates that such strains apparently have a selective advantage in populations vaccinated with aP vaccine (*17*).

To corroborate this hypothesis, we conducted systematic studies on PRN prevalence in Argentina, a country that uses wP vaccine. We monitored and analyzed *B. pertussis* population dynamics in Buenos Aires. Our aim was to assess whether PRN-deficient strains were circulating in Buenos Aires and to analyze the results obtained in relation to the vaccine used and the epidemiologic situation of the disease during 2000–2017.

Materials and Methods

Population Studied, Clinical Case Definition, and Laboratory Diagnosis

We used pertussis epidemiologic data and samples collected during 2000–2017 from the Pertussis Reference

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Laboratory (La Plata, Argentina). We collected data on patient sex, age, duration of symptoms, vaccination status, and laboratory results.

We confirmed clinical cases of pertussis in patients by isolation of *B. pertussis* culture, amplification of *B. pertussis*–specific DNA by using PCR, or serologic analysis (IgG titer to pertussis toxin >120 IU/mL). We defined a confirmed case of pertussis as one that meets the clinical case definition and is epidemiologically linked to a laboratory-confirmed case (*18*–*20*).

Vaccine Schedule in Buenos Aires

The wP vaccine was introduced in Argentina (population 44.9 million) during the 1970s and is still used for the 3 primary doses at 2, 4, and 6 months of age; for the 2 boosters at 18 months of age; and at school entry (5–6 years of age) in the public sector (≈90% of the population). The aP vaccine is used in the private sector and for the recommended boosters in adolescents, healthcare workers in contact with infants <12 months of age, household contacts of low-birthweight infants, and pregnant women.

In most of Argentina, the diphtheria–tetanus–pertussis vaccine is used as a third dose; coverage during recent years ranged from 91.0% to 95.0%; in certain jurisdictions, this value was <80.0% (*21*). Official coverage rates for adolescent boosters were 75.3% during 2015, 81.9% during 2016, and 88.0% during 2017. Official coverage rates for maternal immunization were 61.7% during 2015, 65.6% during 2016, and 67.0% during 2017.

Samples and Bacterial Growth Conditions

The Pertussis Reference Laboratory samples included nasopharyngeal specimens from 16,151 hospitalized patients from Buenos Aires with signs/symptoms of pertussis infection. These samples were routinely screened for *B. pertussis* by culture and PCR. *B. pertussis* culture was performed on Regan-Lowe agar (Difco, https://www.fishersci.com) supplemented with 15% (vol/vol) defibrinated fresh sheep blood at 36.5°C and monitored for 10 days. Suspected colonies were replicated in Bordet-Gengou agar (Difco) supplemented with 15% (vol/vol) defibrinated fresh sheep blood. Colonies exhibiting hemolysis were gram-stained and tested by using agglutination with *B. pertussis*–specific antiserum (Murex Diagnostic Products, https:///www.murex-ph.com) and PCR (*22*,*23*). The isolates were also biochemically typed by using the API-20-NE system (bioMérieux, https://www.biomerieux.com).

Isolates were stored at –80°C in 1% (wt/vol) casamino-acid solution containing 20% (vol/vol) glycerol. *B. pertussis* strain Tohama phase I (Collection de l'Institut Pasteur) was also grown on Bordet-Gengou agar at 36.5°C for 72 h.

B. pertussis Isolate Characterization

Genotyping

A total of 350 *B. pertussis* isolates collected in Buenos Aires during January 2000–December 2017 were included in the analyses (Table 1). For genetic typing, we amplified *ptx*P, pertussis toxin A subunit (*ptx*A), *prn*, and fimbriae type 3 (*fim*3) loci by using PCR with specific primers (Table 2) and sequenced them as described (*24*–*32*). We also screened isolates for an array of mutations causing deficiency in the immunogen PRN by using PCR amplification and molecular sequencing (*26*,*27*). We used primers 5′-CCCATTCTTCCCTGTTCCAT-3′ and 5′-CCTGAGCCTGGAGACTGG-3′ (*27*) to amplify the complete *prn* gene (*27*), and these primers in combination with internal primers to sequence the complete gene.

We calculated by year the discriminatory power of the multilocus sequence typing technique by using the equation reported by Hunter and Gaston (*33*). This equation is based on the probability that 2 unrelated strains sampled from the test population will be placed into different typing groups. Thus, the index can have any value between 0 and 1, with 0 representing the lowest discriminatory capacity, indicating that all the strains are in a single genotyping group (low diversity), and 1 representing the highest discriminatory capacity, indicating high genotypic diversity among the isolates.

PRN Immunoblotting

For this assay, we treated 2 ×10^{10} CFUs of *B. pertussis* isolates with Laemmli sample buffer, and subjected extracts to electrophoresis on 12.5% (wt/vol) sodium dodecyl sulfate–polyacrylamide gels. After electrophoresis, we transferred the proteins to a polyvinylidene phosphate membrane (Immobilon P; Millipore, http://www.emdmillipore.com) and incubated with a 1:2,500 dilution of PRN-specific polyclonal immune serum. This serum was obtained from BALB/c mice immunized with purified *B. pertussis* 69-kDa PRN (National Institute for Biological Standards and Control [NIBSC] code no. 90/654 version 4). We used alkaline phosphatase–labeled sheep anti-mouse immunoglobulins for detecting immune complexes and nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as phosphatase chromogenic substrates (Biodynamics SRL, https://www.biodynamics.com.ar). The *B. pertussis* Tohama strain served as a PRN-positive control.

Serotype Analysis

We performed serotype analysis by using an agglutination assay with monoclonal antibodies (mABs) against fimbriae type 2 (anti-Fim2 mAb; NIBSC code no. 04/154) and fimbriae type 3 (anti-Fim3 mAb, NIBSC code no. 04/156). These analyses followed European Union laboratory group recommendations (*34*).

Table 1. Immunization status of patients infected with *Bordetella pertussis* strains studied, Argentina, 2000–2017*

		Patient information						
		<2 mo of age	<6 mo of age		6–12 mo of age		>12 mo of age	
Year	No. strains	Unvaccinated because of age	Incomplete vaccination schedule	Complete vaccination schedule	Incomplete vaccination schedule	Complete vaccination schedule	Incomplete vaccination schedule	Complete vaccination schedule
2000	7	3	1	1	2	0	0	0
2001	7	2	1	1	3	0	0	0
2002	5	3	1	1	0	0	0	0
2003	9	4	2	2	1	0	0	0
2004	9	3	2	2	2	0	0	0
2005	6	3	1	1	1	0	0	0
2006	6	0	3	0	3	0	0	0
2007	38	10	10	9	6	3	0	0
2008	45	21	10	6	4	0	4	0
2009	7	3	2	2	0	0	0	0
2010	6	4	2	0	0	0	0	0
2011	86	40	20	16	2	4	0	4
2012	59	20	15	10	0	9	5	0
2013	6	3	0	0	0	3	0	0
2014	3	0	2	0	0	1	0	0
2015	6	3	1	1	0	0	1	0
2016	32	12	1	8	2	0	1	8
2017	13	3	1	1	0	2	0	6

*Complete vaccination schedule indicates that a person according to his or her age received the total number of doses indicated in the National Vaccination Calendar. Incomplete vaccination schedule indicates that a person according to his or her age did not receive the total number of doses indicated in the National Vaccine Calendar.

Results

Pertussis Epidemiology in Buenos Aires

During 2000–2017, the Pertussis Reference Laboratory received 75% of total clinical samples (nasopharyngeal swab samples) from pertussis-suspected case-patients identified in Buenos Aires and reported to the Ministry of Health. Of these 16,151 samples, 3,220 (19.9%) were from laboratory-confirmed cases. A total of 2,870 samples were positive by PCR for *B.* pertussis–specific genes, and 350 samples were positive by PCR and culture.

The provincial cases per year distribution reflected the pattern of the entire country; 3 outbreaks were detected, in 2008, 2011, and 2016 (Figure 1). In each year of the period analyzed, most cases were detected in infants <1–2 months of age and those >2–4 months of age (Figure 2). The high proportion of cases recorded in patients <6 months of age was expected because pertussis is most severe in that age group.

We obtained the distribution of confirmed pertussis cases by patient age and vaccination status. Of confirmed cases, data were complete for 72.6% (2,338/3,220) of vaccinated persons and for 26.5% (619/2,338) of nonvaccinated persons <2 months of age. For infants <6 months of age, 45.3% had complete age-specific vaccination schedules. The percentage of patients with complete schedules was 53.7% for children >6 months of age and 6.4% for adolescents >11 years of age. Although this percentage for adolescents was low, this age group contained considerably fewer persons than those <6 months of age (44 infants vs. 1,590 children >6 months of age).

B. pertussis Genotyping

Almost all (99.7%) *B. pertussis* isolates analyzed contained the *ptx*A1 and *prn*2 alleles. Clinical isolates obtained during 2000–2004 had up to 4 different multilocus sequence typing genotypes (Figure 3). The index of discrimination calculated by year for this period ranged from 0.25 to 0.80. The highest value (higher diversity) was detected in 2000. The *ptx*P1 or *ptx*P4 variants were detected before 2004; thereafter, the *ptx*P3 locus prevailed. Most (291/350, 83.1%) isolates obtained after 2004 had the *ptx*P3-*ptx*A1-*prn*2-*fim*3–2 genotype. For 2004–2017, the index of

Table 2. Primers used in PCR for *Bordetella pertussis* strains studied, Argentina, 2000–2017*

Gene†	Primer sequence, 5′→3′	Reference(s)
*ptx*P	F: AATCGTCCTGCTCAACCGCC R: GGTATACGGTGGCGGGAGGA	(*27*,*28*)
*ptx*A	F: CCCCTGCCATGGTGTGATC R: TCAATTACCGGAGTTGGGCG	(*29*)
prn	F: CAATGTCACGGTCCAA R: GCAAGGTGATCGACAGGG	(*26*)
fim3	F: GACCTGATATTCTGATGCCG R: AAGGCTTGCCGGTTTTTTTTGG	(*31*)

*F, forward; R, reverse.
†*fim*3, fimbriae type 3; *prn*, pertactin; *ptx*A, pertussis toxin subunit A, *ptx*P, pertussis toxin promoter.

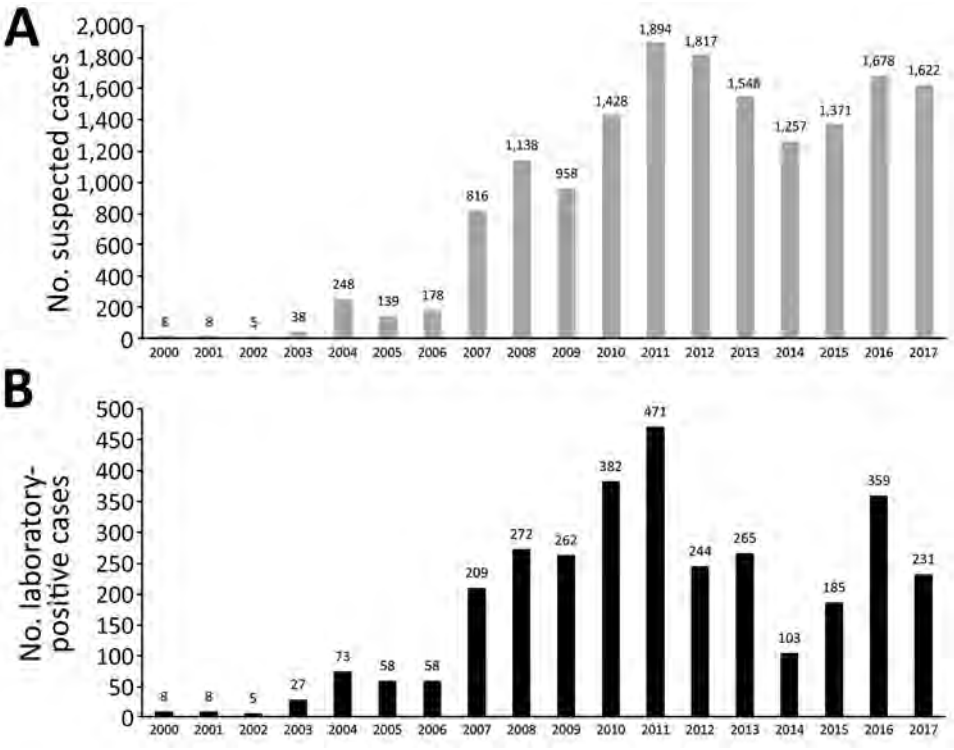

Figure 1. Pertussis cases per year reported to the Pertussis Reference Laboratory, Buenos Aires, Argentina, 2000–2017. A) No. suspected cases. B) No. laboratory-positive cases. Numbers above the bars indicate actual values.

discrimination ranged from 0 to 0.24, indicating the lowest diversity detected.

Fimbriae Serotyping

Of 350 isolates tested, only 1 obtained during 2016 was classified as Fim2. The remaining isolates were classified as Fim3.

PRN Immunoblots

Only 2 of the *B. pertussis* isolates included in this study were PRN deficient. Both strains were obtained from patients <1 year of age who had typical pertussis signs/symptoms. These 2 case-patients were linked in time (2016) but not geographically. One of these patients was born to a mother vaccinated with a PRN-containing aP vaccine and the other to a nonvaccinated mother. For these 2 strains, we detected insertion sequence 481 sequence (forward sense) at position 1613–1614 of *prn*, which disrupted the gene.

Discussion

We conducted molecular genetic characterization of 350 *B. pertussis* isolates obtained during 2000–2017 from hospitalized patients in Buenos Aires, Argentina. Buenos Aires, similar to the entire country of Argentina, uses only wP vaccine for primary series of pertussis vaccinations. Most *B. pertussis* isolates were obtained during the outbreaks detected during 2007–2008 (83), 2011–2012 (145), and 2016–2017 (45). A total of 78% of isolates were obtained from patients <6 months of age, 13.7% from patients 6–12 months of age, and 8.3% from patients >12 months of age. As expected, most *B. pertussis* isolates were from unvaccinated persons. As detected in other countries, we found that almost all isolates characterized had the Fim3 serotype (*35*).

Of 350 isolates, variants *ptx*P1 and *ptx*P4 and the allele *prn*1 were detected before 2004. Starting in 2004, a total of 313 isolates obtained had the *ptx*P3-*ptx*A1-*prn*2 alleles with either *fim*3–1 or *fim*3–2. These genotypes differed from those of the vaccine-production strains (*36*) and were the most common in other countries that had vaccinated populations (*35*).

The polymorphism in PRN and subsequent spread of PRN-deficient isolates have elicited a deep concern in the healthcare system because these changes hypothetically might represent a selective avoidance by the bacteria of the immunity induced by the vaccines. The predominance of *prn*2 detected in more recent isolates from Buenos Aires is consistent with the hypothesis that strains in the vaccinated population with that allele are fitter than those harboring other *prn* alleles (*37*).

Regarding a deficiency in PRN expression, we detected only 2 isolates containing insertion sequence 481 in the coding region of *prn*. These isolates were obtained from infants <1 year of age that were linked in time (2016) but not location. One of these patients was born to a mother vaccinated with a PRN-containing aP vaccine and the other patient was born to a nonvaccinated mother.

There are no reports of pertactin deficiency in a country such as Argentina that includes wP vaccine as the only vaccine for primary pertussis vaccination. Recently,

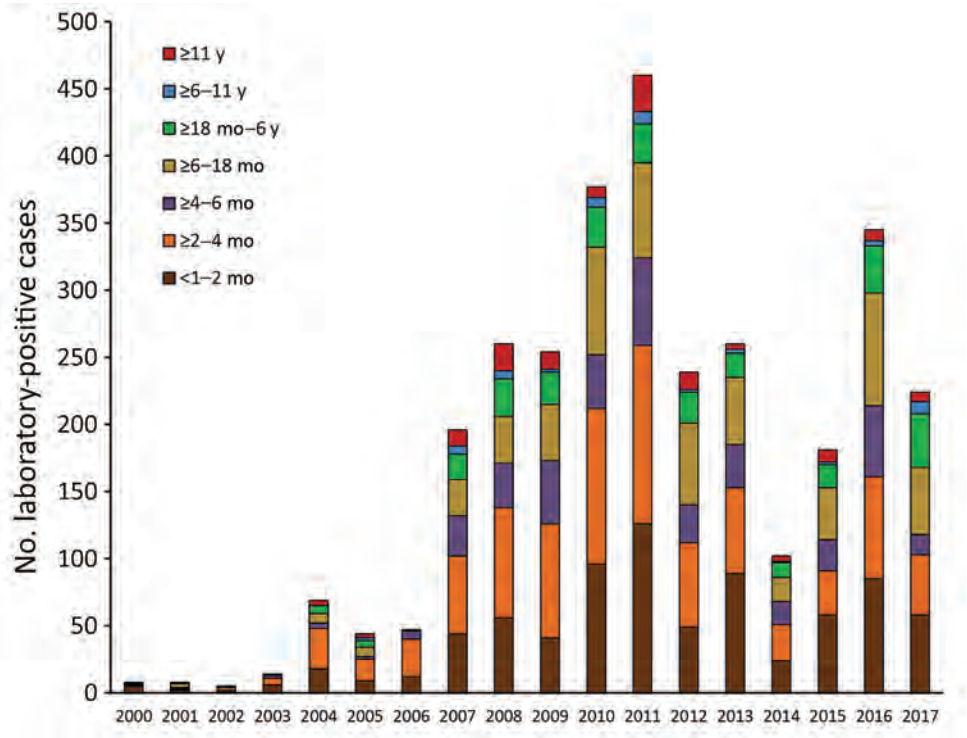

Figure 2. Number of laboratory-positive pertussis cases for 7 age cohorts, Buenos Aires, Argentina, 2000–2017.

Poland, the only country in Europe that still uses the wP vaccine but also the aP vaccine for primary series, has reported detection of PRN-deficient clinical isolates (*38*). The percentage of those isolates was lower (15%) than that detected in the United States, Canada, or Australia (>65%), which only use aP vaccines (*10*,*39*,*40*). Detection of these isolates might be a consequence of the increase in the use of aP vaccines in Poland. Within this context, our study is apparently unique because Argentina uses only wP vaccine for the primary series of pertussis vaccinations.

This low frequency of PRN-deficient strains in regions where wP vaccine is still used supports the hypothesis that PRN-deficient clinical isolates have an advantage in aP vaccine–primed immunity (*41*). Accordingly, PRN-deficient clinical isolates were able to overcome an anti-PRN–mediated inhibition of macrophage cytotoxicity in vitro (*42*). Moreover, this study also showed that recently collected PRN-deficient *B. pertussis* clinical isolates harboring a *ptx*P3 variant and the *prn*2 allele had higher CFUs per lung and were capable of sustaining infection longer in aP vaccine–immunized mice than isolates still producing

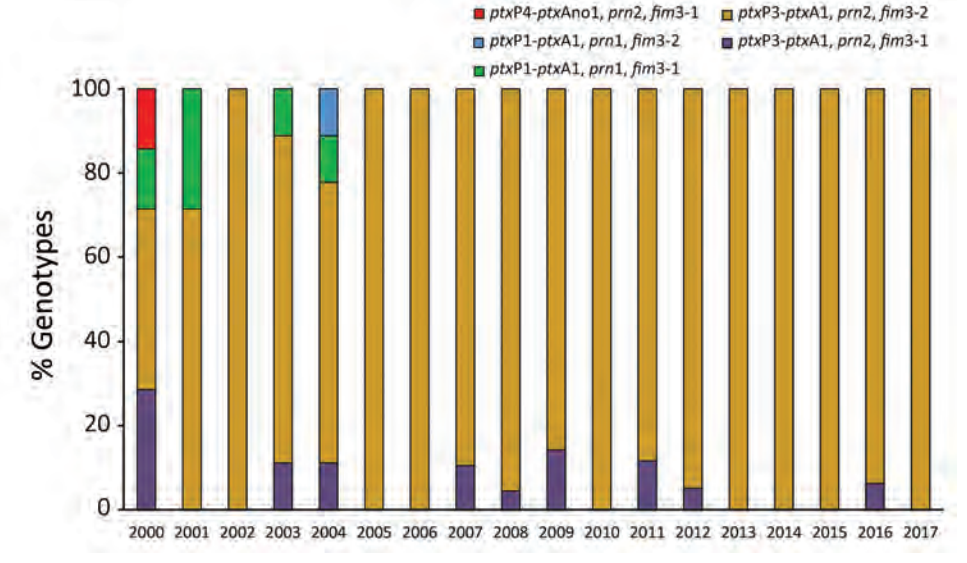

Figure 3. Percentage of multilocus sequence typing genotypes of *Bordetella pertussis* among isolates collected in Buenos Aires, Argentina, 2000–2017. *fim*, fimbriae; *prn*, pertactin; *ptx*A, pertussis toxin subunit A, *ptx*P, pertussis toxin promoter.

the protein. The investigators of that study speculated that these particular isolates might be capable of infecting immunized persons at an earlier stage of waning immunity after aP vaccine immunization or postinfection, thus having an advantage over isolates producing PRN. The findings of Hegerle et al. (*42*) are consistent with those recently reported by Safarchi et al. (*17*), which indicated a higher fitness of Prn-negative strains in aP vaccine–immunized mice. These investigators demonstrated in a mixed-infection model that PRN-negative *B. pertussis* colonized the respiratory tract of aP-immunized mice more effectively than the PRN-positive strain, thus outcompeting that strain (*17*).

Regarding a possible association between clinical findings and the PRN expression of the bacterial isolates that caused the human infections, recent studies suggest that symptoms (with the exception of apneas, which were less likely in PRN-deficient infections) and clinical course were similar regardless of PRN expression (*14*,*41*). Clarke et al. provide new data on this subject, which suggest that rapid emergence of PRN-deficient *B. pertussis* variants is unlikely to contribute to any greater risk for death or severe outcomes from infections in young, vulnerable infants (*43*).

Our study supports the hypothesis regarding pathogen adaptation of *B. pertussis* to the type of vaccine used. A key finding in this study was that use of the wP vaccine in the primary series of vaccinations correlated with a near complete absence of PRN-deficient strains, although the aP vaccine was used in subsequent vaccine regimens. Continued surveillance for PRN production in circulating *B. pertussis* is needed, as well as monitoring of other possible genotypic changes in the *B. pertussis* population, including a lack of expression of other immunogens contained in acellular vaccines.

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D.H. planned the study, interpreted data, and edited the figures and manuscript; D.B. interpreted data and edited the figures and manuscript; F.C., V.R., P.M.A., G.M., and E.B. performed experiments and laboratory analyses. All authors approved the final version of the manuscript.

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References

1. Stephenson JB. Reactions to whooping cough vaccine. BMJ. 1979;2:933. https://doi.org/10.1136/bmj.2.6195.933
2. Pertussis vaccines: WHO position paper. Wkly Epidemiol Rec. 2010;85:385–400.
3. Yeung KH, Duclos P, Nelson EA, Hutubessy RC. An update of the global burden of pertussis in children younger than 5 years: a modelling study. Lancet Infect Dis. 2017;17:974–80. https://doi.org/10.1016/S1473-3099(17)30390-0
4. Clark TA. Changing pertussis epidemiology: everything old is new again. J Infect Dis. 2014;209:978–81. https://doi.org/10.1093/infdis/jiu001
5. Vizzotti C, Juarez MV, Bergel E, Romanin V, Califano G, Sagradini S, et al. Impact of a maternal immunization program against pertussis in a developing country. Vaccine. 2016;34:6223–8. https://doi.org/10.1016/j.vaccine.2016.10.081
6. Falleiros Arlant LH, de Colsa A, Flores D, Brea J, Avila Aguero ML, Hozbor DF. Pertussis in Latin America: epidemiology and control strategies. Expert Rev Anti Infect Ther. 2014;12:1265–75. https://doi.org/10.1586/14787210.2014.948846
7. Wendelboe AM, Van Rie A, Salmaso S, Englund JA. Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J. 2005;24(Suppl):S58–61. https://doi.org/10.1097/01.inf.0000160914.59160.41
8. McGirr A, Fisman DN. Duration of pertussis immunity after DTaP immunization: a meta-analysis. Pediatrics. 2015;135:331–43. https://doi.org/10.1542/peds.2014-1729
9. Mooi FR, Van Der Maas NA, De Melker HE. Pertussis resurgence: waning immunity and pathogen adaptation: two sides of the same coin. Epidemiol Infect. 2014;142:685–94. https://doi.org/10.1017/S0950268813000071
10. Lam C, Octavia S, Ricafort L, Sintchenko V, Gilbert GL, Wood N, et al. Rapid increase in pertactin-deficient *Bordetella pertussis* isolates, Australia. Emerg Infect Dis. 2014;20:626–33. https://doi.org/10.3201/eid2004.131478
11. Advani A, Gustafsson L, Ahrén C, Mooi FR, Hallander HO. Appearance of Fim3 and *ptx*P3-*Bordetella pertussis* strains, in two regions of Sweden with different vaccination programs. Vaccine. 2011;29:3438–42. https://doi.org/10.1016/j.vaccine.2011.02.070
12. Kallonen T, Mertsola J, Mooi FR, He Q. Rapid detection of the recently emerged *Bordetella pertussis* strains with the *ptx*P3 pertussis toxin promoter allele by real-time PCR. Clin Microbiol Infect. 2012;18:E377–9. https://doi.org/10.1111/j.1469-0691.2012.04000.x
13. Hegerle N, Guiso N. Bordetella pertussis and pertactin-deficient clinical isolates: lessons for pertussis vaccines. Expert Rev Vaccines. 2014;13:1135–46. https://doi.org/10.1586/14760584.2014.932254
14. Bodilis H, Guiso N. Virulence of pertactin-negative *Bordetella pertussis* isolates from infants, France. Emerg Infect Dis. 2013;19:471–4. https://doi.org/10.3201/eid1903.121475
15. Pawloski LC, Queenan AM, Cassiday PK, Lynch AS, Harrison MJ, Shang W, et al. Prevalence and molecular characterization of pertactin-deficient *Bordetella pertussis* in the United States. Clin Vaccine Immunol. 2014;21:119–25. https://doi.org/10.1128/CVI.00717-13
16. Tsang RS, Shuel M, Jamieson FB, Drews S, Hoang L, Horsman G, et al. Pertactin-negative *Bordetella pertussis* strains in Canada: characterization of a dozen isolates based on a survey of 224 samples collected in different parts of the country over the last 20 years. Int J Infect Dis. 2014;28:65–9. https://doi.org/10.1016/j.ijid.2014.08.002
17. Safarchi A, Octavia S, Luu LD, Tay CY, Sintchenko V, Wood N, et al. Pertactin negative *Bordetella pertussis* demonstrates higher fitness under vaccine selection pressure in a mixed infection model. Vaccine. 2015;33:6277–81. https://doi.org/10.1016/j.vaccine.2015.09.064

18. Centers for Disease Control and Prevention. Pertussis/whooping Cough (*Bordetella pertussis*). Case definition; 2014 [cited 2019 Aug 4]. https://wwwn.cdc.gov/nndss/conditions/pertussis/case-definition/2014
19. World Health Organization. WHO-recommended surveillance standard of pertussis [cited 2019 Aug 4]. https://wwwwhoint/immunization/monitoring_surveillance/burden/vpd/surveillance_type/passive/pertussis_standards/en
20. Argentina Ministry of Health. Whopping cough and convulsions of pertussis. Case definitions [in Spanish] [cited 2019 Aug 5]. http://www.msal.gob.ar/images/stories/pdf/coqueluche-recomendaciones-definiciones.pdf
21. Gentile A. *Bordetella pertussis* infection [in Spanish]. Arch Argent Pediatr. 2010;108:78–81.
22. Grimprel E, Bégué P, Anjak I, Betsou F, Guiso N. Comparison of polymerase chain reaction, culture, and western immunoblot serology for diagnosis of *Bordetella pertussis* infection. J Clin Microbiol. 1993;31:2745–50.
23. Hozbor D, Fouque F, Guiso N. Detection of *Bordetella bronchiseptica* by the polymerase chain reaction. Res Microbiol. 1999;150:333–41. https://doi.org/10.1016/S0923-2508(99)80059-X
24. Schouls LM, van der Heide HG, Vauterin L, Vauterin P, Mooi FR. Multiple-locus variable-number tandem repeat analysis of Dutch *Bordetella pertussis* strains reveals rapid genetic changes with clonal expansion during the late 1990s. J Bacteriol. 2004;186:5496–505. https://doi.org/10.1128/JB.186.16.5496-5505.2004
25. Mooi FR, van Loo IH, van Gent M, He Q, Bart MJ, Heuvelman KJ, et al. *Bordetella pertussis* strains with increased toxin production associated with pertussis resurgence. Emerg Infect Dis. 2009;15:1206–13. https://doi.org/10.3201/eid1508.081511
26. Fiett J, Letowska I, Gniadkowski M, Hryniewicz W. The new strategy for allele identification of the genes coding for pertussis toxin subunit S1 (*ptx*S1) and pertactin (*prn*) in *Bordetella pertussis*. J Microbiol Methods. 2003;55:651–66. https://doi.org/10.1016/S0167-7012(03)00207-0
27. Mooi FR, Hallander H, Wirsing von König CH, Hoet B, Guiso N. Epidemiological typing of *Bordetella pertussis* isolates: recommendations for a standard methodology. Eur J Clin Microbiol Infect Dis. 2000;19:174–81. https://doi.org/10.1007/s100960050455
28. Borisova O, Kombarova SY, Zakharova NS, van Gent M, Aleshkin VA, Mazurova I, et al. Antigenic divergence between *Bordetella pertussis* clinical isolates from Moscow, Russia, and vaccine strains. Clin Vaccine Immunol. 2007;14:234–8. https://doi.org/10.1128/CVI.00294-06
29. Advani A, Donnelly D, Hallander H. Reference system for characterization of *Bordetella pertussis* pulsed-field gel electrophoresis profiles. J Clin Microbiol. 2004;42:2890–7. https://doi.org/10.1128/JCM.42.7.2890-2897.2004
30. Bottero D, Gaillard ME, Fingermann M, Weltman G, Fernández J, Sisti F, et al. Pulsed-field gel electrophoresis, pertactin, pertussis toxin S1 subunit polymorphisms, and surfaceome analysis of vaccine and clinical *Bordetella pertussis* strains. Clin Vaccine Immunol. 2007;14:1490–8. https://doi.org/10.1128/CVI.00177-07
31. van Loo IH, Heuvelman KJ, King AJ, Mooi FR. Multilocus sequence typing of *Bordetella pertussis* based on surface protein genes. J Clin Microbiol. 2002;40:1994–2001. https://doi.org/10.1128/JCM.40.6.1994-2001.2002
32. Hardwick TH, Plikaytis B, Cassiday PK, Cage G, Peppler MS, Shea D, et al. Reproducibility of *Bordetella pertussis* genomic DNA fragments generated by *Xba*I restriction and resolved by pulsed-field gel electrophoresis. J Clin Microbiol. 2002;40:811–6. https://doi.org/10.1128/JCM.40.3.811-816.2002
33. Hunter PR, Gaston MA. Numerical index of the discriminatory ability of typing systems: an application of Simpson's index of diversity. J Clin Microbiol. 1988;26:2465–6.
34. Network E-Ls. External quality assurance scheme for *Bordetella* identification and *B. pertussis* typing [cited 2019 Aug 4]. https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/EQA-pertussis-Bordetella-ID-B-typing.pdf
35. Barkoff AM, Mertsola J, Pierard D, Dalby T, Hoegh SV, Guillot S, et al. Surveillance of circulating *Bordetella pertussis* strains in Europe during 1998 to 2015. J Clin Microbiol. 2018;56:e01998-17. https://doi.org/10.1128/JCM.01998-17
36. Bottero D, Gaillard ME, Basile LA, Fritz M, Hozbor DF. Genotypic and phenotypic characterization of *Bordetella pertussis* strains used in different vaccine formulations in Latin America. J Appl Microbiol. 2012;112:1266–76. https://doi.org/10.1111/j.1365-2672.2012.05299.x
37. van Gent M, van Loo IH, Heuvelman KJ, de Neeling AJ, Teunis P, Mooi FR. Studies on Prn variation in the mouse model and comparison with epidemiological data. PLoS One. 2011;6:e18014. https://doi.org/10.1371/journal.pone.0018014
38. Polak M, Zasada AA, Mosiej E, Krysztopa-Grzybowska K, Witkowski L, Rzeczkowska M, et al. Pertactin-deficient *Bordetella pertussis* isolates in Poland: a country with whole-cell pertussis primary vaccination. Microbes Infect. 2018;Dec 21:pii: S11286-4579(18)30193-X.
39. Quinlan T, Musser KA, Currenti SA, Zansky SM, Halse TA. Pertactin-negative variants of *Bordetella pertussis* in New York State: a retrospective analysis, 2004–2013. Mol Cell Probes. 2014;28:138–40. https://doi.org/10.1016/j.mcp.2013.12.003
40. Queenan AM, Cassiday PK, Evangelista A. Pertactin-negative variants of *Bordetella pertussis* in the United States. N Engl J Med. 2013;368:583–4. https://doi.org/10.1056/NEJMc1209369
41. Martin SW, Pawloski L, Williams M, Weening K, DeBolt C, Qin X, et al. Pertactin-negative *Bordetella pertussis* strains: evidence for a possible selective advantage. Clin Infect Dis. 2015;60:223–7. https://doi.org/10.1093/cid/ciu788
42. Hegerle N, Dore G, Guiso N. Pertactin deficient *Bordetella pertussis* present a better fitness in mice immunized with an acellular pertussis vaccine. Vaccine. 2014;32:6597–600. https://doi.org/10.1016/j.vaccine.2014.09.068
43. Clarke M, McIntyre PB, Blyth CC, Wood N, Octavia S, Sintchenko V, et al. The relationship between *Bordetella pertussis* genotype and clinical severity in Australian children with pertussis. J Infect. 2016;72:171–8. https://doi.org/10.1016/j.jinf.2015.11.004

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Molecular and Clinical Comparison of Enterovirus D68 Outbreaks among Hospitalized Children, Ohio, USA, 2014 and 2018

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Enterovirus D68 (EV-D68) causes respiratory tract infections and neurologic manifestations. We compared the clinical manifestations from 2 EV-D68 outbreaks in 2014 and 2018 and a low-activity period in 2016 among hospitalized children in central Ohio, USA, and used PCR and sequencing to enable phylogenetic comparisons. During both outbreak periods, infected children had respiratory manifestations that led to an increase in hospital admissions for asthma. The 2018 EV-D68 outbreak appeared to be milder in terms of respiratory illness, as shown by lower rates of pediatric intensive care unit admission. However, the frequency of severe neurologic manifestations was higher in 2018 than in 2014. During the same period in 2016, we noted neither an increase in EV-D68 nor a significant increase in asthma-related admissions. Phylogenetic analyses showed that EV-D68 isolates from 2018 clustered differently within clade B than did isolates from 2014 and are perhaps associated with a different EV-D68 subclade.

Enterovirus D68 (EV-D68) was originally identified in 1962 in children with severe respiratory tract infections in California, USA (*1*). The virus shares biological features with enteroviruses and rhinoviruses and was reported sporadically after these initial reports (*2*). However, EV-D68 gained epidemiologic and clinical relevance in 2014 after it was identified as an important pathogen associated with severe lower respiratory tract infections and, in some cases, with central nervous system disease (i.e., acute flaccid myelitis [AFM]) (*3*–*5*).

Nationwide Children's Hospital (NCH) in Columbus, Ohio, USA, experienced a first outbreak of EV-D68 in 2014 that was associated with respiratory distress and disproportionately affected children with asthma; no case of AFM was identified (*3*). Although EV-D68 reportedly has a biennial seasonality, NCH did not have an EV-D68 outbreak in 2016. EV-D68 emerged again in 2018 and caused respiratory infections and, in some cases, neurologic manifestations.

The objective of this study was to compare differences in clinical characteristics and disease severity among children hospitalized with EV-D68 infection at NCH in 2018 with those identified during the 2014 outbreak and during a low-activity period (2016). We also sought to define the overlap between EV-D68 circulation and hospitalizations for asthma and compare the sequence variation of EV-D68 strains identified during the 2018 outbreak with strains identified in previous years.

Materials and Methods

Sample Collection and Testing Algorithms

We identified children hospitalized at NCH who had EV-D68 infection during the 2018 outbreak and during a non-outbreak period (2016) and compared their clinical manifestations and characteristics with those identified during the 2014 outbreak as previously described (*3*). In brief, during June 1–October 19, 2018, we collected nasopharyngeal samples using flock swabs that went into viral transport media. All samples were obtained in accordance with standard of care for patients <21 years of age who tested positive for rhinovirus/enterovirus (RV/EV) on the FilmArray Respiratory Panel version 1.7 (*6*) and were stored at –80°C for further testing. We retrospectively identified samples

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from 2016, collected during the same period, and retrieved them from –80°C storage for testing. After excluding duplicates, we selected samples on the basis of availability, amount of remnant, and integrity for EV-D68 testing using a laboratory-developed real-time reverse transcription PCR (rRT-PCR) targeting the 5′ nontranslated region of the human enterovirus genome as described (*3*). Because we conducted EV-D68 testing after patient encounters, results were not available to the treating physician.

The sampling selection criteria differed between the 2 outbreak periods (Figure 1). During 2014, we screened a smaller set of samples for EV-D68 and focused on hospitalized patients. In 2018, we screened samples from both outpatients and inpatients. However, for both periods the clinical analyses focused on hospitalized patients only.

We reviewed electronic healthcare records from patients positive for EV-D68 for data collection. Patients in whom EV-D68 was identified but for whom clinical data were not available, those evaluated in the outpatient setting, those ≥21 years of age, and patients who acquired RV/EV infection during hospitalization were excluded from analyses. We compared differences in clinical characteristics and disease severity parameters among patients from the 2018 EV-D68 outbreak, those identified in 2016, and patients during the 2014 outbreak. The clinical characteristics of patients during the 2014 outbreak were previously reported (*3*). The Institutional Review Board of NCH approved the study.

Admissions for Asthma

We retrieved data related to admissions for asthma during the same time periods in 2014, 2016, and 2018 from the electronic data warehouse. We included patients <21 years of age who were hospitalized with an asthma diagnosis in any NCH inpatient unit. We used the following codes from the International Classification Diseases, Ninth (ICD-9) or Tenth (ICD-10) Revision, for asthma: ICD-9, 493.*; ICD-10, J45.20–J45.998.

EV-D68 rRT-PCR Testing and Sequencing

For EV-D68 detection and quantitation, we used a laboratory-developed rRT-PCR as described previously (*3*). We selected a subset of EV-D68–positive samples for partial viral protein (VP) 1 gene sequencing of an 805-bp PCR

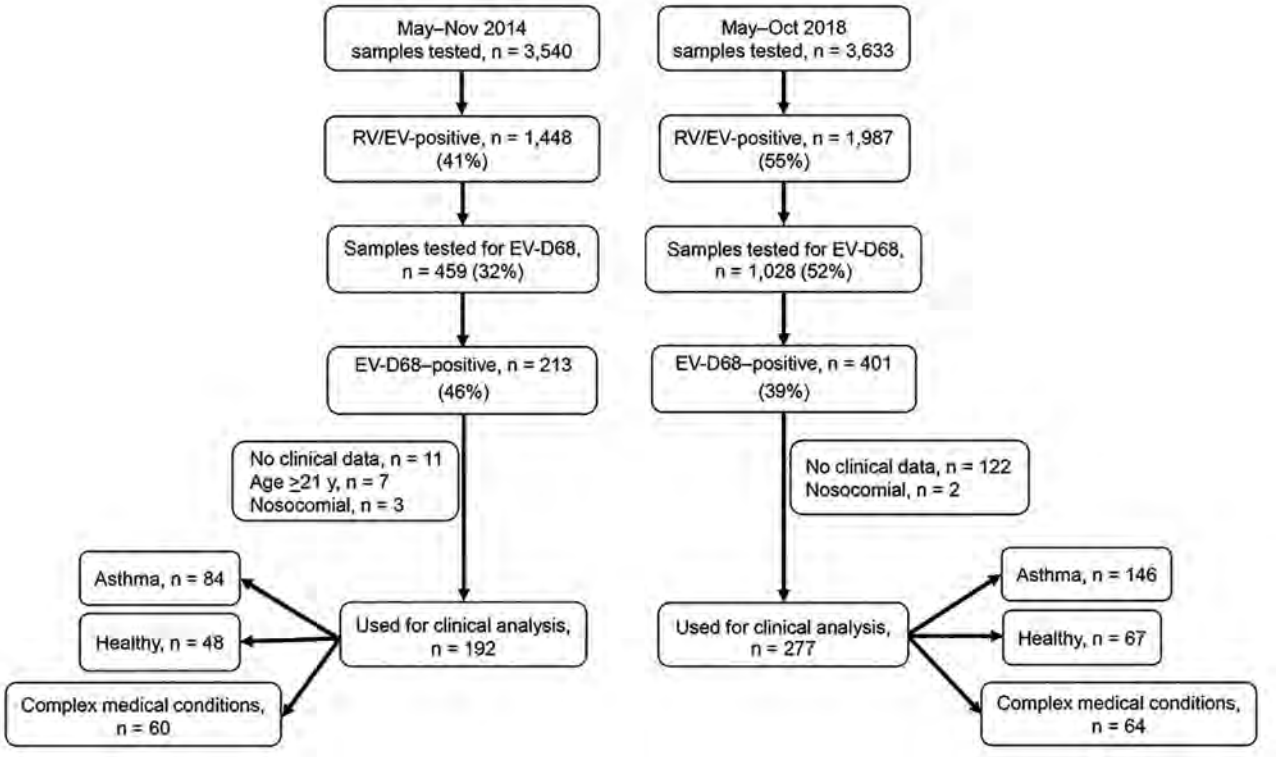

Figure 1. Sample and patient selection for investigation of EV-D68 outbreaks, Columbus, Ohio, USA. Viral testing was conducted at Nationwide Children's Hospital Department of Pathology. During May–November 2014, a total of 3,540 samples underwent viral testing, of which 41% tested positive for RV/EV by a single or multiplex PCR. Four hundred fifty-nine samples were selected randomly on the basis of availability, integrity, and amount of specimen, of which 44% were positive for EV-D68. During May–October 2018, a total of 3,633 samples were tested for RV/EV by FilmArray Respiratory Panel v1.7 (*6*); 1,987 (55%) were positive. Of the 1,025 convenience samples, 401 (39%) were positive for EV-D68. After samples for which clinical data were not available, for which patient age was ≥21 years, or for which EV-D68 was acquired nosocomially were excluded, 192 case-patients from the 2014 outbreak and 278 from the 2018 outbreak were included in the analyses. EV-D68, enterovirus D68; RV/EV, rhinovirus/enterovirus.

product, as described (*7*). We performed cycle sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, https://www.thermofisher.com) on the automated sequencer 3130xl Genetic Analyzer (Applied Biosystems) bidirectionally. We generated multiple sequence alignments and phylogenetic trees and compared the amino acid sequences (including BE, DC, and GH loops) of partial VP1 as described (*3*,*8*,*9*).

Statistical Analysis

We conducted descriptive analyses using frequency distributions or rates and used medians and interquartile ranges to summarize the demographic data and patients' baseline characteristics. We analyzed associations between categorical variables using the Fisher exact or χ^2 test and assessed normality for continuous variables using the Shapiro-Wilk test and 2-tailed Student *t* tests, Mann-Whitney test, 1-way ANOVA (analysis of variance), or Kruskal-Wallis tests where used as appropriate. Two-tailed p values <0.05 were considered significant. We performed statistical analyses using GraphPad Prism 8 (https://www.graphpad.com).

Results

Sample Selection and Clinical Characteristics of EV-D68–Infected Patients during the 2018 Outbreak

During the 2018 outbreak, of 3,633 samples tested by the FilmArray panel 1,987 (55%) were positive for RV/EV. We further evaluated 1,028 samples, of which 401 (39%) tested positive for EV-D68. This number compares with 213 (46%) EV-D68–positive samples of 459 in the 2014 outbreak (Figure 1).

Of the 401 patient samples that tested positive for EV-D68 in 2018, we excluded 124 (122 because patients were evaluated in the outpatient setting or clinical data were not available and 2 from children with nosocomial EV-D68 infection), leaving a total of 277 patients hospitalized with EV-D68 infection. Of those, 67 (24%) children were previously healthy, 146 (53%) had preexisting asthma or a history of wheezing, and 64 (23%) had another underlying chronic medical condition (Table 1). Children with a history of asthma or wheezing were older (median age 4.1 years) than children who had complex medical conditions (median 2.5 years) or were previously healthy (median 1.4 years; p<0.01); findings did not differ by sex or race. Most children had respiratory symptoms (94%–100%) with or without fever, followed by gastrointestinal manifestations (27%–32%). Eight (2.9%) children had neurologic manifestations, 2 AFM, and 1 opsoclonus/myoclonus syndrome (OMS).

Overall, children with asthma required pediatric intensive care unit (PICU) admission more frequently (63%) than did previously healthy children (37%) or children with chronic medical conditions (29%; p<0.0001); however, duration of hospitalization was longer for children with underlying conditions. The EV-D68 semiquantitative viral load (cycle threshold) ranged from an average of 25.1 to 26.1 and did not differ significantly between groups.

Clinical Manifestations during the 2014 and 2018 EV-D68 Outbreaks and the 2016 Low-Activity Period

We compared the demographic and clinical characteristics of children from the 2014 and 2018 EV-D68 outbreaks (Table 2). Overall, children with EV-D68 infection identified in 2018 were significantly younger and more often of white race; we found no differences in sex or presence of asthma or other chronic medical conditions. During the 2018 outbreak, children with EV-D68 infection more commonly had gastrointestinal symptoms than during the 2014 outbreak (28% in 2018 vs. 12% in

Table 1. Demographic and clinical characteristics of children with EV-D68 infection, Nationwide Children's Hospital, Columbus, Ohio, USA, 2018*

Characteristics	Previously healthy, n = 67	Chronic medical condition, n = 64	Asthma, n = 146	p value
Median age, y (IQR)	1.4 (0.9–4.0)	2.5 (1.1–6.9)	4.1 (2.1–7.5)	**<0.0001**
Sex, no. (%)				
M	31 (46.3)	36 (56.2)	84 (57.5)	0.29
F	36 (53.7)	28 (43.8)	62 (42.5)	
Race, no. (%)				
White	42 (62.7)	43 (67.2)	84 (57.5)	0.12
Black	14 (20.9)	13 (20.3)	50 (33.5)	
Other	11 (16.4)	8 (12.5)	13 (8.9)	
PICU admission, no. (%)	25 (37.3)	19 (29.7)	92 (63)	**<0.0001**
Median hospitalization, d (IQR)	1.8 (1–3)	3.2 (1.5–8)	2.5 (1.7–3.5)	**0.003**
Signs/symptoms, no. (%)				
Respiratory	64 (95.5)	60 (93.8)	146 (100)	**0.01**
Fever	39 (58.2)	36 (56.3)	67 (45.8)	0.16
Neurologic	4 (5.9)	3 (4.7)	1 (0.7)	0.06
Gastrointestinal	18 (26.8)	20 (31.3)	40 (27.4)	0.81
Rash	3 (4.5)	0	1 (0.7)	0.05
Median EV-D68 C_t (IQR)	26.1 (22.2–30.3)	25.5 (22.2–28.8)	25.1 (22–38.5)	0.67

*Bold indicates significance. C_t, cycle threshold; EV-D68, enterovirus D68; IQR, interquartile range; PICU, pediatric intensive care unit.

Table 2. Demographic and clinical characteristics of children with EV-D68 infection, Nationwide Children's Hospital, Columbus, Ohio, USA, 2014 and 2018 outbreaks*

Characteristic	2014, n = 192	2018, n = 277	p value
Demographics			
Median age, y (IQR)	5 (2–7.7)	3.1 (1.2–6.8)	0.0008
Sex, no. (%)			
M	112 (58.3)	153 (55.2)	0.50
F	80 41.7)	124 (44.6)	
Race, no. (%)			
White	79 (41.1)	169 (61.0)	
Black	75 (39.1)	76 (27.4)	
Other	38 (19.8)	32 (11.5)	**<0.0001**
Illness, no. (%)			
Previously healthy	48 (25)	67 (24.2)	0.09
Chronic condition†	60 (31.2)	64 (23.1)	
Asthma	84 (43.8)	146 (52.7)	
Clinical presentation and disease severity, no. (%)			
Respiratory	192 (100)	271 (97.8)	0.08
Fever	106 (55.2)	142 (51.3)	0.45
Neurologic	4 (2.1)	8 (2.9)	0.57
AFM/OMS	0	3 (1.1)	0.27
Other: seizures	4 (2.1)	5 (1.8)	>0.99
Gastrointestinal	24 (12.5)	79 (28.5)	**<0.0001**
Rash	10 (5.2)	4 (1.4)	**0.02**
PICU admission, no. (%)	131 (68.2)	136 (49.1)	**<0.0001**
Hospitalization, d (IQR)	2.8 (1.9–4.2)	2.5 (1.6–3.8)	**0.01**

*Bold indicates significance. AFM/OMS, acute flaccid myelitis/opsoclonus/myoclonus syndrome; EV-D68, enterovirus D68; IQR, interquartile range; PICU, pediatric intensive care unit.
†Comprises genetic syndromes, congenital heart disease, history of prematurity, neurologic disorders, neuromuscular disorders.

2014; p<0.001); symptoms included emesis, abdominal pain, and diarrhea. On the other hand, children with EV-D68 infection identified during the 2014 outbreak had respiratory symptoms and skin rashes more frequently than did children during the 2018 outbreak. The proportion of children who required PICU admission was lower in 2018 (49%) than in 2014 (68%; p<0.0001), and duration of hospitalization was shorter in 2018 (2.5 days) than in 2014 (2.8 days; p = 0.01).

Severe neurologic manifestations occurred more often during the 2018 outbreak. In the 2014 cohort, 4 (2%) patients had febrile seizures, but no other neurologic findings were documented, and no case of AFM was identified. In 2018, however, 8 (2.9%) patients had neurologic manifestations; 2 had AFM and 1 OMS. Two of these 3 children were previously healthy; 1 had underlying asthma. Four additional children sought treatment for complex febrile seizures (3 of these patients had a history of epilepsy), and 1 infant had viral meningitis. In this infant, parechovirus was identified by rRT-PCR in cerebrospinal fluid (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/11/19-0973-App1.pdf).

During 2016, of 3,098 samples tested by the FilmArray panel, 1,293 (42%) were positive for RV/EV. Of those, 211 were further tested for EV-D68, and 14 (7%) yielded positive results. Nine of the 14 patients identified with EV-D68 infection were hospitalized; all had respiratory symptoms, and none had neurologic manifestations. Equal proportions of children had asthma or chronic medical conditions or were previously healthy (3 [33%] each).

EV-D68 Seasonality and Asthma

We also compared the proportion of samples in which we detected EV-D68 during June–October 2014, 2016, and 2018 and analyzed the seasonality of EV-D68 in relation to admissions for asthma (Figure 2). The proportion of RV/EV detected from all nasopharyngeal samples analyzed according to the standard of care was 41% in 2014 and 2016 and 55% in 2018; overall EV-D68 detection was 44% in 2014, 7% in 2016, and 39% in 2018. The proportion of EV-D68 detected among RV/EV-positive samples (Figure 2, panel A) and the number of admissions for asthma (Figure 2, panel B) were calculated weekly during the 3 periods. The duration of the 2014 outbreak was shorter (mid-July through early October, 13 weeks), and peaked the last week of August, coinciding with a disproportionate number of admissions for asthma. In 2018, the first cases of EV-D68 were identified earlier (mid-June) and ended the first week of October (total of 16 weeks), peaking the last week of July/first week of August. In parallel, the number of admissions for asthma increased during July and August but peaked during the last week of August, which was delayed in relation to EV-D68 circulation. Admissions for asthma during the same time period in 2016 were substantially lower, as was EV-D68 detection.

Molecular Characteristics of EV-D68 2018 Strains

We sequenced 130 EV-D68–positive samples from 2018 and aligned them to both NCH strains from prior years and to EV-D68 sequences available at the National Center for Biotechnology Information website. The NCH 2018 strains

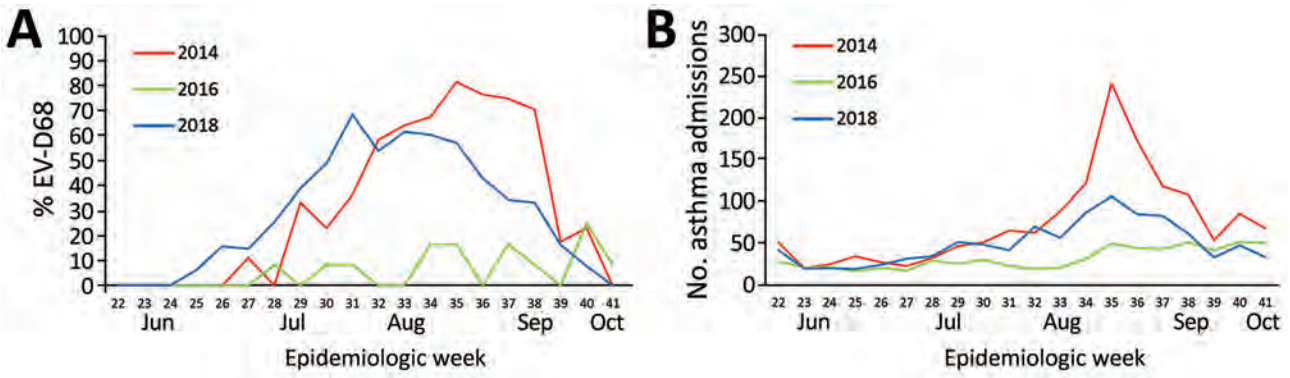

Figure 2. Percentage of EV-D68 (A) and number of admissions for asthma per 1,000 hospital admissions (B) among rhinovirus/enterovirus-positive (RV/EV) samples, Nationwide Children's Hospital, Columbus, Ohio, USA, June–October 2014, 2016, and 2018. EV-D68, enterovirus D68.

were >98.5% identical to each other and demonstrated >85% sequence identity to the VP1 regions of the prototype Fermon strain (GenBank accession no. NC_038308). These 2018 strains were also 92%–94% identical to the 2014 and 2011 NCH strains previously reported (*3*). The most closely related sequences to the NCH 2018 strains were those isolated in 2016 and 2015 from different geographic regions (*10*,*11*).

We used 17 NCH strains (10 from 2018, 5 from 2014, and 2 from 2011) for further genetic characterization. Phylogenetic analyses followed by bootstrap analyses indicated that all NCH strains identified during the 2018 outbreak clustered into a new sublineage within major clade B, differently from the 2011 and 2014 NCH strains (Figure 3). Amino acid sequence alignment for the BC, DE, and GH loops (Figure 4) showed that the NCH 2018 strains displayed a unique amino acid signature, and all contained the amino acid residue (218T) that characterizes the EV-D68 clade B3 (*10*).

Discussion

The recent emergence of EV-D68 as a cause of severe respiratory disease, coupled with its association with AFM, suggests that a deeper understanding of this virus is needed (*12*–*14*). In this study, we examined >1,000 patient specimens from 2 outbreaks and from 1 period with low EV-D68 activity and conducted both molecular and clinical analyses to compare these periods. Although the 2018 EV-D68 outbreak appeared to be milder, as shown by the lower number of hospital admissions for asthma and lower rates of PICU admissions, we observed severe neurologic manifestations only in 2018.

The comparative clinical analyses between outbreaks showed that hospitalized children during 2018 were younger, but the proportion with underling medical conditions, including asthma, was comparable between periods. Symptoms also were similar in the 2 outbreaks, with the notable exceptions of greater gastrointestinal manifestations in 2018, as well as 3 children with severe neurologic manifestations (2 with AFM and 1 with OMS), which we did not observe during 2014 or 2016.

Since 2014, concurrent with the surge of EV-D68 respiratory-associated illness, children with severe neurologic manifestations have been reported in the United States and elsewhere; episodic increases were identified in 2016 and 2018 (*5*,*12*,*14*–*21*). The cause of AFM has not been established in most cases, despite extensive pathogen-specific or metagenomic sequencing tests. Although a direct link between AFM and EV-D68 has not been established, observational and animal studies suggest a strong association. On the one hand, EV-D68 causes paralytic myelitis in mice; AFM cases have been shown to cluster during periods of EV-D68 circulation, and EV-D68 has been the most common virus detected in respiratory specimens from children with AFM, albeit rarely in cerebrospinal fluid (*22*–*25*). Other neurologic conditions associated with EV-D68 have been described, but to our knowledge, no other cases of OMS have been reported in the literature (*26*,*27*). Further studies are ongoing, but our findings agree with others and highlight the importance of comprehensive surveillance and research to further characterize the role of EV-D68 in AFM that will enable pursuit of effective therapies and prevention strategies.

Although the EV-D68 rRT-PCR testing in this study was not designed to determine true incidence, it did help to monitor EV-D68 activity and showed a marked increase in EV-D68 circulation during the summers of 2014 and 2018 and little or no activity during the same period in 2016. The increase in EV-D68 activity during 2014 and 2018 mirrored an increase in the number of admissions for asthma during those periods, and although the 2014 outbreak had a sharper increase at the end of August, the duration of the 2018 outbreak was longer. Nonetheless, in both periods admissions for asthma were significantly

higher than during 2016. Although reported to have a biennial seasonality (*20*,*24*,*28*–*31*), the peaks of EV-D68 in 2014 and 2018 were related to an increase in summertime hospitalizations for asthma, which agrees with a recent study conducted in Japan (*32*). During the same period in 2016, admissions for asthma were low and no peak was

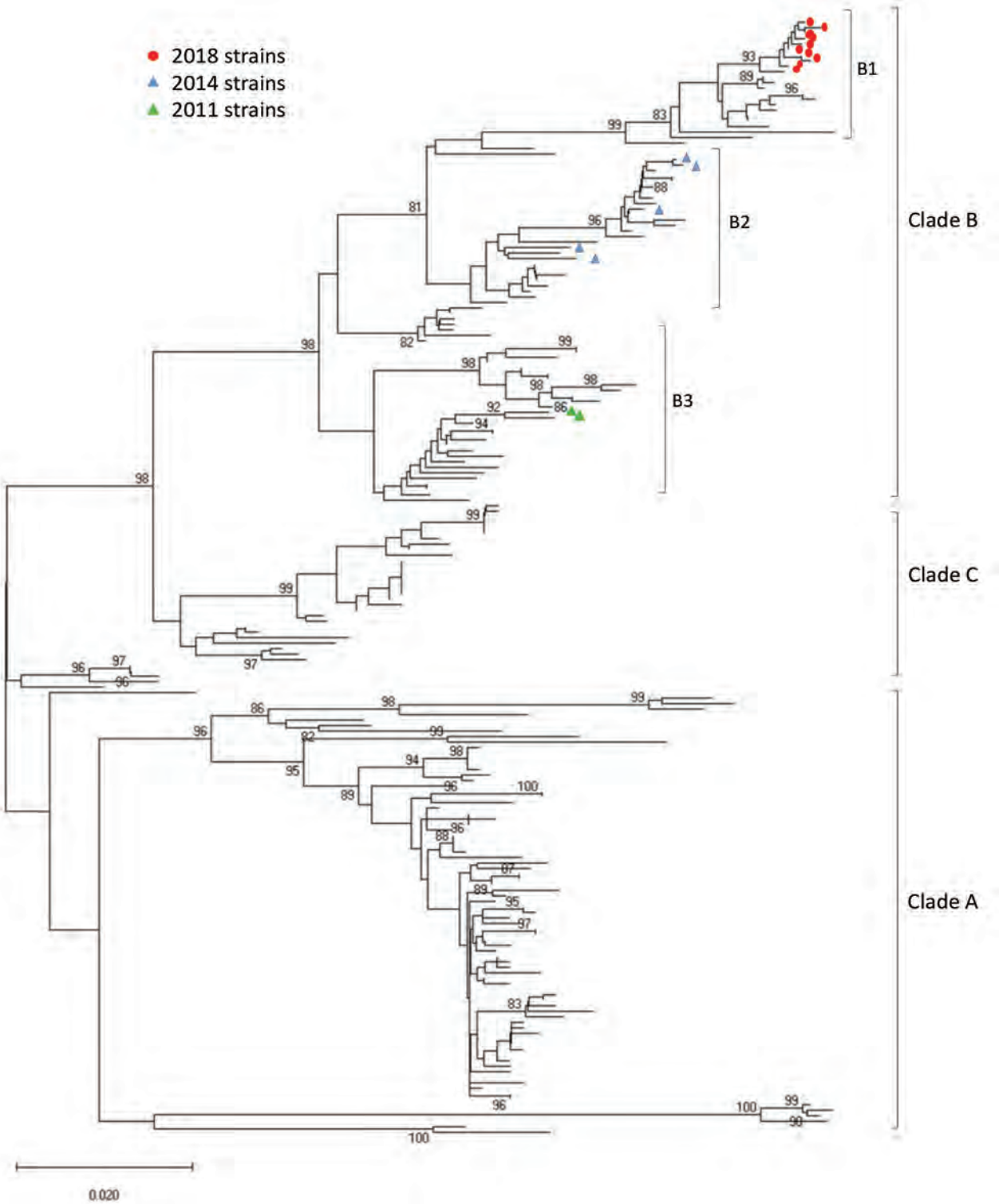

Figure 3. Phylogenetic analysis of EV-D68 from samples from children at Nationwide Children's Hospital, Columbus, Ohio, USA, 2011, 2014, and 2018. Phylogenetic tree was constructed using partial viral protein 1 gene sequences. Scale bar indicates changes in base substitutions per site. EV-D68, enterovirus D68.

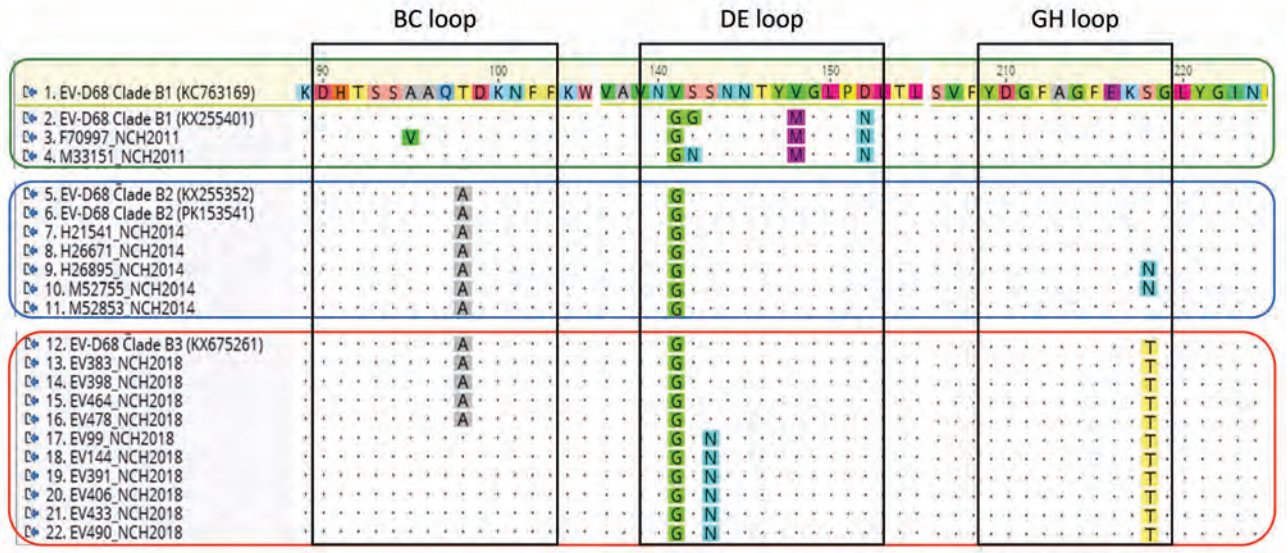

Figure 4. Amino acid analysis of 17 EV-D68 strains from samples from patients at NCH, Columbus, Ohio, USA, 2011, 2014, and 2018. EV-D68 strains representing subclades B1, B2, and B3 were aligned. Black boxes indicate amino acids included in viral protein 1 motifs corresponding to protein loops; colored boxes indicate strains corresponding to each subclade (green, clade B1; blue, clade B2; red, clade B3). GenBank accession numbers are given in parentheses. EV-D68, enterovirus D68; NCH, Nationwide Children's Hospital.

observed, nor was there evidence of high EV-D68 circulation based on surveillance testing in our laboratory, which differs from data reported from other states (*28*,*33*). The magnitude of the increase in asthma hospitalizations was higher in 2014 than in 2018 (peak of 128 admissions during the peak week in 2014 vs. 61 during 2018). Whether these decreases in EV-D68 activity and severity are continued in subsequent EV-D68 outbreaks in the population studied here needs be determined. Nevertheless, EV-D68 should be suspected when summertime admissions related to asthma increase above baseline.

Phylogenetic analyses showed that EV-D68 isolates from 2014 and 2018 clustered differently within clade B (*10*,*29*,*34*,*35*). The relationship of these changes in sequence and the pathogenicity of the virus are unclear (*36*). Nevertheless, at NCH, no AFM cases were identified in 2014, but 2 AFM cases and 1 OMS case occurred in 2018. The pathogenicity and virulence of this new clade needs to be monitored and confirmed by active surveillance, which was implemented at NCH after the 2014 outbreak. Based on our experience, we have made the EV-D68 rRT-PCR test available for respiratory specimens in real time. This test will be used on the basis of clinician orders particularly as it relates to unexplained acute paralysis/muscle weakness.

This study has limitations. We did not test every specimen that was RV/EV-positive for EV-D68; we did, however, test >40% of all samples that tested positive for RV/EV during these 2 outbreaks and a low-activity period, which provides a good representation of EV-D68 circulation during those periods. The clinical analysis was limited to inpatients, thus possibly biasing the apparent severity of the EV-D68 infections; however, data were comparable between the 3 periods, which was the main study objective. Unfortunately, the samples associated with the 2 AFM cases were not available for sequencing, and thus we cannot make any definitive conclusions about different or more pathogenic viral strains.

In summary, EV-D68 circulation was associated with a significant medical burden. By more consistent and specific testing for EV-D68, a better understanding of the epidemiology of this emerging virus will help inform clinical care (*37*).

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References

1. Schieble JH, Fox VL, Lennette EH. A probable new human picornavirus associated with respiratory diseases. Am J Epidemiol. 1967;85:297–310. http://dx.doi.org/10.1093/oxfordjournals.aje.a120693
2. Oberste MS, Maher K, Schnurr D, Flemister MR, Lovchik JC, Peters H, et al. Enterovirus 68 is associated with respiratory illness and shares biological features with both the enteroviruses and the rhinoviruses. J Gen Virol. 2004;85:2577–84. http://dx.doi.org/10.1099/vir.0.79925-0

3. Moyer K, Wang H, Salamon D, Leber A, Mejias A. Enterovirus D68 in hospitalized children: sequence variation, viral loads and clinical outcomes. PLoS One. 2016;11:e0167111. http://dx.doi.org/10.1371/journal.pone.0167111
4. Messacar K, Abzug MJ, Dominguez SR. 2014 outbreak of enterovirus D68 in North America. J Med Virol. 2016;88:739–45. http://dx.doi.org/10.1002/jmv.24410
5. Aliabadi N, Messacar K, Pastula DM, Robinson CC, Leshem E, Sejvar JJ, et al. Enterovirus D68 infection in children with acute flaccid myelitis, Colorado, USA, 2014. Emerg Infect Dis. 2016;22:1387–94. http://dx.doi.org/10.3201/eid2208.151949
6. Leber AL, Everhart K, Daly JA, Hopper A, Harrington A, Schreckenberger P, et al. Multicenter evaluation of BioFire FilmArray Respiratory Panel 2 for detection of viruses and bacteria in nasopharyngeal swab samples. J Clin Microbiol. 2018;56:e01945–17. http://dx.doi.org/10.1128/JCM.01945-17
7. Todd AK, Hall RJ, Wang J, Peacey M, McTavish S, Rand CJ, et al. Detection and whole genome sequence analysis of an enterovirus 68 cluster. Virol J. 2013;10:103. http://dx.doi.org/10.1186/1743-422X-10-103
8. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9. http://dx.doi.org/10.1093/bioinformatics/bts199
9. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol. 2013;30:2725–9. http://dx.doi.org/10.1093/molbev/mst197
10. Piralla A, Principi N, Ruggiero L, Girello A, Giardina F, De Sando E, et al. Enterovirus-D68 (EV-D68) in pediatric patients with respiratory infection: the circulation of a new B3 clade in Italy. J Clin Virol. 2018;99-100:91–6. http://dx.doi.org/10.1016/j.jcv.2018.01.005
11. Pariani E, Pellegrinelli L, Merlone AD, Piralla A, Baldanti F, Binda S. Letter to the editor: need for a European network for enterovirus D68 surveillance after detections of EV-D68 of the new B3 lineage in Sweden and Italy, 2016. Euro Surveill. 2017;22:pii:30440.
12. Knoester M, Helfferich J, Poelman R, Van Leer-Buter C, Brouwer OF, Niesters HGM; 2016 EV-D68 AFM Working Group. 2016 EV-D68 AFM Working Group. Twenty-nine cases of enterovirus-D68-associated acute flaccid myelitis in Europe 2016: a case series and epidemiologic overview. Pediatr Infect Dis J. 2019;38:16–21. http://dx.doi.org/10.1097/INF.0000000000002188
13. Brown DM, Hixon AM, Oldfield LM, Zhang Y, Novotny M, Wang W, et al. Contemporary circulating enterovirus D68 strains have acquired the capacity for viral entry and replication in human neuronal cells. MBio. 2018;9:e01954–18. http://dx.doi.org/10.1128/mBio.01954-18
14. Ruggieri V, Paz MI, Peretti MG, Rugilo C, Bologna R, Freire C, et al. Enterovirus D68 infection in a cluster of children with acute flaccid myelitis, Buenos Aires, Argentina, 2016. Eur J Paediatr Neurol. 2017;21:884–90. http://dx.doi.org/10.1016/j.ejpn.2017.07.008
15. Ayscue P, Van Haren K, Sheriff H, Waubant E, Waldron P, Yagi S, et al.; Centers for Disease Control and Prevention (CDC). Acute flaccid paralysis with anterior myelitis—California, June 2012–June 2014. MMWR Morb Mortal Wkly Rep. 2014;63:903–6.
16. Greninger AL, Naccache SN, Messacar K, Clayton A, Yu G, Somasekar S, et al. A novel outbreak enterovirus D68 strain associated with acute flaccid myelitis cases in the USA (2012–14): a retrospective cohort study. Lancet Infect Dis. 2015;15:671–82. http://dx.doi.org/10.1016/S1473-3099(15)70093-9
17. Sejvar JJ, Lopez AS, Cortese MM, Leshem E, Pastula DM, Miller L, et al. Acute flaccid myelitis in the United States, August–December 2014: results of nationwide surveillance. Clin Infect Dis. 2016;63:737–45. http://dx.doi.org/10.1093/cid/ciw372
18. Bjerin O, Martín Muñoz D, Gerald C, Brytting M, Eriksson M. Acute flaccid myelitis amongst Swedish children with a possible link to an outbreak of enterovirus D68 [in Swedish]. Lakartidningen. 2017;114:pii:ETDZ.
19. Cabrerizo M, García-Iñiguez JP, Munell F, Amado A, Madurga-Revilla P, Rodrigo C, et al. First cases of severe flaccid paralysis associated with enterovirus D68 infection in Spain, 2015–2016. Pediatr Infect Dis J. 2017;36:1214–6. http://dx.doi.org/10.1097/INF.0000000000001668
20. Knoester M, Schölvinck EH, Poelman R, Smit S, Vermont CL, Niesters HGM, et al. Upsurge of enterovirus D68, the Netherlands, 2016. Emerg Infect Dis. 2017;23:140–3. http://dx.doi.org/10.3201/eid2301.161313
21. Kamau E, Harvala H, Blomqvist S, Nguyen D, Horby P, Pebody R, et al. Increase in enterovirus D68 infections in young children, United Kingdom, 2006–2016. Emerg Infect Dis. 2019;25:1200–3. http://dx.doi.org/10.3201/eid2506.181759
22. Messacar K, Asturias EJ, Hixon AM, Van Leer-Buter C, Niesters HGM, Tyler KL, et al. Enterovirus D68 and acute flaccid myelitis—evaluating the evidence for causality. Lancet Infect Dis. 2018;18:e239–47. http://dx.doi.org/10.1016/S1473-3099(18)30094-X
23. Hopkins SE, Elrick MJ, Messacar K. Acute flaccid myelitis—keys to diagnosis, questions about treatment, and future directions. JAMA Pediatr. 2019;173:117–8. http://dx.doi.org/10.1001/jamapediatrics.2018.4896
24. Messacar K, Pretty K, Reno S, Dominguez SR. Continued biennial circulation of enterovirus D68 in Colorado. J Clin Virol. 2019;113:24–6. http://dx.doi.org/10.1016/j.jcv.2019.01.008
25. Hixon AM, Yu G, Leser JS, Yagi S, Clarke P, Chiu CY, et al. A mouse model of paralytic myelitis caused by enterovirus D68. PLoS Pathog. 2017;13:e1006199. http://dx.doi.org/10.1371/journal.ppat.1006199
26. Kuban KC, Ephros MA, Freeman RL, Laffell LB, Bresnan MJ. Syndrome of opsoclonus-myoclonus caused by coxsackie B3 infection. Ann Neurol. 1983;13:69–71. http://dx.doi.org/10.1002/ana.410130115
27. Blaes F, Dharmalingam B. Childhood opsoclonus-myoclonus syndrome: diagnosis and treatment. Expert Rev Neurother. 2016;16:641–8. http://dx.doi.org/10.1080/14737175.2016.1176914
28. Messacar K, Robinson CC, Pretty K, Yuan J, Dominguez SR. Surveillance for enterovirus D68 in Colorado children reveals continued circulation. J Clin Virol. 2017;92:39–41. http://dx.doi.org/10.1016/j.jcv.2017.05.009
29. Wang G, Zhuge J, Huang W, Nolan SM, Gilrane VL, Yin C, et al. Enterovirus D68 subclade B3 strain circulating and causing an outbreak in the United States in 2016. Sci Rep. 2017;7:1242. http://dx.doi.org/10.1038/s41598-017-01349-4
30. Dyrdak R, Grabbe M, Hammas B, Ekwall J, Hansson KE, Luthander J, et al. Outbreak of enterovirus D68 of the new B3 lineage in Stockholm, Sweden, August to September 2016. Euro Surveill. 2016;21:30403. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.46.30403
31. Kramer R, Sabatier M, Wirth T, Pichon M, Lina B, Schuffenecker I, et al. Molecular diversity and biennial circulation of enterovirus D68: a systematic screening study in Lyon, France, 2010 to 2016. Euro Surveill. 2018;23:23. http://dx.doi.org/10.2807/1560-7917.ES.2018.23.37.1700711
32. Korematsu S, Nagashima K, Sato Y, Nagao M, Hasegawa S, Nakamura H, et al. "Spike" in acute asthma exacerbations during enterovirus D68 epidemic in Japan: a nation-wide survey. Allergol Int. 2018;67:55–60. http://dx.doi.org/10.1016/j.alit.2017.04.003
33. Srinivasan M, Niesen A, Storch GA. Enterovirus D68 surveillance, St. Louis, Missouri, USA, 2016. Emerg Infect Dis. 2018;24:2115–7. http://dx.doi.org/10.3201/eid2411.180397

34. Kaida A, Iritani N, Yamamoto SP, Kanbayashi D, Hirai Y, Togawa M, et al. Distinct genetic clades of enterovirus D68 detected in 2010, 2013, and 2015 in Osaka City, Japan. PLoS One. 2017;12:e0184335. http://dx.doi.org/10.1371/journal.pone.0184335
35. Huang YP, Lin TL, Lin TH, Wu HS. Molecular and epidemiological study of enterovirus D68 in Taiwan. J Microbiol Immunol Infect. 2017;50:411–7. http://dx.doi.org/10.1016/j.jmii.2015.07.015
36. Imamura T, Okamoto M, Nakakita S, Suzuki A, Saito M, Tamaki R, et al. Antigenic and receptor binding properties of enterovirus 68. J Virol. 2014;88:2374–84. http://dx.doi.org/10.1128/JVI.03070-13
37. Carrion Martin AI, Pebody RG, Danis K, Ellis J, Niazi S, DE Lusignan S, et al. The emergence of enterovirus D68 in England in autumn 2014 and the necessity for reinforcing enterovirus respiratory screening. Epidemiol Infect. 2017;145:1855–64. http://dx.doi.org/10.1017/S0950268817000590

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Lack of Efficacy of High-Titered Immunoglobulin in Patients with West Nile Virus Central Nervous System Disease

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Release date: October 15, 2019; Expiration date: October 15, 2020

Learning Objectives

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

- Describe safety and tolerability of Omr-IgG-am compared with that of intravenous immunoglobulin (IVIG) and normal saline (NS) in patients with West Nile virus (WNV) neuroinvasive disease in a phase 2, randomized, double-blind, multicenter study
- Determine efficacy of Omr-IgG-am compared with that of IVIG and NS in patients with WNV neuroinvasive disease in a phase 2, randomized, double-blind, multicenter study
- Identify clinical implications of safety and efficacy of Omr-IgG-am compared with that of IVIG and NS in patients with WNV neuroinvasive disease in a phase 2, randomized, double-blind, multicenter study.

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West Nile Virus (WNV) can result in clinically severe neurologic disease. There is no treatment for WNV infection, but administration of anti-WNV polyclonal human antibody has demonstrated efficacy in animal models. We compared Omr-IgG-am, an immunoglobulin product with high titers of anti-WNV antibody, with intravenous immunoglobulin (IVIG) and normal saline to assess safety and efficacy in patients with WNV neuroinvasive disease as part of a phase I/II, randomized, double-blind, multicenter study in North America. During 2003–2006, a total of 62 hospitalized patients were randomized to receive Omr-IgG-am, standard IVIG, or normal saline (3:1:1). The primary endpoint was medication safety. Secondary endpoints were morbidity and mortality, measured using 4 standardized assessments of cognitive and functional status. The death rate in the study population was 12.9%. No significant differences were found between groups receiving Omr-IgG-am compared with IVIG or saline for either the safety or efficacy endpoints.

West Nile virus (WNV) is a mosquitoborne flavivirus that causes a spectrum of human illnesses, ranging from asymptomatic infection to an undifferentiated febrile syndrome (West Nile fever) and potentially lethal neuroinvasive diseases, including encephalitis and myelitis (*1*–*5*). Since its appearance in New York, USA, in 1999, WNV has become a seasonal endemic infection across North America (*5*–*7*). During 1999–2017, a total of 48,183 cases of WNV infection were reported to the Centers for Disease Control and Prevention (CDC), of which 22,999 were defined as neuroinvasive disease (*8*). Among patients with neuroinvasive disease, the mortality rate is 8%–12% (*5*,*8*,*9*). The number of reported cases of WNV disease in the United States averaged ≈2,200 cases annually during 2013–2017, although the true incidence is certainly much higher (*8*,*10*,*11*). Currently, no vaccine or drug has been approved by the Food and Drug Administration for prevention or treatment of human WNV infection.

The National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group initiated a clinical trial of immunotherapy for patients with WNV encephalitis or myelitis using Omr-IgG-am (OMRIX Biopharmaceuticals, Tel Aviv, Israel), an immunoglobulin product that contains high titers of WNV IgG. Murine model experiments demonstrated that anti-WNV globulin administered near the time of infection was highly effective at preventing disease and death (*12*). Anecdotal cases of successful treatment of human WNV with passive immunotherapy have been reported (*13*–*16*). We conducted this phase I/II study to assess the safety and potential efficacy of Omr-IgG-am for treatment for hospitalized adults with WNV neuroinvasive disease.

Methods

Design

During 2003–2006, we enrolled patients into a prospective, randomized, double-blind, placebo-controlled trial of Omr-IgG-am, a human immunoglobulin preparation that had a WNV plaque-reduction neutralization titer of 1:200. We compared Omr-IgG-am with 2 controls: standard intravenous (IV) immunoglobulin (IVIG) (Polygam S/D; Baxter, https://www.baxter.com), derived from US sources and containing no detectable WNV IgG; and normal saline (NS) for IV administration. One hundred patients meeting entry criteria were to be randomized in a 3:1:1 ratio (60 for Omr-IgG-am, 20 for Polygam, and 20 for NS) in blocks of 5. Randomization was implemented with a web-based system developed and maintained by the Data Coordinating Center at the University of Alabama at Birmingham (Birmingham, AL, USA). Randomized patients received a single intravenous dose of study medication on day 1. Patients were followed for 90 days after dosing. All investigators and patients remained blinded for the duration of the study.

The 2 active dosage cohorts (0.5 g/kg and 1.0 g/kg of Omr-IgG-am) were to accrue sequentially. However, because of slow enrollment, impending expiration of Omr-IgG-am stock, and difficulty locating supplies of Polygam free of WNV IgG, the protocol was amended in 2006 to allow continued enrollment in the 0.5 g/kg cohort and to forgo the planned 1.0 g/kg cohort.

Endpoints

The primary endpoint was safety and tolerability of the study medications at day 90 postenrollment. The safety endpoint was defined by the number of serious adverse events (SAEs), regardless of relationship to study drug. The estimated efficacy of Omr-IgG-am in reducing illness and death among patients with confirmed WNV disease (a

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secondary endpoint) was defined by a functional score (on day 90 after randomization) based on the results of 4 standardized assessments of cognitive and functional status: the Barthel Index (BI), the Modified Rankin Scale (MRS), the Glasgow Outcome Score (GOS), and the Modified Mini Mental State Examination (3MS) (*17–19*). We compared outcomes for the patients receiving Omr-IgG-am and those who received control interventions. Other secondary endpoints included the proportion of patients in each group returning to preillness baseline function as assessed by the BI and MRS, and each patient's improvement at 3 months compared with the patient's worst prior evaluation.

Study Population

Participants were enrolled while hospitalized at community or academic medical centers; follow-up visits occurred at outpatient clinics. Two categories of participants were enrolled. The first included hospitalized patients ≥18 years of age with new-onset (≤4 days' duration) encephalitis (altered level of consciousness, dysarthria, or dysphagia), myelitis (asymmetric extremity weakness without sensory abnormality), or both. In addition, the cerebrospinal fluid (CSF) analyses (performed within the previous 96 hours) were required to show pleocytosis (≥4 leukocytes/mm^3) and negative tests for other pathogens. The second eligibility category included adults who were hospitalized without encephalitis or myelitis but who had positive WNV IgM or PCR results, as well as clinical findings compatible with WNV infection and a risk factor for the development of WNV neurologic disease (≥40 years of age or immunocompromised patient ≥18 years of age). Confirmation of acute WNV infection by positive WNV IgM serologic results or PCR detection of WNV RNA in blood or CSF was required for inclusion in the efficacy analyses (*20–22*).

Study Procedures

After verifying inclusion and exclusion criteria and obtaining informed consent, patients were randomized to 1 of the 3 treatment arms. Medical history and physical examination were recorded. Detailed neurologic examinations were conducted (BI, MRS, the GOS, and the 3MS), along with an evaluation of pre-illness functional status.

We obtained CSF samples for WNV serologic testing and PCR before starting the study and performed brain magnetic resonance imaging (MRI) studies before the study and on day 30. We examined participants on days 1–7, 14, 30, and 90. We obtained blood samples for safety laboratory studies (including complete blood count, hemoglobin A1c, platelet count, blood urea nitrogen, creatinine, creatinine phosphokinase, liver enzymes, international normalized ratio, glucose, electrolytes, amylase, and lipase); WNV, HIV, HBV, and parvovirus B19 serologic testing; and PCR for WNV, HIV, HBV, HCV, and parvovirus B19.

The unblinded research pharmacist calculated the volume of study medication. Bottles of study medication and tubing were covered with opaque plastic covers to maintain blinding. We infused study medication intravenously using a 15-μ filter at an initial rate of 0.0083 mL/kg/min, gradually increased to a maximum rate of 3 mL/min.

Site Monitoring and Regulatory Oversight

A total of 71 sites in the United States and Canada completed regulatory requirements for enrollment (although the list of active sites varied from year to year); participants were successfully enrolled at 24 sites. The clinical trial was conducted in accordance with the ethical standards of the Helsinki Declaration. The protocol required approval by a local institutional review board (IRB) or ethics committee before enrollment could proceed; we obtained written informed consent from each participant or a legal guardian. All sites were independently monitored at selected time points and at the completion of the study. A data and safety monitoring board oversaw the study.

Statistical Analyses

We performed statistical analyses using SAS 9.1 software (https://www.sas.com) and StaXact 4.0 (https://www.cytel.com/software/statxact) for exact statistical methods. We analyzed data using standard descriptive statistics and used Fisher's exact test to explore associations for categorical variables between the treatment arm and the 2 control arms. Nonparametric statistical methods used a Wilcoxon test for comparison of continuous variables between the treatment arm and the 2 control arms. No interim analyses were planned.

Sample Size Determinations

Because the study was a phase I/II safety study with a primary objective of estimating the rate of serious adverse events, we did not plan formal tests of hypotheses. Thus, we did not determine the sample size by power analysis. A total sample size of 100 participants was planned (60 Omr-IgG-am, 20 Polygam, and 20 NS). With the assumption that the true adverse event rate was no more than 30%, the 2-sided 95% CIs of the estimated SAE rates were expected to be 18.4%–41.6% for Omr-IgG-am and 9.9%–50.1% for the 2 control arms.

Data Analyses

We included all 62 randomized participants in the safety analysis (intent-to-treat) and calculated estimates of adverse event rates with 2-sided exact 95% CIs for each treatment arm. We performed efficacy analyses on 55 study participants with confirmed WNV infection, although this phase I/II safety study was not sufficiently powered to detect small-to moderate differences in outcome among the

treatment groups. The efficacy endpoint was a combination of illness and death as defined by a functional score calculated 90 days after randomization. The endpoint was based on the results of the BI, GOS, MRS, and 3MS. We placed each participant into 1 of 4 categories: dead, severely impaired, mildly or moderately impaired (but still able to function independently), and normal. We further dichotomized each category into unfavorable and favorable medical outcomes, according to predetermined cut points. Scoring in the unfavorable category on any of the 4 scales placed that participant in the unfavorable category overall.

Conduct of the Study

The first participant was enrolled in September 2003 and the last was enrolled in September 2006. Because of slow accrual and other factors, the study was terminated in December 2006 at the recommendation of the data and safety monitoring board.

Results

Patient Disposition

A total of 242 patients were screened, but only 64 (26%) met the entry criteria. We did not tabulate reasons for study exclusion. Two potential participants were withdrawn before randomization. Thus, we randomized 37 patients to Omr-IgG-am, 12 to Polygam, and 13 to NS (Figure). Thirty-three of the patients in the Omr-IgG-am group, 11 in the Polygam group, and 11 in the NS group were available for follow-up at day 90. Of the 62 patients randomized, 11 terminated prematurely, 8 because of death (mortality rate 12.9%). Three (42.9%) of the 7 participants who did not have laboratory evidence of acute WNV infection died.

Study Population

Most of the patients were Caucasian (73%) and male (66%); mean age was 56 years (Table 1). We noted no baseline differences among any of the 3 randomization groups. Diagnosis of acute WNV infection was serologically confirmed in 55 patients; only 3 were positive for WNV by blood PCR. Most participants already carried a laboratory-confirmed diagnosis of WNV infection at the time of referral to the study.

Clinical Characteristics

Because we found no differences among any of the 3 randomization groups, we summarized the clinical characterization for all 55 patients with confirmed WNV infection. The most common symptoms were fever >38°C (80%), chills/rigors (75%), headache (78%), nuchal rigidity (47%), photophobia (33%), myalgia (78%), arthralgia (33%), nausea (76%), vomiting (60%), diarrhea (46%), shortness of breath (24%), and rash (27%). Detailed descriptions of the neurologic findings in this study cohort were published previously (*23*). Of the 44 participants with abnormal CSF findings at initial evaluation, median values were protein 90 mg/dL, glucose 62 mg/dL, erythrocytes 10 cells/mm^3, and leukocytes 96 cells/mm^3 (lymphocytes 62%, neutrophils 38%).

Safety and Tolerability

As expected for this population of seriously ill patients, large numbers of adverse events (AEs) were recorded (Table 2).

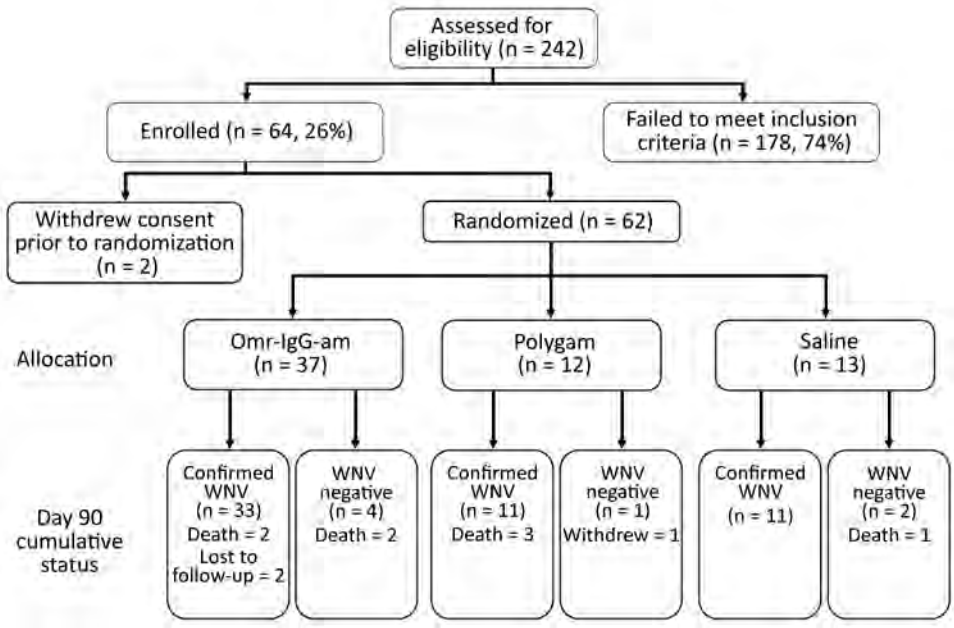

Figure. Patient enrollment, allocation, and final status in study of treatments for West Nile virus central nervous system disease.

Table 1. Demographics and clinical characteristics of patients in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Characteristic	Omr-IgG-am, n = 37	Polygam, n = 12	Normal saline, n = 13	Total, n = 62
Race				
Caucasian/not Hispanic	24 (64.9)	10 (83.3)	11 (84.6)	45 (72.6)
Black/not Hispanic	2 (5.4)	0	1 (7.7)	3 (4.8)
Hispanic	8 (21.6)	1 (8.3)	1 (7.7)	10 (16.1)
Other	3 (8.1)	1 (8.3)	0 (0)	4 (6.5)
Sex				
M	25 (67.6)	7 (58.3)	9 (69.2)	41 (66.1)
F	12 (32.4)	5 (41.7)	4 (30.8)	21 (33.9)
Age, y, mean ± SE	56.2 ± 2.2	54.0 ± 4.1	58.4 ± 4.8	56.2 ± 1.8
Cerebrospinal fluid				
Leukocytes, cells/μL, mean ± SE (median)	207.7 ± 40.3 (124)	187.4 ± 105.5 (58)	146.6 ± 85.8 (37)	192.3 ± 35.3 (95.5)
% Lymphocytes, mean ± SE (median)	53.9 ± 5.0 (52.5)	39.3 ± 10.0 (36.5)	50.2 ± 11.6 (34)	50.1 ± 4.3 (43)
Time from admission to drug infusion, d, mean ± SE (median)	2.9 ± 0.5 (2)	2.8 ± 0.5 (2.5)	2.0 ± 0.4 (2)	2.7 ± 0.3 (2)
Disease group and risk factors				
With encephalitis/myelitis	25 (64.7)	9 (75.0)	11 (84.6)	44 (71.0)
Without encephalitis/myelitis	12 (32.4)	3 (25.0)	2 (15.4)	17 (27.4)
Hematologic malignancy	1 (8.3)	0	0	1 (5.9)
Diabetes mellitus	2 (16.7)	0	0	2 (11.8)
Bone marrow transplant	1 (8.3)	0	0	1 (5.9)
Immunosuppressive medications	1 (8.3)	0	0	1 (5.9)

*Values are no. (%) patients except as indicated. In comparing Omr-IgG-am with Polygam and Omr-IgG-am with normal saline, no significant differences were identified for characteristics listed in this table (all p values >0.05). Denominators vary according to the number of evaluable patients or assessments.

A total of 738 AEs were reported for 58 participants (12.72 AEs/person). The most commonly reported treatment-related AE was hypertension occurring during infusion of the test drug (Table 3). Two grade 3–4 laboratory toxicities (both decreased hematocrit) were reported, both occurring in Omr-IgG-am recipients.

Safety was defined by the total number of SAEs among the 62 randomized participants, regardless of relatedness to study drug administration. Overall, 29 participants (46.8%) experienced 63 SAEs (29 with Omr-IgG-am, 25 with Polygam, 9 with NS; Table 4). The estimated SAE rates (with 2-sided 95% confidence intervals) were 51.4% (range 35.3%–67.7%) for recipients of Omr-IgG-am, 58.3% (range 30.4%–86.2%) for recipients of Polygam, and 23.1% (range 0.2%–46.0%) for those who received NS. The differences in frequency of SAEs among the 3 treatment groups were not statistically significant. A larger number of neurologic SAEs were reported in the Polygam group (Table 5), although the types of events were highly divergent (declining mental status, quadriparesis, cranial nerve palsies, tremor, seizures) and likely attributable to WNV neuroinvasive disease.

Five SAEs were assessed by the investigator to be possibly, probably, or definitely related to the study medication (Table 4). Two events (chest pain and leukopenia, both assessed as possibly) occurred in Omr-IgG-am recipients; both resolved. One SAE (respiratory distress, assessed as probably) occurred in a Polygam recipient and resolved. Two instances of neutropenia (both assessed as possibly) were reported in NS recipients and resolved.

To monitor the possibility of transmission of other viral pathogens by the immunoglobulin preparations, participants

Table 2. Summary of AEs in intent-to-treat population of patients in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Characteristic	Omr-IgG-am, n = 37	Polygam, n = 12	Normal saline, n = 13	Total, n = 62
AEs	514	106	118	738
patients with an AE	36 (97.3)	11 (91.7)	58 (93.5)	58 (93.5)
AEs per patient	14.28	9.64	10.73	12.72
Relationship to treatment†				
Unrelated	482 (93.8)	99 (93.4)	113 (95.8)	694 (94)
Related	29 (5.6)	7 (6.6)	5 (4.2)	41 (5.6)
Not stated	3 (0.6)	0	0	3 (0.4)
Severity of AE†				
Mild	226 (44.0)	45 (42.5)	61 (51.7)	332 (45.0)
Moderate	221 (43.0)	34 (32.1)	45 (38.1)	300 (40.7)
Severe	54 (10.5)	13 (12.3)	9 (7.6)	76 (10.3)
Life-threatening	13 (2.5)	14 (13.2)	3 (2.5)	30 (4.1)

*Values are no. (%) except as indicated. In comparing Omr-IgG-am with Polygam and Omr-IgG-am with normal saline, no significant differences were identified for characteristics listed in this table (all p values >0.05) except for mild severity Omr-IgG-am vs. Polygam (p<0.01). AE, adverse event.
†As assessed by the investigator.

were screened preinfusion and on day 30. No participant was positive for HBV or HIV. Two patients (1 recipient of Omr-IgG-am, 1 recipient of Polygam) had negative parvovirus B19 IgG titers preinfusion but had detectable antibodies at follow-up; both of these patients had negative parvovirus IgM titers and PCR assays. This finding likely represents antibody passively acquired from the immunoglobulin infusion rather than acute parvovirus infection. One patient tested positive for hepatitis C virus by both serologic testing and PCR preinfusion and remained positive on day 90.

Efficacy

For each test instrument (BI, 3MS, GOS, and MRS), composite scores measured at the time of enrollment indicated impaired neuropsychological function, which improved over 90 days of follow-up, consistent with the natural history of resolving WNV neurologic disease (Table 6). No significant differences in outcomes were apparent for the 3 treatment groups; therefore, summary statistics allow assessment of day 90 outcomes for the combined population. By 3MS, 50.9% of patients were determined to be normal/unimpaired, 18.2% had mild or moderate impairment, 12.7% were severely impaired, and 9.1% died. When the BI, GOS, and MRS tests were applied to the same population, the percentage of patients who were evaluated as normal/unimpaired were 47.3% by BI, 36.4% by GOS, and 14.5% by MRS; the proportion classified as severely impaired was 27%–29% by each of these 3 instruments.

We further dichotomized outcomes into favorable and unfavorable (Table 7). We found no significant differences in the proportion of patients experiencing an unfavorable outcome at day 90 between treatment and control (although there was again a nonsignificant trend toward better outcomes in the NS group). Overall, 51% of patients had a favorable outcome. We determined the proportion of patients returning to preillness baseline at day 90 for each randomization group. By the BI and the MRS, the 2 most sensitive indices, 45.9% and 32.8% of patients returned to their preillness status, respectively.

Table 3. Most commonly reported treatment-related adverse events in intent-to-treat population in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Adverse event	Omr-IgG-am, n = 37	Polygam, n = 12	Normal saline, n = 13	Total, n = 62
Hypertension	7	0	2	9
Dosing error	6	0	2	8
Elevated transaminases	3	1	0	4
Fever, chills	3	1	0	4
Shortness of breath	0	3	0	3
Rash, pruritus	2	0	0	2
Chest pain	2	0	0	2
Other	6	2	1	9
Total	29	7	5	41

*Values are no. patients.

The median duration of hospital stay was 10 days for the Omr-IgG-am group, 12 days for the Polygam group, and 8.5 days for the NS group. Of the 62 patients enrolled in the study, 23 (37%) required intensive care unit (ICU) management. We found no differences in the duration of ICU stay (median 13 days) among the 3 treatment groups. Six patients required mechanical ventilation (median duration 5 days).

Virologic Studies

All 55 patients initially had positive WNV IgM serologic test results; 36 (67.9%) of 53 patients were WNV IgG positive preinfusion. Reverse transcription PCR for WNV RNA in blood was positive for only 3 (5.9%) of 51 patients before infusion of study medication; no patient had a positive WNV PCR result from blood on day 3. Of the 49 patients for whom day 90 serologic data were available, 40 (81.6%) were persistently positive for WNV IgM and 47 (95.9%) for WNV IgG.

Table 4. Summary of SAEs in intent-to-treat population in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Characteristic	Omr-IgG-am, n = 37	Polygam, n = 12	Normal saline, n = 13	Total, n = 62
SAEs	29	25	9	63
Patients with an SAE	19 (51.4)	7 (58.3)	3 (23.1)	29 (46.8)
SAEs per patient	1.53	3.57	3.0	2.17
Relationship to treatment†				
Unrelated	27 (93.1)	24 (96.0)	7 (77.8)	58 (92.1)
Related	2 (6.9)	1 (4.0)	2 (22.2)	5 (7.9)
Severity†				
Mild	1 (3.4)	0	0	1 (1.6)
Moderate	4 (13.8)	3 (12.0)	0	7 (11.1)
Severe	9 (31.0)	8 (32.0)	4 (44.4)	21 (33.3)
Life-threatening	11 (37.9)	11 (44.0)	4 (44.4)	26 (41.3)
Death	4 (13.8)	3 (12.0)	1 (11.1)	8 (12.7)

*Values are no. (%) except as indicated. In comparing Omr-IgG-am with Polygam and Omr-IgG-am with normal saline, no significant differences were identified for characteristics listed in this table (all p values >0.05) except for mild severity Omr-IgG-am vs. Polygam (p<0.01). SAE, serious adverse event.
†As assessed by the investigator.

Table 5. Most commonly reported serious adverse events in intent-to-treat population in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Serious adverse event	Omr-IgG-am, n = 37	Polygam, n = 12	Normal saline, n = 13	Total, n = 62
Respiratory failure	8	7	2	17
Neurologic event or mental status decline	1	11	1	13
Cardiac event	4	1	0	5
Anemia	2	1	0	3
Leukopenia	1	0	2	3
Urinary tract infection	1	0	1	2
Pneumonia	2	0	0	2
Pulmonary embolism	1	0	1	2
Atelectasis	0	2	0	2
Pleural effusion	0	1	1	2
Other	9	2	1	12
Total	29	25	9	63

*Values are no. patients.

Discussion

Because preliminary data from animal models and case reports suggested that immunotherapy could alter the outcome of WNV neurologic infection, the National Institute of Allergy and Infectious Diseases Collaborative Antiviral Study Group initiated a clinical study to determine the safety and potential efficacy of a high-titered immunoglobulin product in patients with WNV neuroinvasive disease. The trial was terminated prematurely because of slow accrual and reduced availability of study products. At the time of study termination in 2006, the Polygam supply derived from US sources contained measurable titers of WNV IgG and was no longer an acceptable control.

Looking at recorded SAEs, deaths, and laboratory parameters, we found no differences in safety and tolerability among Omr-IgG-am (0.5 g/kg), Polygam, and NS. Illness outcomes, measured by a panel of 4 neuropsychological test instruments, were not statistically different among the 3 groups. Although the results did not meet statistical significance (in part because of the small sample size), we found a persistent trend toward better outcomes (both illness and death) in the NS group compared with the immunoglobulin groups (Tables 6, 7). Although the validity of this observation is unconfirmed, we do not recommend the administration of immunoglobulin products to patients with neuroinvasive WNV disease until further research can be conducted to establish the relative risk–benefit profile.

The study protocol was designed to capture patients as early as possible in their clinical courses, when immunotherapy was most likely to be beneficial. Unfortunately,

Table 6. Summary of impairment and death at day 90 after randomization for patients with confirmed West Nile virus in study of treatments for West Nile virus central nervous system disease, by treatment arm*

Instrument	Omr-IgG-am, n = 33	Polygam, n = 11	Normal saline, n = 11	Total, n = 55
Modified Mini-Mental Status Examination				
Normal, score >88	14 (42.4)	6 (54.5)	8 (72.7)	28 (50.9)
Mild/moderately impaired but independent, score 78–88	8 (24.2)	0	2 (18.2)	10 (18.2)
Severely impaired, score <78	5 (15.2)	1 (9.1)	1 (9.1)	7 (12.7)
Dead, score 0	2 (6.0)	3 (27.3)	0	5 (9.1)
Not done/lost to follow-up	4 (12.1)	1 (9.0)	0	5 (9.1)
Barthel Index				
Normal, score >94	15 (45.5)	5 (45.5)	6 (54.5)	26 (47.3)
Mild/moderately impaired but independent, score 90–94	1 (3.0)	0	2 (18.2)	3 (5.5)
Severely impaired, score <90	11 (33.3)	2 (18.2)	3 (27.3)	16 (29.1)
Dead, score 0	2 (6.1)	3 (27.3)	0	5 (9.1)
Not done/lost to follow-up	4 (12.1)	1 (9.0)	0	5 (9.1)
Glasgow Outcome Score				
Normal, score 5	10 (30.3)	3 (27.3)	7 (63.6)	20 (36.4)
Mild/moderately impaired but independent, score 4	9 (27.2)	2 (18.2)	1 (9.1)	12 (21.8)
Severely impaired, score 2–3	9 (27.2)	3 (27.3)	3 (27.3)	15 (27.3)
Dead, score 1	2 (6.0)	3 (27.3)	0	5 (9.1)
Not done/lost to follow-up	3 (9.0)	0	0	3 (5.5)
Modified Rankin Scale				
Normal, score 0	4 (12.1)	0	4 (36.4)	8 (14.5)
Mild/moderately impaired but independent, score 1–3	15 (45.5)	5 (45.5)	4 (36.4)	24 (43.6)
Severely impaired, score 4–5	10 (30.3)	3 (27.3)	3 (27.3)	16 (29.1)
Dead, score 6	2 (6.0)	3 (27.3)	0	5 (9.1)
Not done	2 (6.0)	0	0	2 (3.6)

*Values are no. (%) patients. In comparing Omr-IgG-am with Polygam and Omr-IgG-am with normal saline, we found no significant differences with respect to the impairment and death categories (excluding "not done") for all instruments listed on this table ($p > 0.05$ for all values).

Table 7. Summary of unfavorable outcomes at day 90 after randomization of patients with confirmed West Nile virus in study of treatments for West Nile virus central nervous system disease*

Regimen	No. (%) patients			Odds ratio (95% CI)
	Favorable	Unfavorable	Missing	
Omr-IgG-am, n = 33	15 (45.5)	17 (51.5)	1 (3.0)	Referent
Polygam, n = 11	5 (45.5)	6 (54.5)	0	1.012 (0.198–4.975)
Normal saline, n = 11	8 (72.7)	3 (27.3)	0	3.238 (0.606–21.959)
Total confirmed, n = 55	28 (50.9)	26 (47.2)	1 (1.8)	

*Favorable/unfavorable determinations made on the basis of results of 4 standardized assessments of cognitive and functional status: Barthel Index (favorable ≥90, unfavorable <90), Modified Rankin Scale (favorable 0–3, unfavorable 4–6), Glasgow Outcome Score (favorable 4 or 5, unfavorable <4), and the Modified Mini Mental State Examination (favorable ≥78, unfavorable <78).

patients often entered the study pool later, after a diagnosis of WNV infection had been confirmed by laboratory testing. Delays in enrollment and study drug administration could have diminished the potential efficacy of Omr-IgG-am. By the time symptomatic neurologic disease was present, the infection had probably progressed to a point at which the administration of passive immunotherapy was unlikely to be beneficial.

In this study population of relatively healthy middle-aged persons, 15% (as assessed by MRS) to 50% (as assessed by BI) returned to baseline function. Only 33%–46% of patients returned to their preillness state (as defined retrospectively by a family member). These data differ somewhat from proportions of patients experiencing normal or mild to moderate impairment reported by other investigators (*24*–*28*). Other published WNV case series had different demographic and disease characteristics and used different definitions, making interstudy comparisons problematic. Our population had a relatively high percentage of patients requiring ICU care and extended durations of hospitalization.

Effective therapy for WNV neuroinvasive disease remains an unmet medical need. A human monoclonal antibody directed against WNV has shown activity in animal models (*29*,*30*) but remains unproven for human infection (*31*,*32*). Antiviral drugs that can be initiated early in the course of WNV disease are urgently needed (*33*–*35*).

We learned several lessons that will inform the design of future studies of therapies for WNV disease. At various time points, this clinical trial activated 71 individual sites in 28 US states and 3 Canada provinces but was still unable to achieve full enrollment. The challenges encountered during the conduct of the study were numerous. First, and most notably, the precise geographic localization of emerging vectorborne illnesses is difficult to predict. WNV infection occurs seasonally (usually July–October in North America) and in scattered geographic locations. We worked closely with our collaborators at CDC but were unable to project with sufficient precision where the incidence of WNV disease would be highest in the subsequent season. Even when we correctly predicted geographic regions where disease activity was high, it was extremely difficult to activate sufficient study sites quickly. Furthermore, WNV infection is predominantly a rural disease, whereas many of our study sites were located in urban areas. Second, it was often difficult to refer potential participants to active study sites. Investigators received numerous calls regarding WNV patients who were hospitalized at nonparticipating medical centers, some even in the same city. However, logistical and financial constraints prevented most of these patients from being transferred to a site with an IRB-approved protocol in place. Third, most patients were considered for enrollment in the study only after WNV infection had been confirmed. The study was designed to enable enrollment of suspected WNV patients (before laboratory confirmation) to expedite early therapy, but this rarely occurred, as demonstrated by the mean time from admission to study drug administration (2.6 days). Animal model data have indicated that passive immunotherapy of WNV infection with exogenous antibodies is most effective if instituted very early in the course of infection.

Finally, there were regulatory constraints, as we have described previously (*36*). The median time required to obtain IRB approval at US medical centers was ≈6 months (*36*). Consequently, many potential participants could not be enrolled because sites failed to receive IRB approval and activate the protocol in a timely manner. The availability of a central IRB could have shortened site registration time considerably and potentially enhanced patient enrollment. As a result of unpredictable geographic variation, fluctuating incidence, and seasonal enrollment windows, an agile and flexible universal IRB system will be mandatory if future large-scale clinical trials of therapies for emerging vectorborne infectious diseases (e.g., WNV, Chikungunya, dengue, Zika virus) are to be successfully performed in the United States.

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References

1. Watson JT, Pertel PE, Jones RC, Siston AM, Paul WS, Austin CC, et al. Clinical characteristics and functional outcomes of West Nile fever. Ann Intern Med. 2004;141:360–5. https://doi.org/10.7326/0003-4819-141-5-200409070-00010
2. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. West Nile virus. Lancet Infect Dis. 2002;2:519–29. https://doi.org/10.1016/S1473-3099(02)00368-7
3. Hayes EB, Gubler DJ. West Nile virus: epidemiology and clinical features of an emerging epidemic in the United States. Annu Rev Med. 2006;57:181–94. https://doi.org/10.1146/annurev.med.57.121304.131418
4. O'Leary DR, Marfin AA, Montgomery SP, Kipp AM, Lehman JA, Biggerstaff BJ, et al. The epidemic of West Nile virus in the United States, 2002. Vector Borne Zoonotic Dis. 2004;4:61–70. https://doi.org/10.1089/153036604773083004
5. Petersen LR, Brault AC, Nasci RS. West Nile virus: review of the literature. JAMA. 2013;310:308–15. https://doi.org/10.1001/jama.2013.8042
6. Yeung MW, Shing E, Nelder M, Sander B. Epidemiologic and clinical parameters of West Nile virus infections in humans: a scoping review. BMC Infect Dis. 2017;17:609. https://doi.org/10.1186/s12879-017-2637-9
7. Roehrig JT. West nile virus in the United States—a historical perspective. Viruses. 2013;5:3088–108. https://doi.org/10.3390/v5123088
8. Centers for Disease Control and Prevention. Final cumulative maps and data for 1999–2017. 2018 [cited 2018 Nov 13]. https://www.cdc.gov/westnile/statsmaps/cumMapsData.html
9. Sejvar JJ, Lindsey NP, Campbell GL. Primary causes of death in reported cases of fatal West Nile fever, United States, 2002–2006. Vector Borne Zoonotic Dis. 2011;11:161–4. https://doi.org/10.1089/vbz.2009.0086
10. Lindsey NP, Staples JE, Lehman JA, Fischer M. Surveillance for human West Nile virus disease—United States, 1999–2008. MMWR Surveill Summ. 2010;59:1–17.
11. Petersen LR, Carson PJ, Biggerstaff BJ, Custer B, Borchardt SM, Busch MP. Estimated cumulative incidence of West Nile virus infection in US adults, 1999–2010. Epidemiol Infect. 2013;141:591–5. https://doi.org/10.1017/S0950268812001070
12. Ben-Nathan D, Lustig S, Tam G, Robinzon S, Segal S, Rager-Zisman B. Prophylactic and therapeutic efficacy of human intravenous immunoglobulin in treating West Nile virus infection in mice. J Infect Dis. 2003;188:5–12. https://doi.org/10.1086/376870
13. Haley M, Retter AS, Fowler D, Gea-Banacloche J, O'Grady NP. The role for intravenous immunoglobulin in the treatment of West Nile virus encephalitis. Clin Infect Dis. 2003;37:e88–90. https://doi.org/10.1086/377172
14. Hamdan A, Green P, Mendelson E, Kramer MR, Pitlik S, Weinberger M. Possible benefit of intravenous immunoglobulin therapy in a lung transplant recipient with West Nile virus encephalitis. Transpl Infect Dis. 2002;4:160–2.
15. Shimoni Z, Niven MJ, Pitlick S, Bulvik S. Treatment of West Nile virus encephalitis with intravenous immunoglobulin. Emerg Infect Dis. 2001;7:759. https://doi.org/10.3201/eid0704.017432
16. Ben-Nathan D, Gershoni-Yahalom O, Samina I, Khinich Y, Nur I, Laub O, et al. Using high titer West Nile intravenous immunoglobulin from selected Israeli donors for treatment of West Nile virus infection. BMC Infect Dis. 2009;9:18. https://doi.org/10.1186/1471-2334-9-18
17. Mahoney FI, Barthel DW. Functional evaluation: the Barthel Index. Md State Med J. 1965;14:61–5.
18. McDowell I, Kristjansson B, Hill GB, Hébert R. Community screening for dementia: the Mini Mental State Exam (MMSE) and Modified Mini-Mental State Exam (3MS) compared. J Clin Epidemiol. 1997;50:377–83. https://doi.org/10.1016/S0895-4356(97)00060-7
19. Teng EL, Chui HC. The Modified Mini-Mental State (3MS) examination. J Clin Psychiatry. 1987;48:314–8.
20. Martin DA, Noga A, Kosoy O, Johnson AJ, Petersen LR, Lanciotti RS. Evaluation of a diagnostic algorithm using immunoglobulin M enzyme-linked immunosorbent assay to differentiate human West Nile virus and St. Louis encephalitis virus infections during the 2002 West Nile virus epidemic in the United States. Clin Diagn Lab Immunol. 2004;11:1130–3.
21. Zhang W, Wu J, Li Y, Li F, Njoo H. Rapid and accurate in vitro assays for detection of West Nile virus in blood and tissues. Transfus Med Rev. 2009;23:146–54. https://doi.org/10.1016/j.tmrv.2008.12.008
22. Tyler KL, Pape J, Goody RJ, Corkill M, Kleinschmidt-DeMasters BK. CSF findings in 250 patients with serologically confirmed West Nile virus meningitis and encephalitis. Neurology. 2006;66:361–5. https://doi.org/10.1212/01.wnl.0000195890.70898.1f
23. Hart J Jr, Tillman G, Kraut MA, Chiang HS, Strain JF, Li Y, et al.; NIAID Collaborative Antiviral Study Group West Nile Virus 210 Protocol Team. West Nile virus neuroinvasive disease: neurological manifestations and prospective longitudinal outcomes. BMC Infect Dis. 2014;14:248. https://doi.org/10.1186/1471-2334-14-248
24. Haaland KY, Sadek J, Pergam S, Echevarria LA, Davis LE, Goade D, et al. Mental status after West Nile virus infection. Emerg Infect Dis. 2006;12:1260–2. https://doi.org/10.3201/eid1708.060097
25. Klee AL, Maidin B, Edwin B, Poshni I, Mostashari F, Fine A, et al. Long-term prognosis for clinical West Nile virus infection. Emerg Infect Dis. 2004;10:1405–11. https://doi.org/10.3201/eid1008.030879
26. Hughes JM, Wilson ME, Sejvar JJ. The long-term outcomes of human West Nile virus infection. Clin Infect Dis. 2007;44:1617–24. https://doi.org/10.1086/518281
27. Sejvar JJ, Curns AT, Welburg L, Jones JF, Lundgren LM, Capuron L, et al. Neurocognitive and functional outcomes in persons recovering from West Nile virus illness. J Neuropsychol. 2008;2:477–99. https://doi.org/10.1348/174866407X218312
28. Sejvar JJ, Haddad MB, Tierney BC, Campbell GL, Marfin AA, Van Gerpen JA, et al. Neurologic manifestations and outcome of West Nile virus infection. JAMA. 2003;290:511–5. https://doi.org/10.1001/jama.290.4.511
29. Morrey JD, Siddharthan V, Olsen AL, Roper GY, Wang H, Baldwin TJ, et al. Humanized monoclonal antibody against West Nile virus envelope protein administered after neuronal infection protects against lethal encephalitis in hamsters. J Infect Dis. 2006;194:1300–8. https://doi.org/10.1086/508293
30. Morrey JD, Siddharthan V, Wang H, Hall JO, Skirpstunas RT, Olsen AL, et al. West Nile virus-induced acute flaccid paralysis is prevented by monoclonal antibody treatment when administered after infection of spinal cord neurons. J Neurovirol. 2008;14:152–63. https://doi.org/10.1080/13550280801958930
31. Oliphant T, Engle M, Nybakken GE, Doane C, Johnson S, Huang L, et al. Development of a humanized monoclonal antibody with therapeutic potential against West Nile virus. Nat Med. 2005;11:522–30. https://doi.org/10.1038/nm1240
32. Throsby M, Geuijen C, Goudsmit J, Bakker AQ, Korimbocus J, Kramer RA, et al. Isolation and characterization of human

monoclonal antibodies from individuals infected with West Nile virus. J Virol. 2006;80:6982–92. https://doi.org/10.1128/JVI.00551-06
33. Diamond MS. Progress on the development of therapeutics against West Nile virus. Antiviral Res. 2009;83:214–27. https://doi.org/10.1016/j.antiviral.2009.05.006
34. Lim SP, Shi PY. West Nile virus drug discovery. Viruses. 2013;5:2977–3006. https://doi.org/10.3390/v5122977
35. Morrey JD, Taro BS, Siddharthan V, Wang H, Smee DF, Christensen AJ, et al. Efficacy of orally administered T-705 pyrazine analog on lethal West Nile virus infection in rodents. Antiviral Res. 2008;80:377–9. https://doi.org/10.1016/j.antiviral.2008.07.009
36. Jester PM, Tilden SJ, Li Y, Whitley RJ, Sullender WM. Regulatory challenges: lessons from recent West Nile virus trials in the United States. Contemp Clin Trials. 2006;27:254–9. https://doi.org/10.1016/j.cct.2006.02.004

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Serosurvey for Influenza D Virus Exposure in Cattle, United States, 2014–2015

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Influenza D virus has been detected predominantly in cattle from several countries. In the United States, regional and state seropositive rates for influenza D have previously been reported, but little information exists to evaluate national seroprevalence. We performed a serosurveillance study with 1,992 bovine serum samples collected across the country in 2014 and 2015. We found a high overall seropositive rate of 77.5% nationally; regional rates varied from 47.7% to 84.6%. Samples from the Upper Midwest and Mountain West regions showed the highest seropositive rates. In addition, seropositive samples were found in 41 of the 42 states from which cattle originated, demonstrating that influenza D virus circulated widely in cattle during this period. The distribution of influenza D virus in cattle from the United States highlights the need for greater understanding about pathogenesis, epidemiology, and the implications for animal health.

Influenza D virus (IDV; genus Deltainfluenzavirus, family Orthomyxoviridae) is an enveloped, single-stranded, negative sense RNA virus with 7 genome segments and 1 surface glycoprotein, the hemagglutinin-esterase fusion (HEF) protein (*1*,*2*). The first detection of IDV dates back to Oklahoma, USA, in 2011 from pigs exhibiting influenza-like disease (*3*), although retrospective seroprevalence data suggest the presence of IDV in goats in the United States before 2002 (*4*). Subsequently, IDV has been identified in low frequency in pigs in Italy (*5*,*6*) and Luxembourg (*7*). In addition, evidence suggests IDV circulates in other hosts such as small ruminants, camels, and buffalo in Togo, Kenya, and China (*8*,*9*) and small ruminants, feral swine, and equids in the United States (*4*,*10*,*11*).

Although IDV has been detected in other species, cattle appear to be the main reservoir (*1*,*12*). A variety of sample types and methods of detection have been used to determine the prevalence of IDV in different regions, in various ages, breeds, and numbers of cattle evaluated. The lack of consistency between the methods and cattle evaluated may be a contributing factor to variability in prevalence of IDV in different regions. Seroprevalence data have been reported in cattle from Luxembourg (*7*), Japan (*13*,*14*), the United States (*1*,*15*,*16*), Togo, Benin, and Morocco (*9*); the highest reported seropositive rate (80.2%) was in the United States (*16*) and Luxembourg (*7*) and the lowest (1.9%) in Benin (*9*). Serologic testing provides an indication of IDV exposure but is not a measure of active infections. IDV RNA from respiratory samples of cattle has been detected in several countries: the United States (*1*,*15*,*17*,*18*), Italy (*5*), France (*19*), Ireland (*20*), China (*8*,*21*), Japan (*22*), and Mexico (*18*). Studies from Mexico (*18*) reported the highest frequency of positive samples (29.7%) and China the lowest (0.7%) (*21*).

In both experimental and field infections with IDV, mild to moderate respiratory disease has been reported (*23*,*24*). In addition, IDV-positive samples are reported not only from cattle manifesting clinical signs associated with bovine respiratory disease but also from cattle that are asymptomatic and appear to be healthy (*20*–*22*). Experimental infection of calves demonstrated that IDV caused mild to moderate respiratory disease and that peak viral shedding occurred at 4–6 days postinfection; seroconversion was detected as early as day 6 postinfection (*12*,*23*,*24*). Whereas IDV infection by itself has been associated mainly with mild respiratory illness, IDV has also been implicated as a contributor to bovine respiratory disease complex (BRDC), which is the most costly disease affecting the US cattle industry (*17*,*18*,*23*,*25*).

Because there are no commercially available vaccines against IDV, positive serologic assays reflect natural

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exposure. Given the potential of IDV to contribute to BRDC, inclusion of IDV in vaccination programs has been debated. The frequency of IDV RNA–positive samples from US cattle is 4.8%–18% (*1*,*15*,*17*,*18*), and positive samples have been reported in the US cattle population since 2003 (*16*). The seropositive rate has been reported at 13.5%–80.2% (*15*,*16*); the Upper Midwest region has the highest seroprevalence. The wide variation of seroprevalence could be caused by differences in the age of the cattle evaluated or by differences across regions because of limited sample size and the focus on the Midwest and South Central regions of the country. We conducted a national serosurvey of cattle of a similar age to fully evaluate the potential role of IDV in BRDC infections and the effect of IDV on animal health and productivity.

Materials and Methods

Samples

We assessed 1,992 banked bovine serum samples for IDV-specific antibodies. The samples, collected between August 2014 and December 2015 as part of the US brucellosis surveillance program, were previously used to screen for ruminant pestivirus and bovine leukemia virus (BLV) exposure (*26*,*27*). We aimed to determine the seropositivity rate for IDV and retrospectively compare that rate with seropositivity rates for ruminant pestivirus and BLV from the same samples to identify regional patterns or differences in the US cattle population.

The serum samples came from both male and female cattle ≥2 years of age, raised in 42 states, and were randomly collected from 5 slaughter plants. The states were categorized into 6 regions as previously defined (*26*): Pacific West (PW), Mountain West (MW), Upper Midwest (UMW), South Central (SC), Northeast (NE), and Southeast (SE) (Figure 1). The number of samples taken in each slaughter plant, listed by state (California, Florida, Nebraska, Pennsylvania, Minnesota), was proportional to the total annual number of cattle ≥2 years of age that had been processed in that plant. All samples were previously reported as negative for brucellosis.

Virus Selection and Propagation

To select the IDV strain used for the hemagglutination inhibition (HI) assay, we performed phylogenetic analysis on HEF genes with IDV strains that circulated in the United States during the same period in which the samples used for this study were collected (Figure 2). We down-

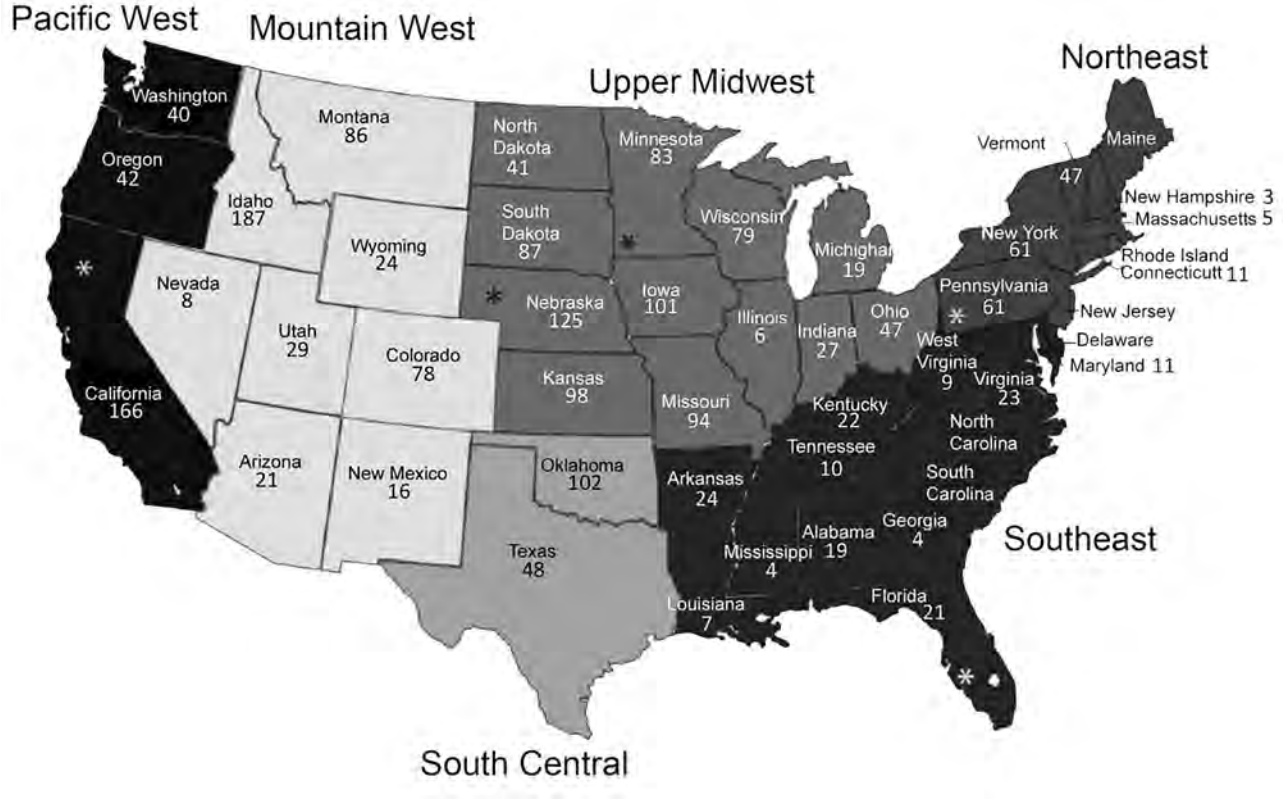

Figure 1. Number of samples collected 42 states in study of influenza D virus in cattle, United States, 2014–2015. Asterisks (*) indicate states with 1 slaughter plant that contributed samples. Alaska, Hawaii, and states without numbers did not contribute samples.

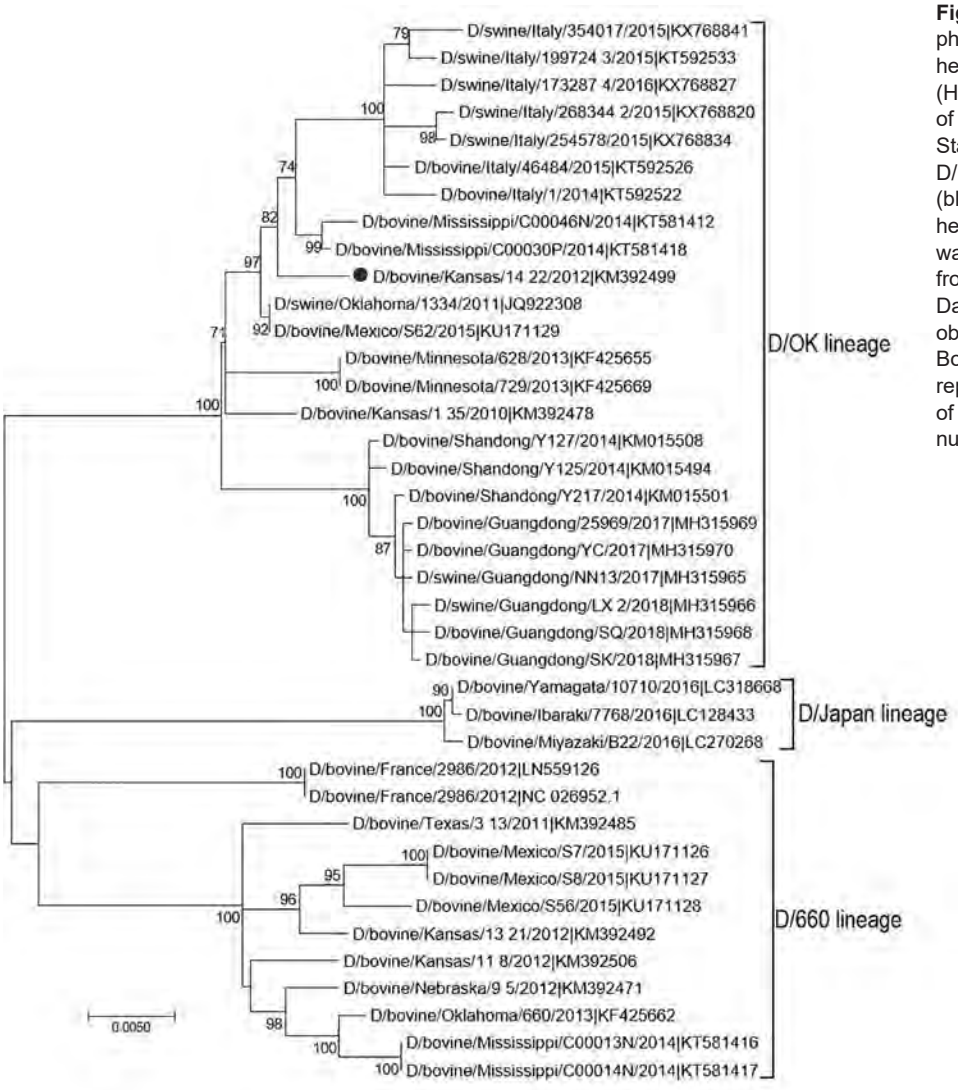

Figure 2. Maximum-likelihood phylogeny of the influenza D virus hemagglutinin-esterase fusion (HEF) gene constructed for study of influenza D virus in cattle, United States. Representative US strain D/bovine/Kansas/14-22/2012 (black dot), used as antigen in hemagglutination inhibition analysis, was aligned with reference strains from the Influenza Research Database (http://www.fludb.org) obtained on September 28, 2018. Bootstrap values >70% (1,000 replicates) are shown to the right of the nodes. Scale bar represents nucleotide substitutions per site.

loaded full-length HEF gene segment sequences (n = 39) from the Influenza Research Database (http://www.fludb.org) on September 28, 2018. We aligned full-length segments using the MAFFT plug-in for Geneious version 9.1.4 (Biomatters Ltd., http://www.geneious.com) with subsequent manual correction. We constructed a maximum-likelihood tree inferred in IQ-tree (http://www.iqtree.org) using a general time-reversible model of nucleotide substitution combined with a gamma-distributed rate variation with statistical support generated through ultrafast bootstrap analysis (*28*,*29*). We chose a representative US strain, D/bovine/Kansas/14-22/12, showing a high amino acid similarity (96%–99.2%) with US strains detected during 2014–2015, and a high hemagglutination (HA) titer.

We maintained swine testicle cells (ATCC CRL-1746) used for propagation of IDV in MEM medium (Sigma Aldrich, https://www.sigmaaldrich.com), supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (PAA Laboratories, Inc., https://www.fishersci.com) and L-glutamine (ThermoFisher Scientific, https://www.thermofisher.com) antibiotic-antimycotic solution incubated at 37°C in a humid atmosphere of 5% CO_2. We propagated the D/bovine/Kansas/14-22/12 strain, diluted 1:1,000 in swine testicle cells cultured in serum-free medium in the presence of TPCK-trypsin (0.1 μg/mL) and 5% bovine serum albumin, and incubated at 37°C for up to 4 days.

Serology

We performed the HI assay for detection of D/bovine/Kansas/14-22/12–specific antibodies in accordance with the specifications in the World Health Organization manual on animal influenza A virus diagnosis and surveillance (*30*). We treated 1:3 serum samples with receptor-destroying enzyme (Denka Seiken UK, http://www.denka-seiken.jp) at 37°C for 18 hours, heat inactivated it at 56°C for 1 h, and diluted it 1:10 with phosphate-buffered saline.

We conducted the assay in duplicate, at room temperature and in V-bottom 96-well plates, starting at 1:10 and doing 2-fold serial dilutions to reach a 1:1,280 dilution. We added the serially diluted samples to the virus (8 hemagglutination units/50 μL) for 1 h, then incubated with 0.5% turkey red blood cells for 30 min. The endpoint titer was the reciprocal of the highest dilution of serum that demonstrated partial to full inhibition of hemagglutination. We determined that a serum with an HI titer ≥40 was seropositive according to previous IDV serosurveillance studies (*4*,*15*). We used a negative control (PBS), as well as a positive control consisting of a rabbit polyclonal antiserum generated against D/swine/OK/1334/2011, in the HI assay (*1*). To exclude the possible presence of nonspecific antibodies, we also performed HI with serum samples from 10 colostrum-deprived calves; all showed titers of 0, which confirmed negativity.

Statistical Analysis

We used GraphPad Prism 7 software (GraphPad Software, LLC, https://www.graphpad.com) to statistically compare seropositive rates of IDV infection by χ^2 test and geometric mean titers (GMT) by the Kruskal-Wallis and Mann-Whitney tests. We considered $p<0.05$ significant.

Results

Of the 1,992 cattle serum samples tested by HI assay for detection of IDV-specific antibodies, 1,545 (77.5%) samples were positive; the overall GMT of positive samples was 230 (titers ranged from 40 to 1,280). We identified positive serum in samples from 41 of the 42 states tested (Table). The seropositivity rate was 25%–93.8% among the states and average GMT was 80–460. However, sample size was small in some of the states with low positivity, low titer, or both, which might have caused bias in the regional distribution.

We categorized the results by geographic region to compare the differences of seropositive rate and GMT. The seropositive rate range was 47.7%–84.6% ($p<0.05$) and GMT 110–260 ($p<0.05$) among the regions (Table). Mountain West region had the highest seropositive rate (84.6%) and GMT (260); Northeast region had the lowest seropositive rate (47.7%) and GMT (110).

Discussion

Although IDV was described in pigs earlier than in cattle in the United States, subsequent reports of retrospective samples suggested that cattle are the natural reservoir (*1*,*12*). Because seroprevalence surveillance in US cattle had been conducted only at state or regional levels, we undertook a nationwide serologic survey to detect IDV antibodies in cattle. Our results clearly demonstrate that IDV circulated with high frequency in cattle in all regions of the United States during 2014–2015.

We observed regional variation in seropositive rate and GMT, although all regions had relatively high frequency. Overall, the Upper Midwest and Mountain West

Table. Serosurveillance results for influenza D virus in cattle, by region and state, United States, 2014–2015*

Region and state	No. samples	Seropositive rate, %†	GMT (range)‡
Mountain West			
Idaho	187	87.2	230 (40–1,280)
Montana	86	84.9	270 (40–1,280)
Colorado	78	88.5	330 (40–1,280)
Utah	29	79.3	240 (80–1,280)
Wyoming	24	79.2	460 (80–1,280)
Arizona	21	57.1	140 (40–1,280)
New Mexico	16	93.8	210 (40–1,280)
Nevada	8	75.0	250 (80–1,280)
Total	449	84.6	260 (40–1,280)
Upper Midwest			
Nebraska	125	91.2	260 (40–1,280)
Iowa	101	92.1	270 (40–1,280)
Kansas	98	86.7	300 (40–1,280)
Missouri	94	86.2	220 (40–1,280)
South Dakota	87	90.8	300 (40–1,280)
Minnesota	83	89.2	280 (40–1,280)
Wisconsin	79	84.8	250 (40–1,280)
Ohio	47	48.9	130 (40–640)
North Dakota	41	56.1	400 (40–1,280)
Indiana	27	37.0	120 (40–640)
Michigan	19	63.2	190 (40–1,280)
Illinois	6	83.3	160 (80–320)
Total	807	84.0	260 (40–1,280)
South Central			
Oklahoma	102	79.4	230 (40–1,280)
Texas	48	75.0	170 (40–1,280)
Total	150	78.0	210 (40–1,280)
Pacific West			
California	166	77.7	190 (40–1,280)
Oregon	42	76.2	300 (40–1,280)
Washington	40	72.5	230 (40–1,280)
Total	248	76.7	210 (40–1,280)
Southeast			
Arkansas	24	83.3	180 (40–640)
Virginia	23	43.5	130 (40–640)
Kentucky	22	68.2	310 (80–1,280)
Florida	21	57.1	170 (40–1,280)
Alabama	19	68.4	140 (40–1,280)
Tennessee	10	50.0	240 (80–640)
West Virginia	9	33.3	200 (80–640)
Louisiana	7	42.9	160 (80–320)
Mississippi	4	25.0	80 (80–80)
Georgia	4	75.0	160 (80–320)
Total	143	59.5	180 (40–1,280)
Northeast			
Pennsylvania	61	50.8	120 (40–1,280)
New York	61	45.9	110 (40–1,280)
Vermont	47	51.1	110 (40–640)
Connecticut	11	0	0
Maryland	7	71.4	110 (40–1,280)
Massachusetts	5	60.0	80 (80–80)
New Hampshire	3	66.7	80 (80–80)
Total	195	47.7	110 (40–1,280)

*GMT, geometric mean titer.
†Seropositive rate was calculated using those samples with hemagglutination inhibition titer ≥40.
‡GMT was calculated using those samples with HI titer ≥40. Lowest and highest titers were measured from those samples with HIT titer ≥40.

regions showed the highest seropositive rates and the highest antibody titers, and also encompassed the states with the highest GMT. A similar result was obtained in a pestivirus serologic study performed with the same serum samples; here too, the Mountain West region showed the highest number of antibody-positive animals and higher titers (*26*). Although it is not possible to establish the cause, both pestivirus and IDV serology follow a similar trend. Potential causes include herd size, which can exceed 1,000 animals in these areas, and the potential for livestock and wildlife species to commingle and facilitate virus transmission. Evidence indicates IDV can infect nonbovine hosts, such as sheep, goats, pigs, and equids, in the United States (*4*,*10*,*31*). However, the full range of susceptible hosts for IDV is unknown, and interspecies transmission has not been demonstrated among the known hosts.

Seroprevalence of IDV in small ruminants was reported in samples collected from the Mountain West and Upper Midwest regions, whereas samples from other regions were negative (*4*). Moreover, in the Upper Midwest region, a high percentage of small ruminants with high titers was described, and the farms where they were located were in close proximity to cattle farms (*4*). This issue needs to be explored further to understand the importance of IDV as a threat for animal health and whether this is an underlying factor for the increased seroprevalence of viral pathogens in regions that have greater potential for interspecies transmission.

In general, we observed lower titers and a lower percentage of positive animals in the Northeast and Southeast regions. These results are similar to those reported from the pestivirus serosurvey that also found these 2 regions to have the lowest titers and lowest number of cattle seropositive for BVDV (*26*). On the other hand, in the BLV serosurvey, the Northeast had the highest seropositive rate for BLV and the Mountain West, the lowest seropositive rate (*27*). Although seroprevalence differences existed between BLV and the other viruses evaluated (pestivirus and IDV), these differences could be caused by limited number of samples collected in these regions, differences in the epidemiology of these viruses, or differences in herd management practices across the regions. Previous data of IDV exposure in cattle of different ages in Mississippi (Southeast region) reported a high seroprevalence in cattle >1 year of age (*15*). Discrepancies between the current study and the previous reports could be explained by the number of samples evaluated in each of these studies; only 4 samples originated from Mississippi in our study, whereas ≥500 cattle were sampled in a previous study (*15*). Although our study encompassed the entire United States, the limited number of samples from several states, and subsequently the regions they represent, may have caused underestimation or overestimation of the seropositive rate of IDV. Despite the limitations of our study, data indicate that IDV is widespread at rates similar to the regional or state data previously reported (*15*,*16*).

Our findings, combined with those from previous serosurveillance studies (*15*,*16*), confirm a high nationwide seroprevalence of IDV in US cattle populations. Because of the potential association of IDV with BRDC (*17*,*18*,*23*,*31*) and the dearth of vaccines to prevent IDV infection (*12*,*32*), concerns have been raised regarding the negative effect of IDV on animal health. A possible explanation for the high seropositive rate is that IDV is common in the respiratory tract of cattle; times of stress, immune attack, or environmental changes that affect the respiratory tract can increase viral shedding but might not cause disease. Unpublished diagnostic data from our laboratory show that IDV is detected more frequently in samples that are also positive for other respiratory pathogens than in those positive for IDV alone. This finding indicates that IDV can either predispose the respiratory tract or act as an opportunistic pathogen in concert with other pathogens to cause BRD. Further research, including co-infection studies, is needed to elucidate the full range of susceptible hosts and the dynamics of interspecies transmission to understand the contribution of IDV to BRDC. In summary, our serosurveillance study of bovine serum samples from 2014–2015 showed a high seropositivity rate for IDV in the United States; 41 of the 42 states from which cattle originated had seropositive animals. No IDV vaccine exists. IDV infection has also been implicated in BRDC, the most costly disease affecting the US cattle industry. Therefore, our findings may indicate an ongoing risk to animal health.

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References

1. Hause BM, Collin EA, Liu R, Huang B, Sheng Z, Lu W, et al. Characterization of a novel influenza virus in cattle and swine: proposal for a new genus in the *Orthomyxoviridae* family. MBio. 2014;5:e00031–14. https://doi.org/10.1128/mBio.00031-14
2. International Committee on Taxonomy of Viruses. Taxonomy. 2017 [cited 2019 Aug 28]. https://talk.ictvonline.org/taxonomy
3. Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. PLoS Pathog. 2013;9:e1003176. https://doi.org/10.1371/journal.ppat.1003176
4. Quast M, Sreenivasan C, Sexton G, Nedland H, Singrey A, Fawcett L, et al. Serological evidence for the presence of influenza D virus in small ruminants. Vet Microbiol. 2015;180:281–5. https://doi.org/10.1016/j.vetmic.2015.09.005
5. Chiapponi C, Faccini S, De Mattia A, Baioni L, Barbieri I, Rosignoli C, et al. Detection of influenza D virus among swine and cattle, Italy. Emerg Infect Dis. 2016;22:352–4. https://doi.org/10.3201/eid2202.151439
6. Foni E, Chiapponi C, Baioni L, Zanni I, Merenda M, Rosignoli C, et al. Influenza D in Italy: towards a better understanding of an emerging viral infection in swine. Sci Rep. 2017;7:11660. https://doi.org/10.1038/s41598-017-12012-3
7. Snoeck CJ, Oliva J, Pauly M, Losch S, Wildschutz F, Muller CP, et al. Influenza D virus circulation in cattle and swine, Luxembourg, 2012–2016. Emerg Infect Dis. 2018;24:1388–9. https://doi.org/10.3201/eid2407.171937
8. Zhai S-L, Zhang H, Chen S-N, Zhou X, Lin T, Liu R, et al. Influenza D virus in animal species in Guangdong Province, southern China. Emerg Infect Dis. 2017;23:1392–6 https://doi.org/10.3201/eid2308.170059
9. Salem E, Cook EAJ, Lbacha HA, Oliva J, Awoume F, Aplogan GL, et al. Serologic evidence for influenza C and D virus among ruminants and camelids, Africa, 1991–2015. Emerg Infect Dis. 2017;23:1556–9. https://doi.org/10.3201/eid2309.170342
10. Nedland H, Wollman J, Sreenivasan C, Quast M, Singrey A, Fawcett L, et al. Serological evidence for the co-circulation of two lineages of influenza D viruses in equine populations of the Midwest United States. Zoonoses Public Health. 2018;65:e148–54. https://doi.org/10.1111/zph.12423
11. Ferguson L, Luo K, Olivier AK, Cunningham FL, Blackmon S, Hanson-Dorr K, et al. Influenza D virus infection in feral swine populations, United States. Emerg Infect Dis. 2018;24:1020–8. https://doi.org/10.3201/eid2406.172102
12. Hause BM, Huntimer L, Falkenberg S, Henningson J, Lechtenberg K, Halbur T. An inactivated influenza D virus vaccine partially protects cattle from respiratory disease caused by homologous challenge. Vet Microbiol. 2017;199:47–53. https://doi.org/10.1016/j.vetmic.2016.12.024
13. Horimoto T, Hiono T, Mekata H, Odagiri T, Lei Z, Kobayashi T, et al. Nationwide distribution of bovine influenza D virus infection in Japan. PLoS One. 2016;11:e0163828. https://doi.org/10.1371/journal.pone.0163828
14. Murakami S, Endoh M, Kobayashi T, Takenaka-Uema A, Chambers JK, Uchida K, et al. Influenza D virus infection in herd of cattle, Japan. Emerg Infect Dis. 2016;22:1517–9. https://doi.org/10.3201/eid2208.160362
15. Ferguson L, Eckard L, Epperson WB, Long LP, Smith D, Huston C, et al. Influenza D virus infection in Mississippi beef cattle. Virology. 2015;486:28–34. https://doi.org/10.1016/j.virol.2015.08.030
16. Luo J, Ferguson L, Smith DR, Woolums AR, Epperson WB, Wan XF. Serological evidence for high prevalence of influenza D viruses in cattle, Nebraska, United States, 2003–2004. Virology. 2017;501:88–91. https://doi.org/10.1016/j.virol.2016.11.004
17. Collin EA, Sheng Z, Lang Y, Ma W, Hause BM, Li F. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. J Virol. 2015;89:1036–42. https://doi.org/10.1128/JVI.02718-14
18. Mitra N, Cernicchiaro N, Torres S, Li F, Hause BM. Metagenomic characterization of the virome associated with bovine respiratory disease in feedlot cattle identified novel viruses and suggests an etiologic role for influenza D virus. J Gen Virol. 2016;97:1771–84. https://doi.org/10.1099/jgv.0.000492
19. Ducatez MF, Pelletier C, Meyer G. Influenza D virus in cattle, France, 2011–2014. Emerg Infect Dis. 2015;21:368–71. https://doi.org/10.3201/eid2102.141449
20. Flynn O, Gallagher C, Mooney J, Irvine C, Ducatez M, Hause B, et al. Influenza D virus in cattle, Ireland. Emerg Infect Dis. 2018;24:389–91. https://doi.org/10.3201/eid2402.170759
21. Jiang W-M, Wang S-C, Peng C, Yu J-M, Zhuang Q-Y, Hou G-Y, et al. Identification of a potential novel type of influenza virus in bovine in China. Virus Genes. 2014;49:493–6. https://doi.org/10.1007/s11262-014-1107-3
22. Mekata H, Yamamoto M, Hamabe S, Tanaka H, Omatsu T, Mizutani T, et al. Molecular epidemiological survey and phylogenetic analysis of bovine influenza D virus in Japan. Transbound Emerg Dis. 2018;65:e355–60. https://doi.org/10.1111/tbed.12765
23. Ferguson L, Olivier AK, Genova S, Epperson WB, Smith DR, Schneider L, et al. Pathogenesis of influenza D virus in cattle. J Virol. 2016;90:5636–42. https://doi.org/10.1128/JVI.03122-15
24. Salem E, Hägglund S, Cassard H, Corre T, Näslund K, Foret C, et al. Pathogenesis, host innate immune response and aerosol transmission of Influenza D virus in cattle. J Virol. 2019; 93:JVI.01853-18. https://doi.org/10.1128/JVI.01853-18.
25. Ng TFF, Kondov NO, Deng X, Van Eenennaam A, Neibergs HL, Delwart E. A metagenomics and case-control study to identify viruses associated with bovine respiratory disease. J Virol. 2015;89:5340–9 https://doi.org/10.1128/JVI.00064-15
26. Bauermann FV, Ridpath JF, Dargatz DA. A serosurvey for ruminant pestivirus exposure conducted using cattle sera collected for

brucellosis surveillance in the United States. J Vet Diagn Invest. 2017;29:76–82. https://doi.org/10.1177/1040638716680251
27. Bauermann FV, Ridpath JF, Dargatz DA. Bovine leukemia virus seroprevalence among cattle presented for slaughter in the United States. J Vet Diagn Invest. 2017;29:704–6. https://doi.org/10.1177/1040638717702183
28. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32:268–74. https://doi.org/10.1093/molbev/msu300
29. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. UFBoot2: Improving the ultrafast bootstrap approximation. Mol Biol Evol. 2018;35:518–22. https://doi.org/10.1093/molbev/msx281
30. World Health Organization. Manual for the laboratory diagnosis and virological surveillance of influenza. 2011 [cited 2019 Aug 28]. http://www.who.int/influenza/gisrs_laboratory/manual_diagnosis_surveillance_influenza
31. US Department of Agriculture. Feedlot 2011 part IV: health and health management on US feedlots with a capacity of 1,000 or more head. 2013 [cited 2019 Aug 28]. https://www.aphis.usda.gov/animal_health/nahms/feedlot/downloads/feedlot2011/Feed11_dr_PartIV.pdf
32. Wan Y, Kang G, Sreenivasan C, Daharsh L, Zhang J, Fan W, et al. A DNA vaccine expressing consensus hemagglutinin-esterase fusion protein protected guinea pigs from infection by two lineages of influenza D virus. 2018. J Virol. 2018;92:e00110-18. https://doi.org/10.1128/JVI.00110-18

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High Prevalence of *Mansonella ozzardi* Infection in the Amazon Region, Ecuador

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We reviewed Giemsa-stained thick blood smears, obtained through the national malaria surveillance program in the Amazon region of Ecuador, by light microscopy for *Mansonella* spp. microfilariae. Of 2,756 slides examined, 566 (20.5%) were positive. Nested PCR confirmed that the microfilariae were those of *M. ozzardi* nematodes, indicating that this parasite is endemic to this region.

Although mansonelliases is probably the most prevalent filarial infection worldwide, it is the least studied and is considered a neglected parasite infection (*1*,*2*). Human infection with members of the filarial nematode genus *Mansonella*, including *M. ozzardi*, *M. perstans*, and *M. streptocerca* nematodes, is common and widespread in the Western Hemisphere and Africa. *M. ozzardi* nematodes are found exclusively in the Western Hemisphere from southern Mexico to northwestern Argentina but have not been reported in Ecuador, Chile, Uruguay, and Paraguay (*1*,*2*). *M. perstans* infections are found mainly in sub-Saharan Africa, with sporadic cases in a few countries in South America, whereas *M. streptocerca* infections are found only in Africa (*1*–*4*).

Epidemiologic studies have reported that *M. ozzardi* nematodes are highly prevalent in the Amazon Basin (Brazil, Colombia, Peru, Bolivia, Venezuela, and Argentina) and on some Caribbean islands. In the general population, the prevalence rate of infection ranges from 0% to 46%; however, in some areas it is 92.3% (*2*).

Community health workers from the malaria control program in the Amazon region of Ecuador have reported that filaria-like nematodes are seen in thick blood smears, suggesting the presence of a *Mansonella* sp. in this area. *Mansonella* spp. nematodes are transmitted by dipteran flies. In particular, *M. ozzardi* nematodes are transmitted by biting midges of the genera *Culicoides* and by black flies from the genus *Simulium* (*1*). Both of these vectors are present in the Amazon region of Ecuador (Renato Leon, Universidad San Francisco de Quito, pers. comm., 2018 Dec 5).

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Endemic areas for malaria and *M. ozzardi* infection often overlap; thus, microfilariae might be found on thick blood smears prepared for malaria diagnosis (*2*). Microfilariae of *M. ozzardi* can be easily distinguished morphologically from those of *M. perstans* by examination of the tail end: unlike *M. ozzardi* nematodes, the tails of *M. perstans* nematodes are blunt and have nuclei extending to the tail end (*5*). PCR-based amplification of species-specific target sequences results in increased diagnostic sensitivity and reliable differentiation between *M. ozzardi* nematodes and coendemic filarial species, such as *M. perstans* and *Onchocerca volvulus* nematodes (*6*). Therefore, we conducted a retrospective study to document presence of human infections with *Mansonella* spp. nematodes in the Amazon region of Ecuador. The study protocol was approved by the Ethics Committee of the Instituto Nacional de Investigación en Salud Pública (CEISH-INSPI-013).

The Study

We conducted a study in 5 provinces (Sucumbíos, Orellana, Napo, Pastaza, and Morona Santiago) in the Amazon rainforest region of Ecuador for which malaria slides were available. The Amazon region of Ecuador covers ≈40% of the area of this country, extends from the eastern Andes to the lowlands of the Amazon basin, and borders Colombia and Peru.

The study population was composed of mestizos and Kichwa, Shuar, and Achuar indigenous groups. Community health workers collected blood samples by finger prick from 7:00 AM until 7:00 PM from persons suspected of having malaria. Malaria centers are located throughout the rainforest in an ongoing program for malaria control under the guidance of the Ministry of Public Health.

Thick and thin blood smears were stained with Giemsa and viewed by microscopy at 100× magnification under oil immersion for *Plasmodium* spp. parasites. We retrospectively screened all slides obtained during 2014–2015 for *Mansonella* spp. nematodes.

Although >5,000 stained blood smears were reviewed, only 2,756 slides could be read because of poor preservation. We examined thick and thin blood smears by using light microscopy and a 20× objective lens to detect microfilariae and a 40× objective lens for species identification. No epidemiologic data were available because we conducted a retrospective analysis.

Of 2,756 Giemsa-stained blood smears examined, we detected microfilariae of *Mansonella* spp. nematodes in 566 (20.5%). Microfilariae were unsheathed (average length 155 μm–212 μm) and had tapered, nonnucleated tails; anterior extremities ended in cephalic spaces. On the basis of these morphologic characteristics, we identified all filarial infections as *M. ozzardi* nematodes. Many microfilariae appeared damaged and partially destroyed. No microfilariae with the characteristics of *M. perstans* nematodes were observed. Infection rates between provinces ranged from 5.2% to 36.5%; infections were most prevalent in Morona Santiago, which borders Peru (Figure 1).

We then extracted DNA from all microscope slides positive for microfilariae. We scraped blood films off the slides into 70% ethanol. After microcentrifugation at 8,000 rpm for 2 min, we subjected supernatants to DNA extraction by using Chelex treatment (*7*), followed by proteinase K digestion. We performed a nested PCR according to the method of Tang et al. (*6*). This PCR amplifies the internal transcribed spacer region 1 of the rDNA gene of filarial species. The size of this region varies among *O. volvulus*, *M. ozzardi*, and *M. perstans* nematodes, and the PCR yields amplicons of different sizes for each species (*6*). Expected product sizes were 305 bp for *M. ozzardi* nematodes and 312 bp for *M. perstans* nematodes. PCR products after the second amplification were subjected to electrophoresis on 2% agarose gels (Figure 2).

Conclusions

We report a high frequency of autochthonous human mansonelliasis caused by *M. ozzardi* nematodes in the Amazon region of Ecuador. These parasites were identified by microscope identification of characteristic microfilariae and detection of species-specific DNA.

This study showed that human infections with *M. ozzardi* nematodes are highly prevalent throughout the Amazon region of Ecuador. High rates of circulating microfilariae are strongly suggestive of active local transmission, particularly because known vectors (biting midges of the genus *Culicoides* and black flies of the genus *Simulium*) are present in the surveyed region. Further entomologic studies are needed to identify specific Diptera species involved in local transmission of *M. ozzardi* nematodes in Ecuador.

Prevalence of *M. ozzardi* infection ranged from 5.2% to 36.5% in this study, similar to infection rates reported in bordering regions of Colombia and Peru, indicating that this region is probably a large focus of infection extending throughout several countries in the Amazon region (*2*). *M. ozzardi* infection rates were higher than those reported from Brazil, but considerably lower than those for the indigenous population of El Vaupés in Colombia, where the infection rate was 96% (*8*). However, in estimating prevalence by using stored, partially degraded blood smears and light microscopy, we might have underestimated the true prevalence. Use of FTA cards (Whatman, https://www.sigmaaldrich.com) has been demonstrated to be more sensitive for detection of *M. ozzardi* nematodes (*9*).

The morphologic features of microfilariae we observed were typical for *M. ozzardi* nematodes, and nested PCR confirmed detection of a DNA fragment (305 bp) known to be specific for this parasite. This assay used universal filariae PCR primers to amplify a variable portion of filarial parasite internal transcribed spacer 1 rDNA gene and enabled subsequent differentiation of species on the basis of the size of the

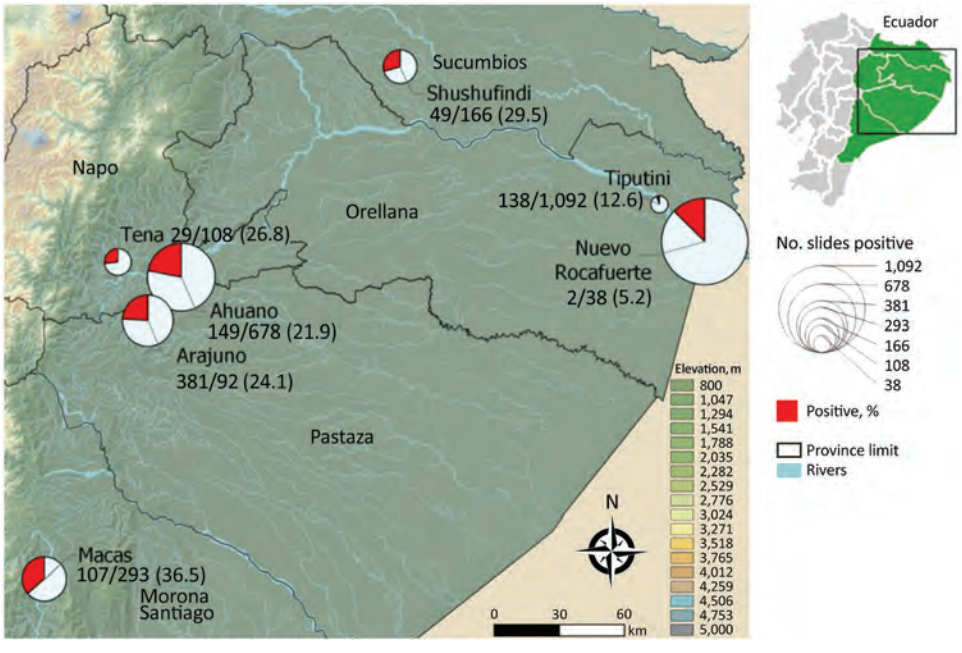

Figure 1. Amazon region of Ecuador where testing for *Mansonella ozzardi* microfilariae in humans was conducted. Of 2,756 archived slides from human infections, 566 (20.5%) were positive for this parasite. Values are no. positive/no. tested (%). Inset shows location of study area within Ecuador.

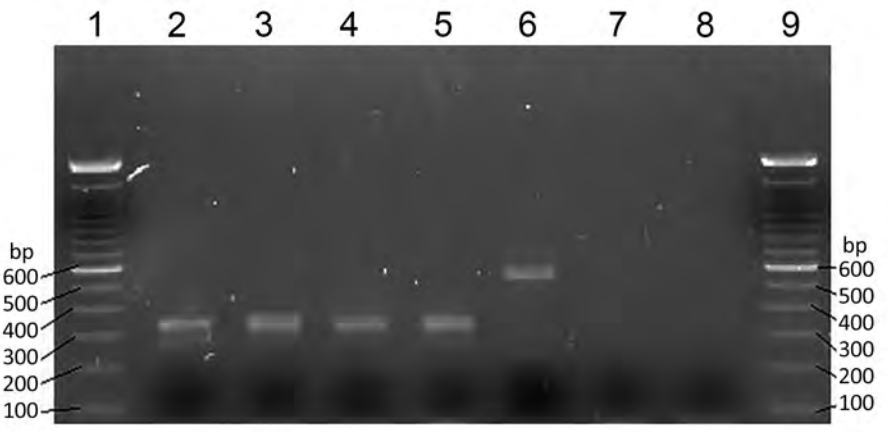

Figure 2. Nested PCR amplification products for *Mansonella ozzardi* microfilariae obtained from archived human samples in the Amazon region of Ecuador. Samples were subjected to electrophoresis on a 2% agarose gel. Lanes 1 and 9, 100-bp molecular mass ladders; lanes 2, 3, 4, and 5, *M. ozzardi* nematode-positive samples (sample nos. 14, 53, 27, and 25, respectively) that yielded a 305-bp fragment; lane 6, *Toxocara canis* roundworm (610-bp fragment); lane 7, *M. ozzardi* nematode–negative thick blood smear; lane 8, negative control. These PCR results confirmed data obtained by microscopy. Morphologic characteristics of and DNA findings for the microfilariae indicated that this parasite was an *M. ozzardi* nematode.

amplified fragment (*6*). In addition, DNA was successfully extracted from scrapings of thick and thin dried blood films, even after long storage periods of 2–3 years.

We observed no microfilariae of *M. perstans* nematodes, and no positive samples showed DNA fragment sizes representative of this nematode (*6*). *M. perstans* nematodes have been reported only in the northern part of the Amazon rainforest from equatorial Brazil to the Caribbean coast of South America (*1*). In Colombia, *M. perstans* nematodes have been observed in a restricted area among the Curripacos Amerindians in the Comisaría del Guainia region bordering Venezuela (*10*), but have not been found in the Peruvian Amazon (*1*,*2*). However, further prospective studies using molecular techniques are needed to clarify the epidemiologic status of this parasite.

In summary, we retrospectively identified human infection with *M. ozzardi* nematodes in the Amazon region of Ecuador. Our findings confirm that this parasite is endemic to this region.

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References

1. Ta-Tang TH, Crainey JL, Post RJ, Luz SL, Rubio JM. Mansonellosis: current perspectives. Res Rep Trop Med. 2018; 9:9–24. https://doi.org/10.2147/RRTM.S125750
2. Lima NF, Veggiani Aybar CA, Dantur Juri MJ, Ferreira MU. *Mansonella ozzardi*: a neglected New World filarial nematode. Pathog Glob Health. 2016;110:97–107. https://doi.org/10.1080/20477724.2016.1190544
3. Gobbi F, Beltrame A, Buonfrate D, Staffolani S, Degani M, Gobbo M, et al. Imported Infections with *Mansonella perstans* nematodes, Italy. Emerg Infect Dis. 2017;23:1539–42. https://doi.org/10.3201/eid2309.170263
4. Tavares da Silva LB, Crainey JL, Ribeiro da Silva TR, Suwa UF, Vicente AC, Fernandes de Medeiros J, et al. Molecular verification of New World *Mansonella perstans* parasitemias. Emerg Infect Dis. 2017;23:545–7. https://doi.org/10.3201/eid2303.161159
5. Centers for Disease Control and Prevention. DPDx: laboratory identification of parasites of public health concern [cited 2019 Aug 13]. https://www.cdc.gov/dpdx/index.html
6. Tang TH, López-Vélez R, Lanza M, Shelley AJ, Rubio JM, Luz SL. Nested PCR to detect and distinguish the sympatric filarial species *Onchocerca volvulus, Mansonella ozzardi* and *Mansonella perstans* in the Amazon Region. Mem Inst Oswaldo Cruz. 2010;105:823–8. https://doi.org/10.1590/S0074-02762010000600016
7. Suenaga E, Nakamura H. Evaluation of three methods for effective extraction of DNA from human hair. J Chromatogr B Analyt Technol Biomed Life Sci. 2005;820:137–41. https://doi.org/10.1016/j.jchromb.2004.11.028
8. Marinkelle CJ, German E. Mansonelliasis in the police station of Vaupes, Colombia [in Spanish]. Trop Geogr Med. 1970;22:101–11.
9. Medeiros JF, Almeida TA, Silva LB, Rubio JM, Crainey JL, Pessoa FA, et al. A field trial of a PCR-based *Mansonella ozzardi* diagnosis assay detects high-levels of submicroscopic *M. ozzardi* infections in both venous blood samples and FTA card dried blood spots. Parasit Vectors. 2015;8:280. https://doi.org/10.1186/s13071-015-0889-z
10. Kozek WJ, D'Alessandro A, Hoyos M. Filariasis in Colombia: presence of *Dipetalonema perstans* in the Comisaría del Guainía. Am J Trop Med Hyg. 1982;31:486–9. https://doi.org/10.4269/ajtmh.1982.31.486

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Clinical REsearch During Outbreaks (CREDO) Training for Low- and Middle-Income Countries

Nzelle Delphine Kayem, Amanda Rojek, Emmanuelle Denis, Alex Salam, Andreas Reis, Piero Olliaro, Peter Horby

We describe a pilot of the Clinical REsearch During Outbreaks (CREDO) initiative, a training curriculum for researchers in epidemic-prone low- and middle-income countries who may respond to disease outbreaks. Participants reported improved confidence in their ability to conduct such research and overall satisfaction with the course structure, content, and training.

Clinical research is an essential component of outbreak response because it underpins clinical management and public health measures (*1*). However, conducting clinical research during epidemics is challenging (*2*,*3*). The Ebola epidemic in West Africa underscored the need for improved research infrastructure and capacity in epidemic-prone vulnerable low- and middle-income countries (LMICs) (*4*). To address this need, a training curriculum, Clinical REsearch During Outbreaks (CREDO), was jointly developed by the Special Programme for Research and Training in Tropical Diseases (TDR), hosted by the World Health Organization, the International Severe Acute Respiratory and Emerging Infections Consortium, and the United Kingdom Public Health Rapid Support Team. The main objective of the CREDO training curriculum is to strengthen the national capacities of LMICs to design and implement clinical research during outbreaks of infectious diseases and to improve team capacity and team effort. We describe the development and piloting of the CREDO training curriculum and present the results of the formal evaluation.

The Study

The learning objectives for the CREDO curriculum (Table 1) were developed using the TDR Global Competency Framework for Clinical Research (*5*,*6*), which describes all areas of competency required to conduct clinical research (*6*). The content of the modules addressed both observational studies and clinical trials. Observational studies were included because they are essential for understanding the etiology, natural history, and pathophysiology of epidemic infectious diseases, many of which are poorly understood. Clinical trials address questions on therapeutics, diagnostics, and other topics, and CREDO covers a range of trial designs including adaptive designs, which may be better suited to the evolving nature of outbreaks.

CREDO is structured as a blended-learning format, with face-to-face sessions (workshops) and asynchronous, downloadable online sessions (e-learning). Our training model was designed with the workshops occurring before and after the e-learning component to introduce and then solidify knowledge gained in the online component. Our assessment model combined multiple-choice questions in the e-learning sessions with simulation exercises in the workshop sessions to provide a balance in testing between recall and critical or creative thinking (*7*).

The curriculum has 12 modules (Table 2): 1 workshop and 11 e-learning modules, 2 of which are prerequisites that must be completed before the workshop date. Modules are freely available online through a digital platform suited to the low bandwidths found in LMICs. The e-learning component is hosted on the Global Health Network website (https://isaric.tghn.org/credo). It is self-paced over a period of 5–6 months, and each module takes an average of 1–2 hours, with some taking as many as 4–5 hours, to complete. The e-learning is completed individually; participants complete the final assessment during the second workshop as a team, each team member actively using the knowledge learned from the online modules to contribute to the design of a clinical trial during a hypothetical outbreak. Each participant who successfully completes all course components earns a CREDO certificate.

We designed the curriculum for team training with multidisciplinary and intact teams (*8*); that is, teams that are already working together to develop and implement clinical studies. Team training refers to the training of an entire team and has resulted in improved patient outcomes in various areas of medicine, such as trauma (*8*,*9*).

During March–August 2017, we conducted a pilot of the CREDO training curriculum. An informal call invited team applications from sub-Saharan Africa, targeted because populations in the region are at risk for outbreaks from high-threat infectious diseases (*10*). We selected 19

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Table 1. Learning objectives for the Clinical REsearch During Outbreaks (CREDO) training course

No.	Objective
1.	Define what emerging and epidemic infections are and discuss their importance
2.	Critique the clinical research response to emerging infections
3.	Understand the key elements in a rapid systematic review
4.	Critically appraise literature and identify gaps in the literature
5.	Select an appropriate study design
6.	Outline the ethical implications of the selection of study design
7.	Describe the ethical considerations required to ensure that informed consent is obtained, particularly in traditional communities or low-resource settings
8.	Describe ethical principles of incorporating special groups (pregnant women, children, etc.) in research during epidemics
9.	Describe ways of minimizing participant loss to follow-up
10.	Identify logistical and operational factors affecting the implementation of clinical research during an outbreak
11.	Formulate project management timelines for a research project
12.	Plan efficient data collection methods
13.	Describe special considerations for community engagement in outbreak research
14.	Assemble a communications team and develop a crisis communications plan
15.	Identify potential sources of study funding and prepare grant applications
16.	Identify a study sponsor and describe the role and responsibilities of the sponsor
17.	Describe the basic elements of the different kinds of contracts used in clinical research
18.	Explain the benefits of streamlined data collection
19.	Explain the important role of data sharing, harmonization, and collaboration in outbreak research

Table 2. Curriculum structure of Clinical REsearch During Outbreaks (CREDO) training course*

Module title	Module summary and website link
Prerequisite modules	
Good clinical practice	A framework of principles to ensure the safety of research participants and integrity and validity of data: https://globalhealthtrainingcentre.tghn.org/ich-good-clinical-practice
The global health research process map	A pragmatic interactive tool provides step-by-step guidance for each stage that needs to be considered when planning a new study: https://processmap.tghn.org/about
Workshop: Evidence-based medicine for epidemic infections and key issues in study design	Delivered in 3 presentations, it provides an introduction to epidemic and emerging infections, critiques the clinical research responses to previous outbreaks, highlights the challenges to clinical research during outbreaks, and discusses possible mitigating strategies: https://isaric.tghn.org/credo/credo-workshop-1
E-learning component: self-paced, in any order, completed individually before the second workshop	
Rapid evidence-needs appraisal	A guide for the conduct of rapid reviews in the event of an outbreak: https://globalhealthtrainingcentre.tghn.org/credo-rapid-evidence-needs-appraisal
Research study planning and governance	An overview of how to set up a clinical research study and find and apply for funding, and important concepts for study managements: https://globalhealthtrainingcentre.tghn.org/credo-research-study-planning-and-governance
Study design	An introduction to the challenges to research design during outbreaks and some mitigating strategies: https://globalhealthtrainingcentre.tghn.org/credo-study-design
Statistics	Background of statistical principles relevant to clinical research and trial design and challenges in outbreaks and some solutions: https://globalhealthtrainingcentre.tghn.org/credo-statistics
Logistics and operational planning	Pragmatic solutions to common logistical and operational challenges to research in outbreaks: https://globalhealthtrainingcentre.tghn.org/credo-logistical-and-operational-planning
Data sharing and harmonization	General outline of data management, sharing, and harmonization to guide the conduct of research during outbreaks: https://isaric.tghn.org/credo/credo-data-sharing-and-harmonisation
Ethics	WHO course on ethics in outbreaks; modules 2, 4, and 6 on the TGHN platform: https://globalhealthtrainingcentre.tghn.org/research-ethics-epidemics-pandemics-and-disaster-situations
Communications and engagement	Effective communication and engagement during outbreaks: https://globalhealthtrainingcentre.tghn.org/credo-communications-and-community-engagement
Special groups: Children, pregnant women, mother/child	A consensus statement on the inclusion of children and pregnant women in research in disease outbreaks to help facilitate including these important groups in future research and clinical trials: https://isaric.tghn.org/credo/ethics-special-groups
Workshop: final assessment, completed in teams	

*Prerequisite modules must be completed online individually before the first workshop. TGHN, The Global Health Network; WHO, World Health Organization.

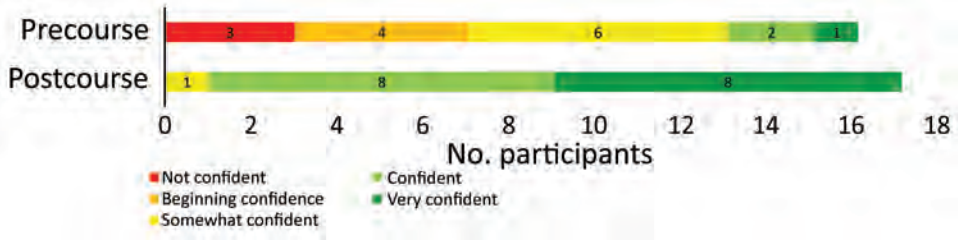

Figure 1. Self-assessed level of confidence with learning objectives of Clinical REsearch During Outbreaks (CREDO) before and after course. Participants' level of confidence in their ability to implement a clinical research study during an outbreak changed substantially: in the precourse assessment, 3 of 17 participants rated themselves as confident or very confident; in postcourse assessment, 16 of 17 did.

participants from 4 multidisciplinary teams in 4 countries (Ethiopia, Ghana, Côte d'Ivoire, and Uganda) on the basis of their clinical research experience and team diversity. Of the selected participants, 8 were female and 11 male. Teams consisted of 11 medical doctors, 2 research nurses, 2 data managers, 1 clinical trial manager, and 3 biomedical scientists.

We conducted the first workshop in Entebbe, Uganda. Its purpose was to appraise the challenges of outbreak research; describe the fundamental concepts of generating clinical evidence; introduce the online component of the training; and assess, using simulated team-based exercises, the participants' confidence in planning and conducting clinical research during an outbreak. After the workshop, participants had time to complete the e-learning modules.

The closing workshop was conducted in Addis Ababa, Ethiopia. Two weeks before this workshop, we gave participants an assignment to develop a clinical trial protocol for a hypothetical outbreak (available on the course website, https://isaric.tghn.org/credo/credo-overall-assignment). This workshop increased and assessed the participants' knowledge and understanding of observational research and clinical trials. The clinical trial protocols in the assignment were assessed using role play; facilitators played the roles of statisticians, community representatives, scientific experts, ethical reviewers, and government representatives. The teams then incorporated suggestions made by the faculty. The revised protocols then underwent review by peers using an adapted scoring tool (Appendix, https://wwwnc.cdc.gov/EID/article/25/11/18-0628-App1.pdf).

Pilot participants completed an online precourse and postcourse evaluation form anonymously. The questions were based on the learning objectives (Table 1) and sought the participants' views on the training received, the structure and the content of the individual course modules, and their ability to conduct clinical research during an outbreak. The responses were structured on a Likert scale from strongly agree to strongly disagree or not confident to very confident, with free-text space to clarify any responses (Appendix).

Of the 19 participants, 16 (84.2%) completed the precourse questionnaire and 17 (89.5%) completed the postcourse questionnaire. Most participants were satisfied with the course; 16 participants rated their level of satisfaction as satisfied or very satisfied. Self-assessed levels of confidence increased after the course; 16 participants rated their level of confidence in implementing the course objectives as confident or very confident after the course, compared with only 3 participants before the course (Figure 1). However, another evaluation is required during or after an outbreak to better assess the effectiveness of the course.

An assessment of the content of CREDO showed largely positive reviews, particularly with respect to the relevance and adequacy of the material covered in each of the course modules (Figure 2). Time for completion of each module was generally sufficient except in the rapid evidence appraisal module, for which 3 participants indicated time was inadequate. Some of the major strengths of CREDO were its structure and content, which most participants considered "holistic" and "comprehensive."

Conclusions

Although clinical research is becoming recognized as an important component of outbreak management (*1*,*11*), few resources exist to assess or build capacity in this area. Most

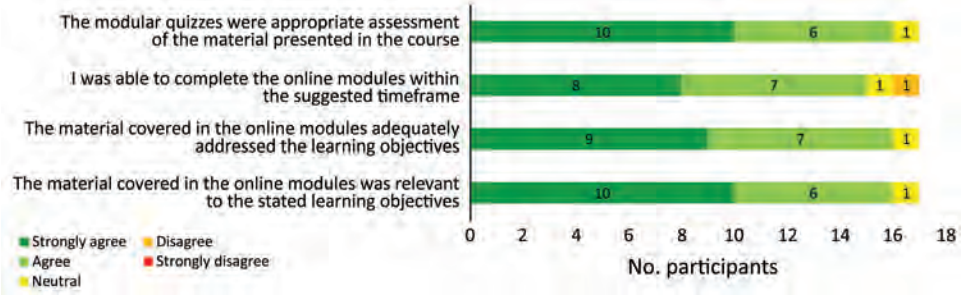

Figure 2. Participant level of agreement with postcourse assessment questions about the quality of the online component of the Clinical REsearch During Outbreaks (CREDO) curriculum.

of the available outbreak training programs or courses focus on surveillance, epidemiology, outbreak investigation, or laboratory investigation (*12*–*15*). CREDO offers a unique perspective with its focus on clinical research and clinical trials.

The strengths of the CREDO training curriculum lie in its format of blended learning, team-based approach, and content tailored to outbreak conditions. Its combination of training in observational studies and clinical trials builds capacity for collecting the much-needed information on pathophysiology and natural history of poorly known conditions, essential components to identify the right interventions to test and the design of the intervention trials.

In addition, CREDO, as an open-access resource, lends itself to adoption and adaptation by interested sites and to creating a community of science by sharing additional or modified materials through the Global Health Network website. CREDO resources can be incorporated into medical or public health training curricula or used as a standalone course for continuous professional development. Some ways in which CREDO will become sustainable are by broadening the number of trainers from among course participants, supporting country ownership, and franchising the course through a variety of providers.

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References

1. Lurie N, Manolio T, Patterson AP, Collins F, Frieden T. Research as a part of public health emergency response. N Engl J Med. 2013;368:1251–5. https://doi.org/10.1056/NEJMsb1209510
2. Rojek AM, Horby PW. Modernising epidemic science: enabling patient-centred research during epidemics. BMC Med. 2016;14:212. https://doi.org/10.1186/s12916-016-0760-x
3. Rojek AM, Horby PW. Offering patients more: how the West Africa Ebola outbreak can shape innovation in therapeutic research for emerging and epidemic infections. Philos Trans R Soc Lond B Biol Sci. 2017;372:20160294. https://doi.org/10.1098/rstb.2016.0294
4. Koita OA, Murphy RL, Fongoro S, Diallo B, Doumbia SO, Traoré M, et al. Clinical research and the training of host country investigators: essential health priorities for disease-endemic regions. Am J Trop Med Hyg. 2016;94:253–7. https://doi.org/10.4269/ajtmh.15-0366
5. Julé A, Furtado T, Boggs L, van Loggerenberg F, Ewing V, Vahedi M, et al. Developing a globally applicable evidence-informed competency framework to support capacity strengthening in clinical research. BMJ Glob Health. 2017;2:e000229. https://doi.org/10.1136/bmjgh-2016-000229
6. World Health Organization Special Programme for Research and Training in Tropical Diseases. Using the TDR global competency framework for clinical research: a set of tools to help develop clinical researchers. 2016 [cited 2019 Aug 19]. https://www.who.int/tdr/publications/year/2016/competency-framework-clinical-res
7. Hift RJ. Should essays and other "open-ended"-type questions retain a place in written summative assessment in clinical medicine? BMC Med Educ. 2014;14:249. https://doi.org/10.1186/s12909-014-0249-2
8. Salas E, DiazGranados D, Klein C, Burke CS, Stagl KC, Goodwin GF, et al. Does team training improve team performance? A meta-analysis. Hum Factors. 2008;50:903–33. https://doi.org/10.1518/001872008X375009
9. Capella J, Smith S, Philp A, Putnam T, Gilbert C, Fry W, et al. Teamwork training improves the clinical care of trauma patients. J Surg Educ. 2010;67:439–43. https://doi.org/10.1016/j.jsurg.2010.06.006
10. Pigott DM, Deshpande A, Letourneau I, Morozoff C, Reiner RC Jr, Kraemer MUG, et al. Local, national, and regional viral hemorrhagic fever pandemic potential in Africa: a multistage analysis. Lancet. 2017;390:2662–72. https://doi.org/10.1016/S0140-6736(17)32092-5
11. National Academies of Sciences, Engineering, and Medicine. Integrating clinical research into epidemic response: the Ebola experience. Washington, DC: The National Academies Press; 2017. https://doi.org/10.17226/24739.
12. Public Health England. United Kingdom Field Epidemiology Training Programme prospectus. 2019 [cited 2019 July 8]. https://www.gov.uk/guidance/field-epidemiology-training-programme-fetp
13. Lescano AG, Salmon-Mulanovich G, Pedroni E, Blazes DL. Outbreak investigation and response training. Science. 2007;318:574–5. https://doi.org/10.1126/science.1146837
14. Centers for Disease Control and Prevention. Field Epidemiology Training Program (FETP) training modules and resources. 2019 [cited 2019 July 8]. https://www.cdc.gov/globalhealth/healthprotection/fetp/training_modules/index.html
15. Centers for Disease Control and Prevention; Society for Healthcare Epidemiology of America. CDC/SHEA Outbreak Response Training Program (ORTP). 2019 [cited 2019 July 8]. http://ortp.shea-online.org/

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Non-*Leishmania* Parasite in Fatal Visceral Leishmaniasis–Like Disease, Brazil

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Through whole-genome sequencing analysis, we identified non-*Leishmania* parasites isolated from a man with a fatal visceral leishmaniasis–like illness in Brazil. The parasites infected mice and reproduced the patient's clinical manifestations. Molecular epidemiologic studies are needed to ascertain whether a new infectious disease is emerging that can be confused with leishmaniasis.

Leishmaniases are caused by ≈20 *Leishmania* species transmitted to humans through sand-fly bites and have been classified into 3 main forms: cutaneous leishmaniasis, mucocutaneous or mucosal leishmaniasis, and visceral leishmaniasis (VL; also known as kala-azar) (*1*,*2*). VL is the most severe form of the disease and can be fatal if misdiagnosed or untreated (*3*). Cases of VL in Brazil account for >90% of annual reported cases in Latin America (*4*), where the causative species is *L. infantum*.

Since 1980, sporadic co-infections of *Leishmania* with apparently monoxenous trypanosomatids have been described (Kaufer et al. [*5*]); these reports are sometimes associated with immunocompromised hosts. More recently, co-infections with *Crithidia*-like (*6*) or *Leptomonas*-like (*7*) parasites have been reported. Whether these co-infections are occasional findings or are evidence for new parasites with the potential to threaten public health remains uncertain. To investigate this problem, we performed whole-genome sequencing of 2 clinical isolates from a patient with a fatal illness with clinical characteristics similar to those of VL.

The Study

During 2011–2012, we characterized 2 parasite strains, LVH60 and LVH60a, isolated from an HIV-negative man when he was 64 years old and 65 years old (Table; Appendix, https://wwwnc.cdc.gov/EID/article/25/11/18-1548-App1.pdf). Treatment-refractory VL-like disease developed in the man; signs and symptoms consisted of weight loss, fever, anemia, low leukocyte and platelet counts, and severe liver and spleen enlargements. VL was confirmed by light microscopic examination of amastigotes in bone marrow aspirates and promastigotes in culture upon parasite isolation and by positive rK39 serologic test results. Three courses of liposomal amphotericin B resulted in no response. At the third hospital admission, the illness resembled diffuse cutaneous leishmaniasis, in which several disseminated papular skin lesions were observed (Appendix Figure 1, panel A), and a skin biopsy revealed macrophages filled with amastigotes (Appendix Figure 1, panel B), which his liver biopsy results also showed (Appendix Figure 1, panel C). During this third admission, the LVH60a strain was isolated from the skin. Dermal lesions known as post–kala-azar dermal leishmaniasis (PKDL) have rarely been reported in Brazil (*13*), and the clinical aspect of the disseminated papular skin lesions on this patient differed from the clinical presentation of PKDL. Because his illness did not respond to therapy, the patient underwent splenectomy. He died of disease and surgical complications.

We used cryopreserved parasite stocks isolated from bone marrow (LVH60) and skin lesions (LVH60a) to obtain promastigotes for DNA isolation. We obtained clonal colonies and analyzed them to confirm the homogeneity of parasite cultures. For species identification, we amplified the small subunit rRNA (SSU rRNA), ribosomal internal transcribed spacer 1 (ITS1) regions, and glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH) by PCR, sequenced them, and analyzed them. We used a laboratory reference *L. infantum* strain (HU-UFS14) used in

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Table. Non-*Leishmania* parasites isolated from 2 patients with visceral leishmaniasis–like illness used for whole-genome sequencing, Brazil*

Clinical isolate	Year isolated	Tissue source	Patient age, y/sex	Treatment	Recidivism	Healing time	Serologic test result (rK39)	MLEE	Experimental assays
LVH60	2011	BM	64/M	Liposomal amphotericin B	Yes, 3	Fatal case	Positive	Inconclusive	Mouse infection (this study)
LVH60a (DPSLs)	2012	SL	65/M	Liposomal amphotericin B	Yes, 3	Fatal case	Positive	Inconclusive	Mouse infection (this study)
HU-UFS14	2009	BM	15/M	Antimony, amphotericin B	NA	NA	Positive	*L. infantum*	NO- and antimony-resistant (*8*); murine model of infection (*9*–*12*).

*BM, bone marrow; DPSL, disseminated popular skin lesions; MLEE, multilocus enzyme electrophoresis; NA, not available or not applicable; NO, nitrite oxide; SL, skin lesion.

experimental infections elsewhere (*9*–*12*) as control. A PCR using primers for HSP70 gene (specific to discriminate *Leishmania* species [*14*]) resulted in no amplification. Amplicon sequence analyses of SSU rRNA, ITS1, and GAPDH revealed that the LVH60 and LVH60a strains are more closely related to *Crithidia fasciculata* than to *Leishmania*. Only the HU-UFS14 clustered within the *Leishmania* group on a branch composed of *L. infantum* and *L. donovani*.

To characterize the organisms LVH60 and LVH60a, we determined their complete genome sequences with >400× coverage (BioProject accession no. PRJNA398352; related accession numbers in Appendix, Tables 7,9). We assembled the reads into ≈4,500 scaffolds. More than 9,000 coding sequences were deduced per isolate. Only HU-UFS14 presented a predicted haploid genome size similar to that of a known *Leishmania* species (≈33 Mb).

To ascertain the phylogenetic relationships between these isolates, we developed a comprehensive strategy to compare all available trypanosomatid orthologous proteins, in which we calculated a pairwise distance matrix based on the median distance of orthologous genes found using the RSD algorithm (S.R. Maruyama et al., unpub. data). We identified an average of 6,093 orthologs for all considered pairs. Corroborating the phylogenies of single sequences (SSU rRNA, ITS1, and GAPDH), both clinical isolates (except HU-UFS14) clustered apart from the *Leishmania* clade (Figure 1), fitting into another Leishmaniinae subfamily group composed of the monoxenous genera *Leptomonas*, *Lotmaria*, and *Crithidia*, which infect only insect hosts (*5*). These results revealed that the LVH60 and LVH60a isolates do not belong to the *Leishmania* genus. Instead, these isolates form a robust clade including *C. fasciculata* but excluding 2 other *Crithidia* and *Lotmaria* bee parasites.

Because LVH60 and LVH60a were more closely related to monoxenous trypanosomatids, we performed experimental intravenous infections in BALB/c mice with these non-*Leishmania* clinical isolates or the HU-UFS14 strain to evaluate their infectious capacity. We analyzed parasite load in the spleen and liver. We found the LVH60 and LVH60a strains in the liver, although at much lower levels than HU-UFS14. However, in the spleen, we detected only LVH60 (Figure 2, panel A). Because LVH60a was isolated from the skin and both LVH60 and LVH60a were barely detected in organs, we infected BALB/c mice with these parasites through the intradermal route on the ears to evaluate their capacity to generate skin lesions and compared the results with those obtained with *L. major* LV29, the positive control.

Only the LVH60a strain was able to establish infection and cause ear lesions (Figure 2, panel B), as measured by parasite load (Figure 2, panel C) and ear thickness (Figure 1, panel D). The injury caused by LVH60a to the ear skin was more extensive than that resulting from the *L. major* LV29-positive control. Thus, the phenotypes observed with experimental infection corroborate the clinical manifestations in the patient; that is, the LVH60a strain isolated from skin lesions injured the skin tissue of mice under experimental cutaneous infection. Thus, these parasite strains closely related to *C. fasciculata* can be considered a new dixenous parasite able to infect mammals, such as humans and mice.

Conclusions

Our study showed that non-*Leishmania*, *Crithidia*-related parasites were involved in an atypical manifestation similar to VL in this patient. Because few drugs exist with which to treat leishmaniasis, this identification of a new trypanosomatid strain refractory to treatment that can cause disease either as a single infection or as a co-infection with *Leishmania* is serious and might increase the problem of disease control. This scenario highlights the urgent need for studies of new drugs to treat this new strain. Moreover, the fact that this parasite appeared in a sister phylogenetic position to *C. fasciculata* focuses attention on potential vectors because leishmaniasis is transmitted by female sand flies, whereas *C. fasciculata* infects only anopheline and *Culex* mosquitoes. Recently, both *C. fasciculata* and *L. infantum* sequences were detected in phlebotomine *Nyssomyia whitmani* samples collected in the northern region of Brazil (*15*). Our findings raise concerns about the need to isolate

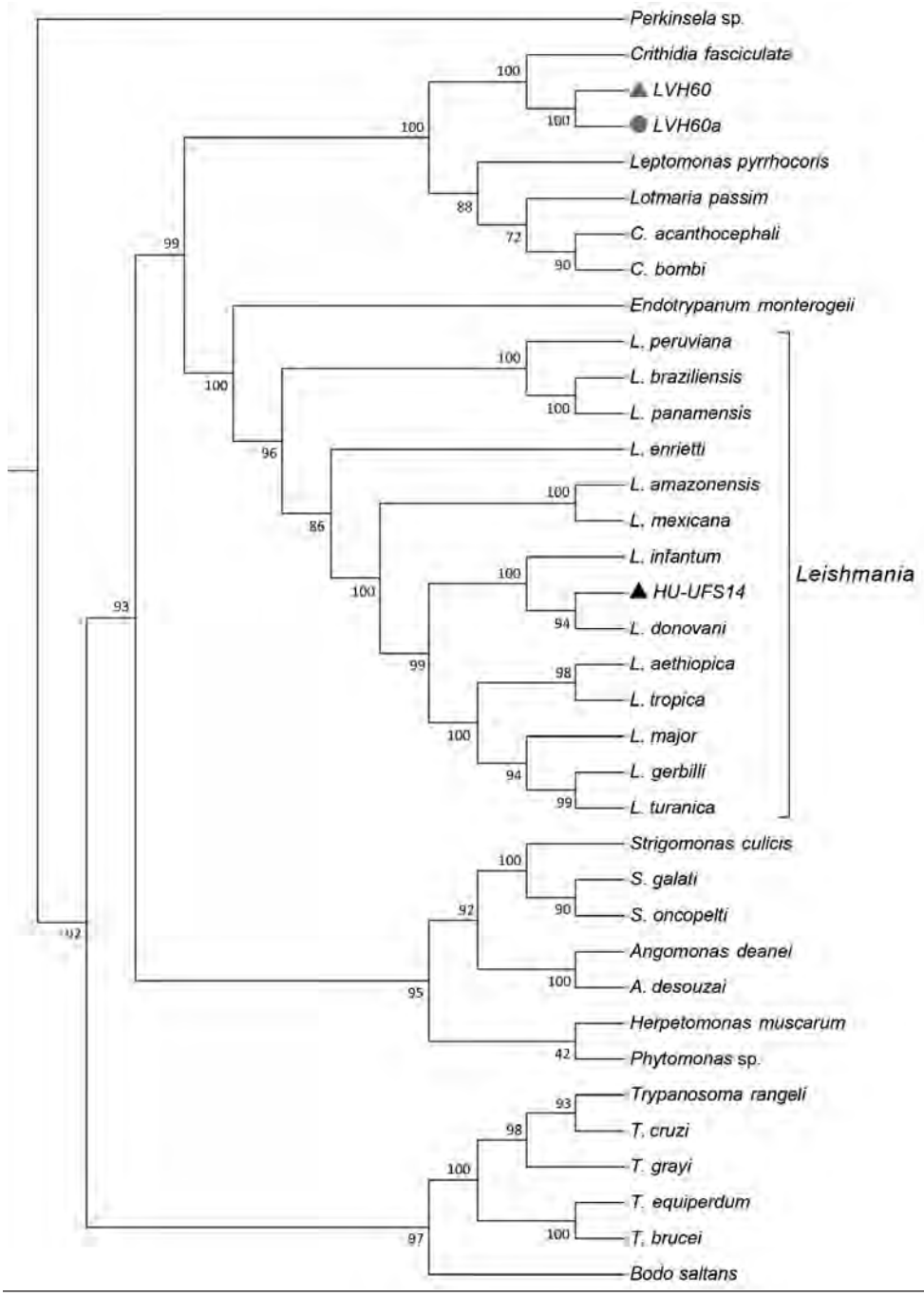

Figure 1. Phylogenomic analysis of genomewide orthologous coding sequences from LVH60 and LVH60a clinical isolates from a 64-year-old man with fatal visceral leishmaniasis–like illness, Brazil, and 33 Trypanosomatida species. Dendrogram shows the genetic relationships among all species investigated in the current study. Hierarchical clustering was performed with a set of ≈6,400 orthologous genes across 33 trypanosomatids, designated as the total orthologous median matrix. HU-UFS14 (black triangle; *L. infantum* laboratory reference strain) is placed in the same branch with *L. infantum* and *L. donovani*, whereas the LVH60 and LVH60a clinical isolates are placed in sister positions with *Crithidia fasciculata*. LVH60 was isolated from bone marrow (gray triangle), LVH60a from a skin lesion (gray circle) biopsy, both from the same patient. Numbers next to the branches represent the percentages of approximate unbiased support probabilities for 10,000 bootstraps, calculated using the pvclust R package (https://cran.r-project.org/web/packages/pvclust). Branch relationships were defined by their median amino acid evolutionary distance (Appendix, https://wwwnc.cdc.gov/EID/article/25/11/18-1548-App1.pdf).

and characterize parasites from more humans, reservoirs, and vectors; map trypanosomatid distribution and epidemiologic control measures; study the sensitivity of these parasites to drugs and design new treatment options; and develop new epidemiologic/ecologic strategies to control *Crithidia*-related species.

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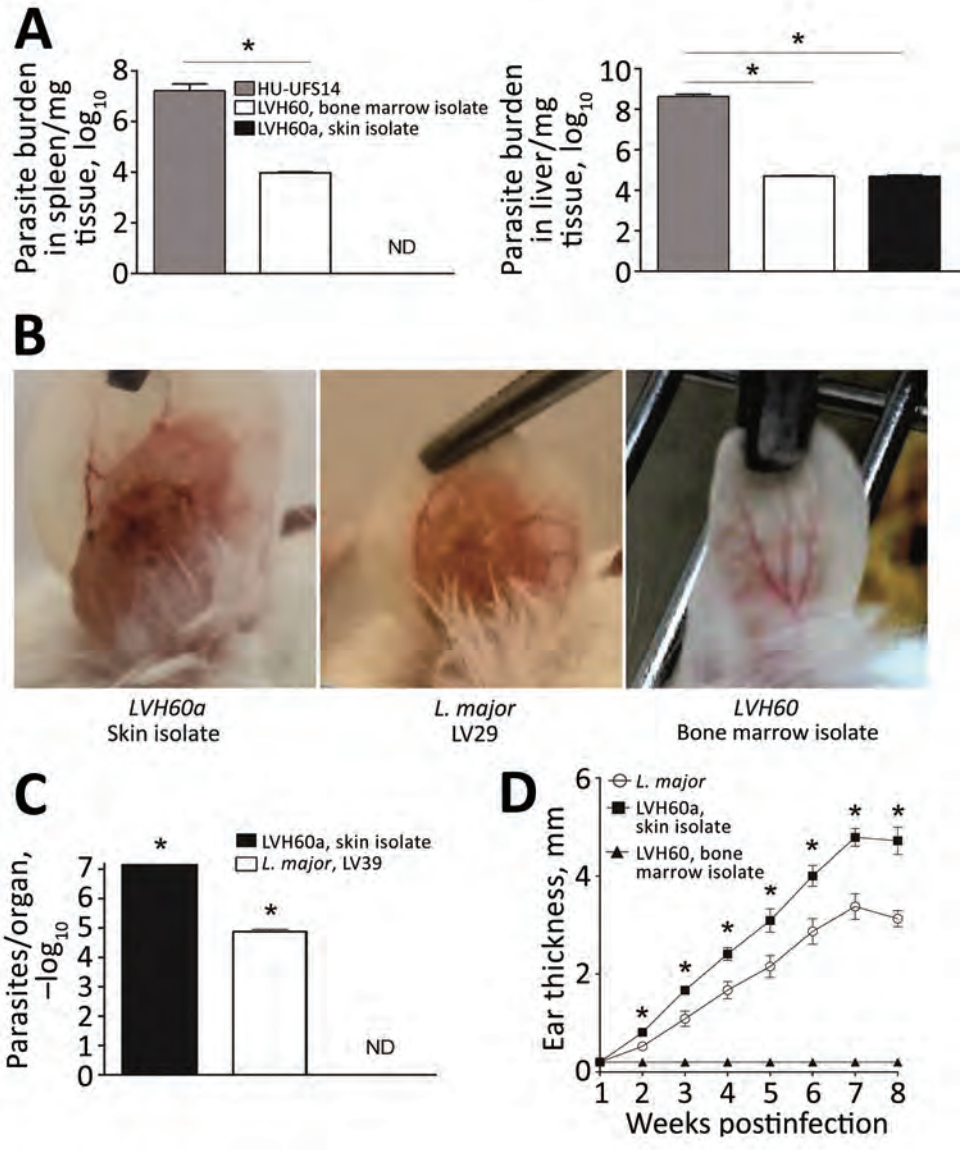

Figure 2. Experimental infection of BALB/c mice with LVH60 and LVH60a clinical isolates obtained from a 64-year-old man with fatal visceral leishmaniasis–like illness, Brazil. LVH60 was isolated from bone marrow, LVH60a from a skin lesion biopsy. Female BALB/c mice were infected intravenously with 10^7 stationary-phase promastigotes. After 4 weeks of infection, spleen and liver samples were collected. Parasite loads were determined by a limiting dilution assay of spleen and liver homogenates and are expressed as the mean ± SD. A) LVH60 strain infection in mice resulted in parasite detection in the spleen and liver; the LVH60a strain was not detected in the spleen. B) For cutaneous infection, BALB/c mice were injected subcutaneously in the right ear dermis with 10^6 stationary phase promastigotes. Infected ears were collected and imaged. C) Parasite burden in ears was assessed by a limiting dilution assay. D) Ear thickness was measured weekly with a digital caliper. The HU-UFS14 strain (*L. infantum*) was used as a positive control for experimental visceral leishmaniasis (A), whereas the LV29 strain (*L. major*) was used as a positive control for experimental cutaneous leishmaniasis. The results represent 3 independent experiments. Error bars indicate SD. ND, not detected. *p<0.05.

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References

1. World Health Organization. Leishmaniasis [cited 2019 Mar 26]. http://www.who.int/leishmaniasis/en
2. Centers for Disease Control and Prevention. Parasites—leishmaniasis [cited 2019 Apr 23]. https://www.cdc.gov/parasites/leishmaniasis
3. El Hajj R, El Hajj H, Khalifeh I. Fatal visceral leishmaniasis caused by *Leishmania infantum*, Lebanon. Emerg Infect Dis. 2018;24:906–7. https://doi.org/10.3201/eid2405.180019
4. Alvar J, Vélez ID, Bern C, Herrero M, Desjeux P, Cano J, et al.; WHO Leishmaniasis Control Team. Leishmaniasis worldwide and global estimates of its incidence. PLoS One. 2012;7:e35671. https://doi.org/10.1371/journal.pone.0035671
5. Kaufer A, Ellis J, Stark D, Barratt J. The evolution of trypanosomatid taxonomy. Parasit Vectors. 2017;10:287. https://doi.org/10.1186/s13071-017-2204-7
6. Ghobakhloo N, Motazedian MH, Naderi S, Ebrahimi S. Isolation of *Crithidia* spp. from lesions of immunocompetent patients with suspected cutaneous leishmaniasis in Iran. Trop Med Int Health. 2019;24:116–26.

7. Ghosh S, Banerjee P, Sarkar A, Datta S, Chatterjee M. Coinfection of *Leptomonas seymouri* and *Leishmania donovani* in Indian leishmaniasis. J Clin Microbiol. 2012;50:2774–8. https://doi.org/10.1128/JCM.00966-12
8. de Azevedo AF, de Lisboa Dutra JL, Barbosa Santos ML, de Alexandria Santos D, Alves PB, de Moura TR, et al. Fatty acid profiles in *Leishmania* spp. isolates with natural resistance to nitric oxide and trivalent antimony. Parasitol Res. 2014;113:19–27. https://doi.org/10.1007/s00436-013-3621-y
9. Nascimento MSL, Ferreira MD, Quirino GFS, Maruyama SR, Krishnaswamy JK, Liu D, et al. NOD2-RIP2–mediated signaling helps shape adaptive immunity in visceral leishmaniasis. J Infect Dis. 2016;214:1647–57. https://doi.org/10.1093/infdis/jiw446
10. Lima MHF, Sacramento LA, Quirino GFS, Ferreira MD, Benevides L, Santana AKM, et al. *Leishmania infantum* parasites subvert the host inflammatory response through the adenosine $A2_A$ receptor to promote the establishment of infection. Front Immunol. 2017;8:815. https://doi.org/10.3389/fimmu.2017.00815
11. Nascimento MS, Carregaro V, Lima-Júnior DS, Costa DL, Ryffel B, Duthie MS, et al. Interleukin 17A acts synergistically with interferon γ to promote protection against *Leishmania infantum* infection. J Infect Dis. 2015;211:1015–26. https://doi.org/10.1093/infdis/jiu531
12. Sacramento LA, da Costa JL, de Lima MH, Sampaio PA, Almeida RP, Cunha FQ, et al. Toll-like receptor 2 is required for inflammatory process development during *Leishmania infantum* infection. Front Microbiol. 2017;8:262. https://doi.org/10.3389/fmicb.2017.00262
13. Carnaúba D Jr, Konishi CT, Petri V, Martinez ICP, Shimizu L, Pereira-Chioccola VL. Atypical disseminated leishmaniasis similar to post-kala-azar dermal leishmaniasis in a Brazilian AIDS patient infected with *Leishmania* (*Leishmania*) *infantum chagasi*: a case report. Int J Infect Dis. 2009;13:e504–7. https://doi.org/10.1016/j.ijid.2009.01.022
14. Espada CR, Ortiz PA, Shaw JJ, Barral AMP, Costa JML, Uliana SRB, et al. Identification of *Leishmania* (*Viannia*) species and clinical isolates of *Leishmania* (*Leishmania*) *amazonensis* from Brazil using PCR-RFLP of the heat-shock protein 70 gene reveals some unexpected observations. Diagn Microbiol Infect Dis. 2018;91:312–8. https://doi.org/10.1016/j.diagmicrobio.2018.03.004
15. Machado TDO, Minuzzi-Souza TTC, Ferreira TS, Freire LP, Timbó RV, Vital TE, et al. The role of gallery forests in maintaining Phlebotominae populations: potential *Leishmania* spp. vectors in the Brazilian savanna. Mem Inst Oswaldo Cruz. 2017;112:681–91. https://doi.org/10.1590/0074-02760170126

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Secondary Autochthonous Outbreak of Chikungunya, Southern Italy, 2017

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In 2017, a chikungunya outbreak in central Italy later evolved into a secondary cluster in southern Italy, providing evidence of disease emergence in new areas. Officials have taken action to raise awareness among clinicians and the general population, increase timely case detection, reduce mosquito breeding sites, and promote mosquito bite prevention.

In 2007 (*1*) and 2017 (*2*), local outbreaks of human chikungunya infection occurred in Italy; both outbreaks were caused by the East/Central/South African strain of chikungunya virus (CHIKV). Both outbreaks were sustained by the invasive mosquito *Aedes albopictus*, largely established in Italy and other countries in southern Europe (*3*). In 2017, France and Italy reported local transmission of CHIKV (*2*,*4*). However, in France, the number of cases was limited; whereas in Italy, 499 probable and confirmed cases of locally acquired CHIKV infection occurred, of which 270 were laboratory confirmed as per the European Union (EU) case definition of June 22, 2018 (https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945&from=EN#page=13).

After local CHIKV transmission was confirmed in the seaside city of Anzio (Lazio region, central Italy) in 2017, the outbreak spread within the region, including in the city of Rome (*5*–*7*). The beginning of the outbreak was traced back to June 2017 (Figure 1). Subsequently, an outbreak developed in Guardavalle Marina, a small village of 2,346 inhabitants in the Calabria region of southern Italy, causing 100 probable/confirmed cases (Figures 1, 2).

The Cases

In August 2017, a patient from Anzio arrived in Guardavalle Marina the day before onset of symptoms that met the clinical criteria of the EU case definition for chikungunya (suspected case); subsequently, more cases in the village were reported. For most case-patients, the clinical course of the disease was fairly mild. All patients with confirmed/probable cases reported fever, and 99% reported severe and persistent joint pain, which seemed to be the most indicative symptom of chikungunya (Appendix Table 2, https://wwwnc.cdc.gov/EID/article/25/11/18-0949-App1.pdf). Phylogenetic analysis of isolates from patients with confirmed cases and from mosquito pools showed that the virus strains from Lazio and Calabria were similar to the East/Central/South African strains detected in Pakistan and India (*2*,*8*). Neither strain contained the A226V mutation that was detected in the strain responsible for the 2007 outbreak in Italy (*1*).

The epidemiologic and microbiological evidence (Appendix) supports the hypothesis that the Guardavalle Marina outbreak originated from Lazio rather than from an independent introduction. The overall clinically observed attack rate in Guardavalle Marina was 4.3%, similar to the 5.4% rate reported during the 2007 outbreak in Castiglione di Cervia but much lower than the 34% rate reported in La Réunion (*9*) or the high attack rates typically reported by other affected tropical countries (*10*). The duration of the Guardavalle Marina outbreak (2 months) was also closer to the duration of the outbreak in Castiglione di Cervia (July–

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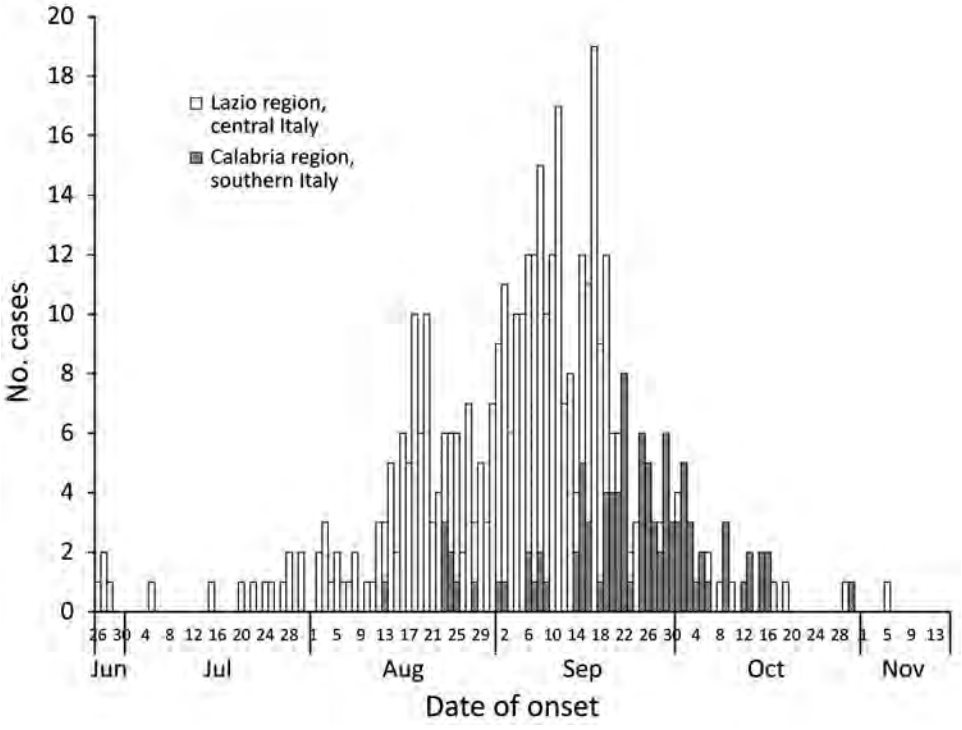

Figure 1. Epidemic curve for 499 cases of chikungunya (probable and confirmed) in central and southern Italy, June 26–November 15, 2017.

September 2007) than to the duration of the outbreak in La Réunion (March 2005–April 2006) (*11*).

Clinically observed attack rates progressively increased with patient age (Appendix Table 1). This pattern was also observed during the 2007 outbreak in Italy (*1*) and was mainly attributed to older age being a proxy for specific behavior linked to higher exposure to bites from *Ae. albopictus* mosquitoes (*12*). We cannot exclude underestimation of the observed attack rate in Guardavalle Marina, even though extensive door-to-door case finding was feasible and performed, given the small size of the village.

Notwithstanding the lack of the A226V mutation, the 2017 strain was introduced and rapidly spread with evidence of disease emergence in new areas. Statistically significant case clustering was confirmed by spatiotemporal data analysis in Guardavalle Marina (Appendix Figure 2), and *Ae. albopictus* mosquito vector competence for the 2017 strain was recently found to be comparable to competence for the 2007 mutated strain (*13*).

Delayed clinical detection of cases, possibly resulting from lack of CHIKV infection awareness among clinicians (*14*), and hence delayed testing for laboratory confirmation, could explain the size and extension of the 2017 outbreak. The fact that the outbreak was contained in a relatively short time after detection could be the result of the combination of targeted vector control interventions (Appendix), cooling temperatures, and increased rainfall during October–November 2017.

Conclusions

Since 2011, Italy has had a yearly updated plan for the surveillance and control of human infections caused by CHIKV; the plan includes designated reference laboratories, vector control, and blood/transplant safety measures (Appendix). After the 2017 outbreak, a "presumed local cluster" was defined as occurring when local transmission of CHIKV is suspected for ≥2 cases, of which only 1 case needs to be laboratory confirmed. It is sufficient for the second case to be suspected on clinical and epidemiologic grounds pending laboratory confirmation. This new definition, aimed at triggering more timely blood/transplant safety and outbreak response measures, was successfully implemented in 2018. Actions to raise awareness among clinicians and the general population are being designed as part of a joint effort of animal and human public health institutions and universities coordinated by the Italy Ministry of Health. These actions are aimed at increasing timely case detection, reducing the number of mosquito breeding sites, and promoting individual prevention of mosquito bites.

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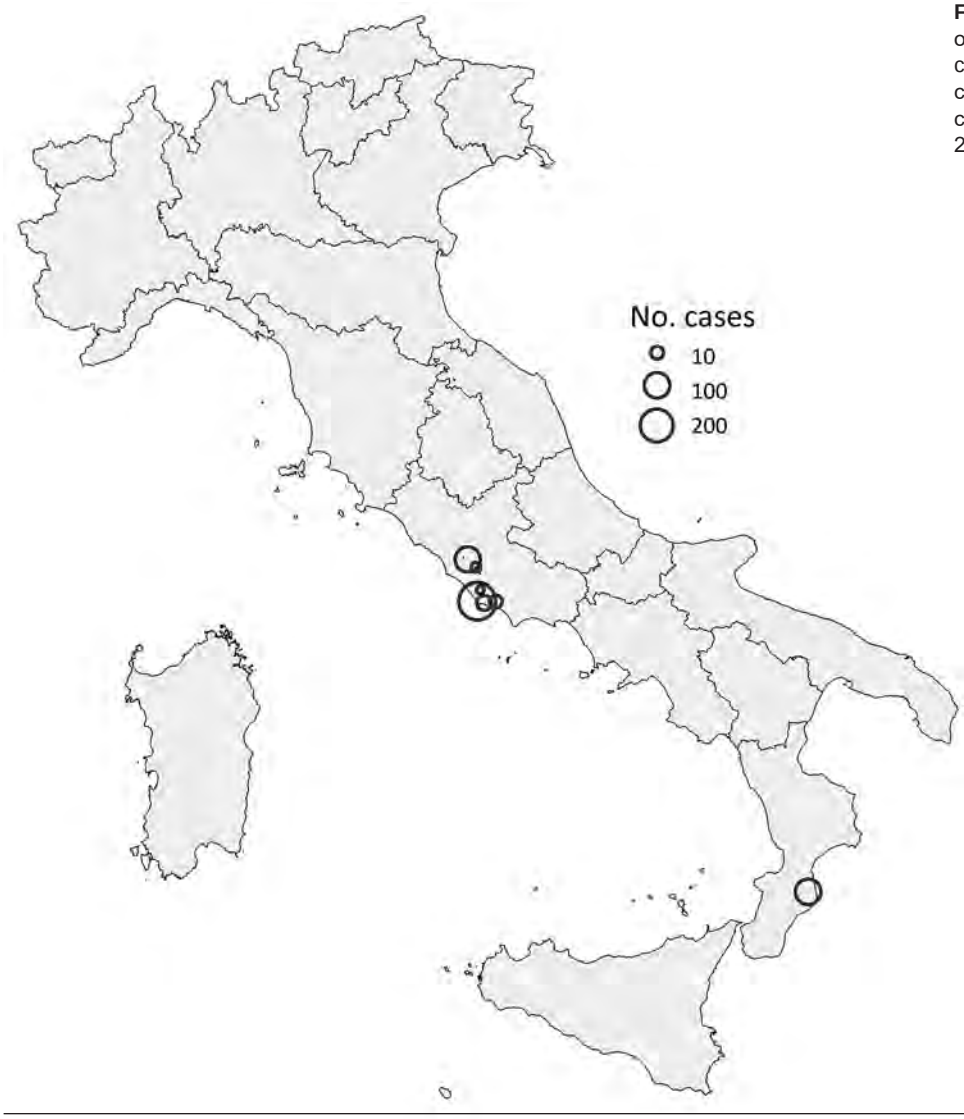

Figure 2. Location and size of clusters of 499 cases of chikungunya (probable and confirmed), by municipality, in central and southern Italy, June 26–November 15, 2017.

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References

1. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, Panning M, et al.; CHIKV Study Group. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet. 2007;370:1840–6. https://doi.org/10.1016/S0140-6736(07)61779-6
2. Venturi G, Di Luca M, Fortuna C, Remoli ME, Riccardo F, Severini F, et al. Detection of a chikungunya outbreak in central Italy, August to September 2017. Euro Surveill. 2017;22:pii=17-00646. https://doi.org/10.2807/1560-7917.ES.2017.22.39.17-00646
3. European Centre for Disease Prevention and Control. *Aedes albopictus*–current known distribution in Europe, April 2017 [cited 2019 Jan 8]. https://ecdc.europa.eu/en/publications-data/aedes-albopictus-current-known-distribution-europe-april-2017
4. Calba C, Guerbois-Galla M, Franke F, Jeannin C, Auzet-Caillaud M, Grard G, et al. Preliminary report of an autochthonous chikungunya outbreak in France, July to September 2017. Euro Surveill. 2017;22:pii=17-00647. https://doi.org/10.2807/1560-7917.ES.2017.22.39.17-00647
5. European Centre for Disease Prevention and Control. Rapid risk assessment. Clusters of autochthonous chikungunya cases in Italy: first update 9 October 2017 [cited 2019 Jan 8]. https://ecdc.europa.eu/ sites/portal/files/documents/RRA-chikungunya-Italy-update-9-Oct-2017.pdf
6. Manica M, Guzzetta G, Poletti P, Filipponi F, Solimini A, Caputo B, et al. Transmission dynamics of the ongoing chikungunya outbreak in central Italy: from coastal areas to the metropolitan city of Rome, summer 2017. Euro Surveill. 2017;22:pii=17-00685. https://doi.org/10.2807/1560-7917.ES.2017.22.44.17-00685

7. Vairo F, Mammone A, Lanini S, Nicastri E, Castilletti C, Carletti F, et al.; Chikungunya Lazio Outbreak Group. Local transmission of chikungunya in Rome and the Lazio region, Italy. PLoS One. 2018;13:e0208896. https://doi.org/10.1371/journal.pone.0208896
8. Lindh E, Argentini C, Remoli ME, Fortuna C, Faggioni G, Benedetti E, et al. The Italian 2017 outbreak chikungunya virus belongs to an emerging *Aedes albopictus* adapted virus cluster introduced from the Indian subcontinent [cited 2019 Jan 7]. https://academic.oup.com/ofid/advance-article/doi/10.1093/ofid/ofy321/5239584
9. Larrieu S, Balleydier E, Renault P, Baville M, Filleul L. Epidemiological surveillance of chikungunya on Reunion Island between 2005 and 2011 [in French]. Med Trop (Mars). 2012;72:38–42.
10. Staples JE, Breiman RF, Powers AM. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clin Infect Dis. 2009;49:942–8. https://doi.org/10.1086/605496
11. Josseran L, Paquet C, Zehgnoun A, Caillere N, Le Tertre A, Solet JL, et al. Chikungunya disease outbreak, Reunion Island. Emerg Infect Dis. 2006;12:1994–5. https://doi.org/10.3201/eid1212.060710
12. Moro ML, Gagliotti C, Silvi G, Angelini R, Sambri V, Rezza G, et al.; Chikungunya Study Group. Chikungunya virus in North-Eastern Italy: a seroprevalence survey. Am J Trop Med Hyg. 2010;82:508–11. https://doi.org/10.4269/ajtmh.2010.09-0322
13. Fortuna C, Toma L, Remoli ME, Amendola A, Severini F, Boccolini D, et al. Vector competence of *Aedes albopictus* for the Indian Ocean lineage (IOL) chikungunya viruses of the 2007 and 2017 outbreaks in Italy: a comparison between strains with and without the E1:A226V mutation. Euro Surveill. 2018;23:1800246. 10.2807/1560-7917.ES.2018.23.22.1800246 https://doi.org/10.2807/1560-7917.ES.2018.23.22.1800246
14. Russo G, Caputo B, Di Lascio A, Gatto G, Solimini AG. Awareness towards chikungunya virus infection risk by general practitioners in Rome: a questionnaire based survey before the 2017 outbreak. Infectious Diseases and Tropical Medicine. 2018;4:e451.

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Fatal Case of Nosocomial *Legionella pneumophila* Pneumonia, Spain, 2018

Diego Vicente, Jose M. Marimón, Itziar Lanzeta, Tania Martin, Gustavo Cilla

A nosocomial case of *Legionella pneumophila* pneumonia likely caused by a serogroup 3 strain was detected by a urinary antigen test in Spain in 2018. Although *Legionella* bacteria could not be isolated from respiratory samples, molecular methods implicated the sink faucet of the patient's room as the probable infection source.

Legionnaires' disease (LD) is a severe form of pneumonia caused primarily by the inhalation of *Legionella* spp. bacteria from colonized natural and artificial water systems. The annual incidence of LD in developed countries is ≈1 case/100,000 inhabitants and has been increasing in recent years (*1*,*2*). This increase is attributed in part to the widespread use of the urinary antigen test (UAT) as a diagnostic method. UAT is a fast and simple method, with good sensitivity and specificity to confirm the clinical suspicion of LD; however, most commercial tests only detect *Legionella pneumophila* serogroup 1. In many countries, UAT is used as the only LD diagnostic method. This strategy leads to the loss of detection of episodes caused by non–serogroup 1 *L. pneumophila*, including *L. pneumophila* serogroups 2–14, and other *Legionella* species (*1*,*2*).

We describe an episode of nosocomial *L. pneumophila* pneumonia probably caused by a serogroup 3 strain, which was diagnosed by UAT and genomic detection of *Legionella* DNA in respiratory samples. The episode was linked to the detection of the causative agent in the water of the sink of the patient's room.

The Study

In February 2018, a 66-year-old man sought care at Hospital Donostia-Instituto Biodonostia (San Sebastian, Spain) because of progressive loss of strength, accompanied by dysarthria and altered state of consciousness. A cranial computed tomography scan performed at hospital admission showed a deep intraparenchymal hematoma and a substantial surrounding edema. After a 1-month hospitalization in the neurology department, the patient was transferred to the long-stay unit of the internal medicine department, where he occupied the same room until the end of the episode. During this period, he was treated with high doses of dexamethasone to reduce the cerebral edema and different cycles of antibiotics (piperacillin/tazobactam and ceftriaxone) because of the presence of abundant respiratory secretions.

In April 2018, the patient had acute worsening of respiratory function, requiring high oxygen flow rates and mechanical ventilation. A chest radiograph showed the appearance of bilateral pulmonary infiltrates, and we observed elevated sepsis-associated markers in the blood analysis.

The patient was given a presumptive diagnosis of nosocomial pneumonia. We obtained a urine sample, 2 blood cultures, and 2 respiratory samples (sputum and tracheal aspirate) for microbiologic analysis. Blood cultures were negative. Results of a fluorescent immunoassay (Sofia *Legionella* FIA, https://www.quidel.com) detected *L. pneumophila* antigen in the urine, a result that was confirmed in a second sample obtained 12 hours later. Both urine samples had a negative result when tested with the Alere BinaxNOW *Legionella* Antigen Test Kit (Fisher Scientific, https://www.fishersci.com), which only detects *L. pneumophila* serogroup 1 (*3*). In the microbiologic culture of respiratory samples, we observed a scanty growth of saprophytic bacteria, but a culture on selective media *Legionella* (BCYE agar) was negative. The multiplex PCR for detection of *Legionella* spp., *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae* (BioGX, https://biogx.com) performed on the BD MAX System (https://www.bd.com) was positive for *Legionella* spp., both in the sputum and in the tracheal aspirate. The patient received levofloxacin but died 48 hours later.

After establishing the diagnosis of LD, we conducted an investigation to determine the origin of the episode and monitored the appearance of more cases. No episodes of *Legionella* spp. pneumonia were detected among patients admitted to the same unit during the previous month and during the month after the episode. We obtained samples of water from 23 different points of the internal medicine department unit where the patient had stayed, including his room's sink faucet and shower as well as another 5 rooms, an office, spillways, and refrigeration equipment.

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Non–serogroup 1 *L. pneumophila* (i.e., serotyped 2–14 in our microbiology department) was isolated in glycine, vancomycin, polymyxin, cycloheximide agar plates from the sink faucet of the patient's room (1,250 cfu/L) and from the sink faucet (275 cfu/L) and shower faucet (1,250 cfu/L) of the contiguous room. Disinfection of the affected facilities through thermal shock was performed, and the disappearance of *Legionella* was verified by using the same methods described. Monoclonal antibody subgrouping conducted at Spain's National Center for Microbiology identified *Legionella* isolates from these 3 environmental samples as serogroup 3. No more *L. pneumophila* was detected in any of the other 20 water samples we analyzed.

We performed sequence-based typing on DNA extracted from the sputum and the tracheal aspirate of the patient and from the 3 environmental isolates. We sequenced and amplified fragments of 7 genes in accordance with a protocol established by the European Working Group for Legionella Infections (EWGLI) (*4*), which includes the use of specific primers for the *neuA* homologue allele *neuAh* (*5*). We applied a nested PCR–derived sequence based typing method directly to DNA extracted from respiratory samples (*6*) and assigned allele and sequence type by using the online EWGLI SBT Database (*7*). The *Legionella* spp. detected in the 2 respiratory samples of the patient, as well as in the water of the patient's and a contiguous room, were identified as sequence type 1341.

Conclusions

Most LD episodes reported worldwide are attributed to *L. pneumophila* serogroup 1. Nevertheless, several studies suggest that episodes caused by *L. pneumophila* other than serogroup 1 might be underestimated because the main current method used for microbiologic diagnosis of LD is the UAT, which in most commercial test kits is limited to the detection of *L. pneumophila* serogroup 1 (*8*–*10*). The analysis of isolates from patients with *Legionella* pneumonia, both in Europe and the United States, shows that up to 20% were caused by *L. pneumophila* serogroups 2–14 or *Legionella* other than *L. pneumophila* (*1*,*2*,*11*), and this rate is higher among hospital-associated cases (*12*). However, *Legionella* culture is rarely used as a routine diagnostic method (*1*,*2*) because of the low efficiency and difficulties in isolation even if the bacterial DNA is present in the sample, as in the case we have described.

In our study, antigen detection in urine was performed by using a method able to detect, in addition to *L. pneumophila* serogroup 1, lipopolysaccharide of most *L. pneumophila* serogroups, including serogroup 3, although with a higher limit of detection (*3*). In addition, a nucleic acid amplification technique that enables detection of all *Legionella* species was used in respiratory samples, and secondarily, helped indicate an epidemiologic link with the infectious source without microbiologic culture of the causative strain. Although strains of *L. pneumophila* with identical sequence type can be of different serogroups, our results strongly suggest that the *L. pneumophila* serogroup 3 ST1341 strain isolated from the water in the sink faucet of the patient's room was the source of the nosocomial pneumonia episode. The *L. pneumophila* serogroup 3 ST1341, probably responsible for the nosocomial pneumonia episode we describe, was also reported in the EWGLI SBT Database in 3 LD episodes (2 in Germany and 1 in Catalonia, Spain) (*7*) and in an environmental sample in Greece (*8*). The strain does not seem to be a frequent genotype, although the case we have described supports the potential of nearly all *Legionella* genotypes to cause fatal infection.

Even though *L. pneumophila* serogroup 1 is responsible for most LD episodes, it is advisable to have techniques to detect other serogroups and species of *Legionella*, especially in the hospital environment, where the presence of non–serogroup 1 *L. pneumophila* is frequent (*8*,*12*) and a high number of susceptible immunosuppressed patients are present.

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References

1. Beauté J; The European Legionnaires' Disease Surveillance Network. Legionnaires' disease in Europe, 2011 to 2015. Euro Surveill. 2017;22:22. https://doi.org/10.2807/1560-7917.ES.2017.22.27.30566
2. Dooling KL, Toews KA, Hicks LA, Garrison LE, Bachaus B, Zansky S, et al. Active Bacterial Core Surveillance for Legionellosis—United States, 2011–2013. MMWR Morb Mortal Wkly Rep. 2015;64:1190–3. https://doi.org/10.15585/mmwr.mm6442a2
3. Ranc AG, Carpentier M, Beraud L, Descours G, Ginevra C, Maisonneuve E, et al. *Legionella pneumophila* LPS to evaluate urinary antigen tests. Diagn Microbiol Infect Dis. 2017;89:89–91. https://doi.org/10.1016/j.diagmicrobio.2017.06.013

4. Mentasti M, Fry NK. European Society of Clinical Microbiology and Infectious Diseases Study Group for *Legionella* Infections. Sequence-based typing protocol for epidemiological typing of *Legionella pneumophila*. 2012 [cited 2019 Sep 11]. http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/protocols/ESGLI%20SBT%20GUIDELINE%20v5.0.pdf
5. Mentasti M, Underwood A, Lück C, Kozak-Muiznieks NA, Harrison TG, Fry NK. Extension of the *Legionella pneumophila* sequence-based typing scheme to include strains carrying a variant of the N-acylneuraminate cytidylyltransferase gene. Clin Microbiol Infect. 2014;20:O435–41. https://doi.org/10.1111/1469-0691.12459
6. Ginevra C, Lopez M, Forey F, Reyrolle M, Meugnier H, Vandenesch F, et al. Evaluation of a nested-PCR-derived sequence-based typing method applied directly to respiratory samples from patients with Legionnaires' disease. J Clin Microbiol. 2009;47:981–7. https://doi.org/10.1128/JCM.02071-08
7. European Working Group for Legionella Infections. EWGLI Sequence-Based Typing (SBT) Database for *Legionella pneumophila* [cited 2019 Sep 11]. http://www.hpa-bioinformatics.org.uk/legionella/legionella_sbt/php/sbt_homepage.php
8. Katsiaflaka A, Pournaras S, Kristo I, Mouchtouri VA, Kyritsi M, Velonakis E, et al. Epidemiological investigation of *Legionella pneumophila* serogroup 2 to 14 isolates from water samples by amplified fragment length polymorphism and sequence-based typing and detection of virulence traits. Appl Environ Microbiol. 2016;82:6102–8. https://doi.org/10.1128/AEM.01672-16
9. European Centre for Disease Prevention and Control. Legionnaires disease in Europe, 2014. 2016 [cited 2019 Sep 11]. https://ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/legionnares-disease-europe-2014.pdf
10. Benson RF, Tang PW, Fields BS. Evaluation of the Binax and Biotest urinary antigen kits for detection of Legionnaires' disease due to multiple serogroups and species of *Legionella.* J Clin Microbiol. 2000;38:2763–5.
11. Quero S, Párraga-Niño N, Barrabeig I, Sala MR, Pedro-Botet ML, Monsó E, et al. Population structure of Environmental and Clinical *Legionella pneumophila* isolates in Catalonia. Sci Rep. 2018;8:6241. https://doi.org/10.1038/s41598-018-24708-1
12. Lévesque S, Lalancette C, Bernard K, Pacheco AL, Dion R, Longtin J, et al. Molecular typing of *Legionella pneumophila* isolates in the province of Quebec from 2005 to 2015. PLoS One. 2016;11:e0163818. https://doi.org/10.1371/journal.pone.0163818

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Swimming Pool–Associated *Vittaforma*-Like Microsporidia Linked to Microsporidial Keratoconjunctivitis Outbreak, Taiwan

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We analyzed 2 batches of environmental samples after a microsporidial keratoconjunctivitis outbreak in Taiwan. Results indicated a transmission route from a parking lot to a foot washing pool to a swimming pool and suggested that accumulation of mud in the foot washing pool during the rainy season might be a risk factor.

Microsporidia are obligate intracellular parasites that are generally found in aquatic environments (*1*,*2*). The main symptoms of microsporidiosis are keratoconjunctivitis, diarrhea, muscular infection, and acalculous cholecystitis, among which keratoconjunctivitis and diarrhea are the most common (*3*,*4*). As a result of improved diagnostic methods and increased awareness, microsporidia are now considered emergent pathogens worldwide (*4*,*5*).

Vittaforma corneae has been considered a major risk factor for ocular microsporidiosis. In a previous study, we provided molecular evidence for the presence of *V. corneae* in hot springs in Taiwan (*6*). However, recent evidence indicates that ocular microsporidiosis might be underreported in keratoconjunctivitis (*5*,*7*). In our previous study, we hypothesized that *V. corneae* or *Vittaforma*-like microsporidia might spread from adjacent land environments (e.g., soil or mud) to aquatic environments (*2*).

In June 2017, the New Taipei City Health Bureau (New Taipei City, Taiwan) was notified of a keratoconjunctivitis outbreak at a resort. The patients, healthy teenagers from a high school wrestling team, were found to contain DNA and spores from *V. corneae*, thus indicating that it was a microsporidial keratoconjunctivitis (MK) outbreak (*8*). Water contamination at the pool was suspected to be responsible for the outbreak. To identify the source of the pathogen, the transmission route, and the risk factors, water samples from various facilities at the swimming resort were collected for further evaluation. Moreover, because past studies have indicated that soil exposure is an important risk factor for MK, both soil and water samples were collected in a follow-up field survey. We describe results based on the 2 field surveys and provide information on the important risk factors for MK.

The Study

This study was initially conducted because of a request of the New Taipei City Health Bureau in response to the MK outbreak (*8*). The swimming pools at the resort had been filled with tap water. Unfortunately, we received information about the MK outbreak 1 day after cleaning and disinfection of the swimming pools had taken place (*8*). Before water samples were collected, the resort was temporarily closed by the health authorities, and the facility was cleaned and disinfected as recommended by the health authorities (i.e., treatment with 5 ppm free available chlorine for 3 hours). The cleaning and disinfection procedures included draining of all water reservoirs, pools, and tubs, followed by surface scrubbing to remove any potential biofilms and disinfection with sodium hypochlorite. The pretreatment methods of samples, PCR conditions, phylogenetic analysis, and all protocols were performed as described in our previous studies (*2*,*6*).

We collected 19 water samples and 8 soil samples from the swimming resort and its surrounding environment (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/25/11/18-1483-App1.pdf). We sequenced all 17 amplicons of the 15 test-positive samples, analyzed them by using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and compared them with reference species from GenBank to determine the most closely related species. All the amplicons were homologous to microsporidia, with identity ranging from 89% to 99% (Table). Only 5 (29.4%) amplicons showed a high degree of homology (>97% identity) to the reference strain.

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Table. Comparison of BLAST results from environmental strains in a field study conducted after a microsporidial keratoconjunctivitis outbreak, Taiwan*

Strain	Sample source	Top BLAST hit (GenBank accession no.)	Maximum identity, study strain/reference (%)	Reference source
Xindian_1_Water	Standard swimming pool	*Vittaforma corneae* strain HotSpring-E1-o (KY245918)	470/473 (99)	Environmental
Xindian_3_Water	Standard swimming pool	*Vittaforma corneae* strain HotSpring-E1-o (KY245918)	467/473 (99)	Environmental
Xindian_10_Water	Foot wash pool	Microsporidium sp. BVOR4 (FJ756182)	438/472 (93)	Environmental
Xindian_14_Water	River	*Enterocytospora artemiae* isolate Ea_monica20 (JX915755)	477/483 (99)	*Artemia franciscana*
Xindian_15_Soil	Park near the resort	*Vittaforma corneae* strain HotSpring-C3-o (KY245925)	430/467 (92)	Environmental
Xindian_16_Water	Sink in the park	Uncultured microsporidia clone Chula_Myositis 1 (JN619406)	420/469 (90)	Clinical
Xindian_17_Soil	Park near the resort	*Nosema* sp. FCG-1468 (LC033883)	429/450 (95)	Honey bee
Xindian_18_Soil	Park near the resort	*Sporanauta perivermis* (KC172651)	432/485 (89)	Marine nematode
Xindian_20_Water	Wastewater from the resort	*Unikaryonidae* sp. JI-2011 (JF960137)	456/486 (94)	Curculionidae
Xindian_21_Water	Wastewater from the resort	Uncultured microsporidia clone Chula_Myositis 1 (JN619406)	425/474 (90)	Clinical
Xindian_22_Soil(U)	Soil in the wastewater flow	*Vittaforma corneae* strain HotSpring-F2-o (KY245925)	453/473 (96)	Environmental
Xindian_22_Soil(D)	Soil in the wastewater flow	*Vittaforma corneae* strain HotSpring-F2-o (KY245925)	450/472 (95)	Environmental
Xindian_23_Soil(U)	Park near the resort	*Vittaforma corneae* strain HotSpring-F3-o (KY245925)	458/472 (97)	Environmental
Xindian_23_Soil(D)	Park near the resort	*Vittaforma corneae* strain HotSpring-F2-o (KY245925)	448/474(95)	Environmental
Xindian_24_Soil	Parking lot near the resort	*Vittaforma corneae* strain LVPEI.BP235FR_11 (KP099409)	453/475 (95)	Clinical
Xindian_25_Water	Pavement rainwater	*Endoreticulatus* sp. Melnik (KU900486)	455/471 (97)	*Euproctis chrysorrhoea*
Xindian_26_Water	Foot wash pool	*Vittaforma corneae* strain LVPEI.BP235FR_11 (KP099409)	453/475 (95)	Clinical
Sw_MK_outbreak	Patient	*Vittaforma corneae* strain LVPEI.BP235FR_11 (KP099409)	472/472(100)	Clinical

*Top BLAST hit indicates the closest reference species that matched the environmental strains using BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Maximum identity represents the percentage identity between the environmental strains and the closest reference species. The fifth column shows the isolated source of the closest reference species.

In the initial survey (10 days after site disinfection), *V. corneae* was not detected. However, other *Vittaforma*-like microsporidia were identified in the standard swimming pool and foot washing pool. According to the Enforcement Rules for Swimming Pool Management in Taiwan, swimming pool water should be chlorinated (with a concentration of free available chlorine of ≈0.3–0.7 ppm) to prevent the spread of waterborne diseases. The presence of these other *Vittaforma*-like microsporidia indicates that some problems might have occurred during the disinfection process.

According to the New Taipei City Health Bureau, all pools, water source tanks, waterlines, and tubs in this facility were drained and scrubbed during the cleanup and disinfection process. The source of *V. corneae* infection remains debatable. Chlorine disinfection studies have shown that residual chlorine is capable of inactivating and reducing the number of microsporidians. However, microsporidian spores are relatively resistant to the typical concentrations of chlorine used for swimming pool disinfection (depending on the species, spores are inactivated after an exposure time of 10–120 min [i.e., inactivation of *Encephalitozoon intestinalis* spores does not reach 100% under 5 ppm of free available chlorine even after 120 minutes of treatment]) (*9*–*11*). Many clinical studies have indicated that soil or mud exposure, visits to hot springs, and outdoor activities, especially after rainfall, are all risk factors for ocular microsporidiosis (*12*,*13*). In addition, our previous study provided evidence for the presence of *V. corneae* in hot springs, thus indicating that pools in outdoor environments were associated with the presence of *V. corneae* (*6*). Therefore, we considered that the contaminating microsporidia in the swimming resort might have been brought to the resort from outside by human activities.

Our data show that many clade IV microsporidia were present in the soil and water samples from the resort site (Appendix Figure 2). Most of the microsporidia in clade IV are of terrestrial origin, according to small subunit rRNA gene phylogenetic analysis (*14*,*15*). Therefore, given that previous studies have shown that rainfall is an

important risk factor for ocular microsporidiosis (*5*,*12*,*13*), we believe that water contamination might originate from soil environments after rainfall. Rainfall occurred near the sampling location during June 15–19 and again during July 1–4 (as recorded by the Taiwan Central Weather Bureau). We conducted a follow-up site survey after the July 1–4 rainfall and found *Vittaforma*-like microsporidians were found in pavement rainwater and the parking lot of the resort. Hence, we hypothesized that the swimming pool water was contaminated through soil or water brought in by human activity during the rainfall. *Vittaforma*-like microsporidians were found in the swimming pool and foot washing pool in the initial survey, which was conducted after a careful disinfection procedure, but microsporidia were not found in tap water or other pools. Therefore, these results suggest that the contamination was not from the waterlines or water sources, and the pools may have been contaminated from an outside source owing to human activities and poor facility configuration. In the follow-up survey, we found 100% identical amplicons in the parking lot and foot washing pool, suggesting a possible transmission pathway for *Vittaforma*-like microsporidians from the outside environment to the swimming pool (Appendix Figure 1).

Conclusions

Our study demonstrated the presence of *Vittaforma*-like microsporidia in a swimming resort and nearby environments in Taiwan. Human activities, rainy weather, and soil-rich or park environments might have been possible sources of microsporidia in the waters at the facility. The foot washing pool and shoe cabinet area are possible contamination areas and might facilitate transmission of microsporidia throughout the swimming resort. We suggest several precautions, including improving the frequency and efficacy of disinfection procedures at the facility, using a continuous water flow facility in foot washing pools, and paying attention to the disinfection and cleaning of the shoe cabinet area, especially during the rainy season. In addition, for swimming resorts that are located in a park, enhanced monitoring of the environment surrounding the swimming pool is warranted.

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References

1. Dowd SE, Gerba CP, Pepper IL. Confirmation of the human-pathogenic microsporidia *Enterocytozoon bieneusi, Encephalitozoon intestinalis*, and *Vittaforma corneae* in water. Appl Environ Microbiol. 1998;64:3332–5.
2. Chen JS, Hsu BM, Tsai HC, Chen YP, Huang TY, Li KY, et al. Molecular surveillance of *Vittaforma*-like microsporidia by a small-volume procedure in drinking water source in Taiwan: evidence for diverse and emergent pathogens. Environ Sci Pollut Res Int. 2018;25:18823–37. https://doi.org/10.1007/s11356-018-2081-4
3. Didier ES. Microsporidiosis: an emerging and opportunistic infection in humans and animals. Acta Trop. 2005;94:61–76. https://doi.org/10.1016/j.actatropica.2005.01.010
4. Keeling P. Five questions about microsporidia. PLoS Pathog. 2009;5:e1000489. https://doi.org/10.1371/journal.ppat.1000489
5. Sharma S, Das S, Joseph J, Vemuganti GK, Murthy S. Microsporidial keratitis: need for increased awareness. Surv Ophthalmol. 2011;56:1–22. https://doi.org/10.1016/j.survophthal.2010.03.006
6. Chen JS, Hsu TK, Hsu BM, Huang TY, Huang YL, Shaio MF, et al. Surveillance of *Vittaforma corneae* in hot springs by a small-volume procedure. Water Res. 2017;118:208–16. https://doi.org/10.1016/j.watres.2017.04.029
7. Joseph J, Vemuganti GK, Sharma S. Microsporidia: emerging ocular pathogens. Indian J Med Microbiol. 2005;23:80–91. https://doi.org/10.4103/0255-0857.16045
8. Wang WY, Chu HS, Lin PC, Lee TF, Kuo KT, Hsueh PR, et al. Outbreak of microsporidial keratoconjunctivitis associated with water contamination in swimming pools in Taiwan. Am J Ophthalmol. 2018;194:101–9. https://doi.org/10.1016/j.ajo.2018.07.019
9. Wolk DM, Johnson CH, Rice EW, Marshall MM, Grahn KF, Plummer CB, et al. A spore counting method and cell culture model for chlorine disinfection studies of *Encephalitozoon* syn. *Septata intestinalis*. Appl Environ Microbiol. 2000;66:1266–73. https://doi.org/10.1128/AEM.66.4.1266-1273.2000
10. Johnson CH, Marshall MM, DeMaria LA, Moffet JM, Korich DG. Chlorine inactivation of spores of *Encephalitozoon* spp. Appl Environ Microbiol. 2003;69:1325–6. https://doi.org/10.1128/AEM.69.2.1325-1326.2003
11. Li X, Fayer R. Infectivity of microsporidian spores exposed to temperature extremes and chemical disinfectants. J Eukaryot Microbiol. 2006;53(Suppl 1):S77–9. https://doi.org/10.1111/j.1550-7408.2006.00180.x

12. Loh RS, Chan CM, Ti SE, Lim L, Chan KS, Tan DT. Emerging prevalence of microsporidial keratitis in Singapore: epidemiology, clinical features, and management. Ophthalmology. 2009; 116:2348–53. https://doi.org/10.1016/j.ophtha.2009.05.004
13. Reddy AK, Balne PK, Garg P, Krishnaiah S. Is microsporidial keratitis a seasonal infection in India? Clin Microbiol Infect. 2011;17:1114–6. https://doi.org/10.1111/j.1469-0691.2010.03084.x
14. Vossbrinck CR, Debrunner-Vossbrinck BA. Molecular phylogeny of the Microsporidia: ecological, ultrastructural and taxonomic considerations. Folia Parasitol (Praha). 2005;52:131–42, discussion 130. https://doi.org/10.14411/fp.2005.017
15. Sokolova Y, Pelin A, Hawke J, Corradi N. Morphology and phylogeny of *Agmasoma penaei* (Microsporidia) from the type host, *Litopenaeus setiferus*, and the type locality, Louisiana, USA. Int J Parasitol. 2015;45:1–16. https://doi.org/10.1016/j.ijpara.2014.07.013

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Isolation of *Legionella pneumophila* by Co-culture with Local Ameba, Canada

Rafik Dey, Harley Mount, Alex W. Ensminger, Greg J. Tyrrell, Linda P. Ward, Nicholas J. Ashbolt

Legionellosis was diagnosed in an immunocompromised 3-year-old girl in Canada. We traced the source of the bacterium through co-culture with an ameba collected from a hot tub in her home. We identified *Legionella pneumophila* serogroup 6, sequence type 185, and used whole-genome sequencing to confirm the environmental and clinical isolates were of common origin.

Legionella pneumophila is a waterborne bacterium responsible for Legionnaires' disease, a potentially fatal respiratory disease acquired through environmental exposure to aerosolized water. According to the World Health Organization, *L. pneumophila* is the most common cause of legionellosis worldwide (*1*). For unknown reasons, cases reported in the United States and Europe have risen sharply over the past decade (*2*,*3*). Most legionellosis cases identified are caused by *L. pneumophila* serogroup 1 (*4*), possibly because of extensive use of initial urinary antibody screening that focuses on serogroup 1.

Free-living amebae are known natural environmental reservoirs for *L. pneumophil*a (*5*). Amebae particularly play a role in legionellae growth in warm and stagnant engineered environments at 35°–45°C and in the bacterium's persistence in high temperatures, biocides, and pH extremes (*6*). Co-culture with amebae is an efficient tool to detect *L. pneumophila* from human and environmental samples (*7*). However, amebae are rarely used in environmental investigations.

The Study

In December 2016, an immunocompromised 3-year-old girl was admitted to Alberta Children's Hospital in Calgary, Alberta, Canada, with acute respiratory distress syndrome and septic shock requiring extracorporeal membrane oxygenation. She was transferred to a pediatric intensive care unit in Edmonton, Alberta, Canada, where examination of the lungs confirmed pneumonia in the left lower lobe segment with a lung abscess. Empiric treatment for infections was started immediately with meropenem, vancomycin, tobramycin, azithromycin, and trimethoprim/sulfamethoxazole. Subsequent bronchoalveolar lavage and bronchoscopy were performed, along with microbial culture for identification of fungi, mycobacteria, mycoplasma, viruses, and *Legionella*. Public Health Laboratory (ProvLab), Edmonton, successfully isolated *Legionella* sp. from clinical samples by using buffered charcoal yeast extract (BCYE) medium, with and without antimicrobial drugs, including polymyxin B, cycloheximide, and vancomycin. The clinical isolate was identified as *L. pneumophila* by using *Legionella pneumophila* Direct DFA Kit (Pro-Lab Diagnostics, https://pro-lab.com) direct fluorescent antibody assay. The National Microbiology Laboratory in Winnipeg, Manitoba, typed the isolate as serogroup 6, sequence type 185 (ST185), confirming Legionnaires' disease.

The patient was treated with levofloxacin and a prophylactic dose of trimethoprim/sulfamethoxazole. She also received vancomycin, meropenem, and tobramycin for 8 days and azithromycin for 5 days. Her condition steadily improved, and she was discharged from the hospital a few days after treatment.

To investigate possible *L. pneumophila* sources, the Infection Prevention Control Research Laboratory of Alberta Health Services collected several first-flush water samples from sinks, a shower head, and a hot tub at the patient's house and from sinks in the admitting hospital. All samples were negative for *L. pneumophila* by culture, but quantitative PCR results indicated the home hot tub was the likely source of the bacterium.

We initially attempted co-culture with *Acanthamoeba polyphaga* (ATCC30461) (*8*) but failed to isolate *Legionella* spp. Because growth of *L. pneumophila* in the environment is hypothesized to be dependent partly on the composition of local amebic populations (*9*), we isolated free-living amebae hosts from the hot tub samples. We then identified ameba-resisting bacteria (ARB) by using the isolated ameba in co-culture with the hot tub samples (*7*).

We isolated free-living ameba hosts by filtering water from different environmental sites. We grew amebae at 30°C on nonnutrient agar supplemented with a thin film of viable *Escherichia coli* (ATCC25922). We isolated 2 free-living amebae, an *Acanthamoeba* sp. and a *Vermamoeba vermiformis*, and identified species by morphology (Figure 1, panel A) and 18S rRNA gene sequencing.

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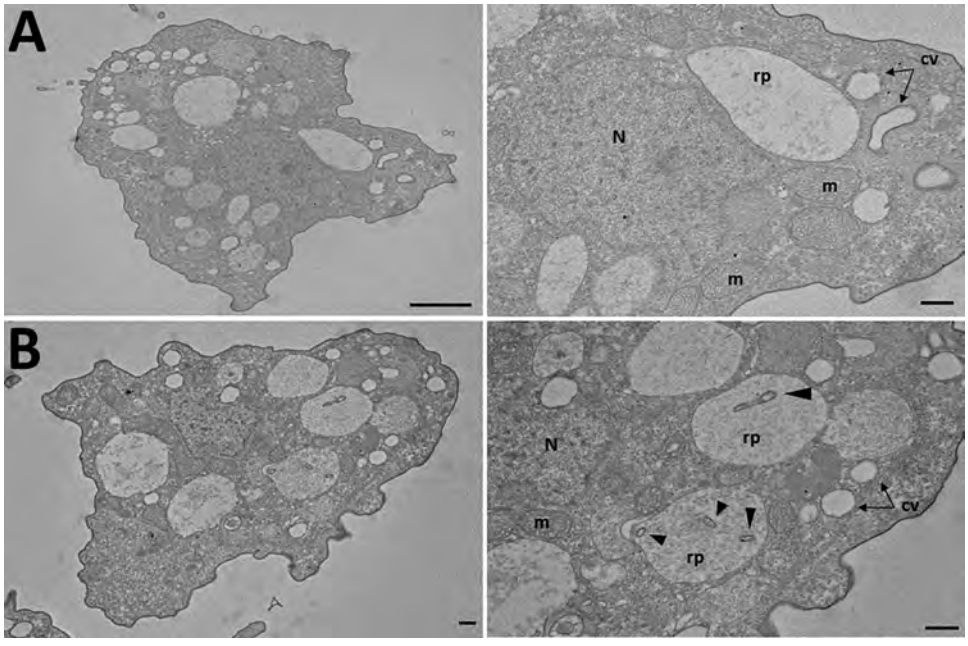

Figure 1. Transmission electron micrograph at of amebae isolated from the home hot tub of a an immunocompromised 3-year-old girl with legionellosis before and after co-culture with *Legionella pneumophila*, Calgary, Alberta, Canada. A) Trophozoites of *Vermamoeba vermiformis* before co-culture. Note the absence of intracellular bacteria in the replicative phagosome. B) *V. vermiformis* replicative phagosome containing *L. pneumophila* serogroup 6 after 6 h of co-culture. Arrows indicate *L. pneumophila* contained within replicative phagosomes. Scale bars in left panels indicate 2 μm; scale bars in right panels indicate 500 nm. cv, contractile vacuoles; m, mitochondria; N, nucleus; rp, replicative phagosome.

For co-culture experiments, we established amebae in axenic cultures in Nunc 25-cm^2 tissue culture flasks (ThermoFisher Scientific, https://www.thermofisher.com) containing 5 mL serum casein glucose yeast extract medium at 37°C with 10% fetal calf serum. Before experiments, we performed subcultures of amebae every 3–4 days to ensure that trophozoites were in an exponential growth phase.

In brief, we co-cultured each environmental water sample with its isolated ameba by using several dilutions and incubating samples at 30°C for 12 h. When we observed amebal lysis, we recovered ARB on BCYE agar. We identified 1 of the ARB isolates from the ameba–hot tub culture as *L. pneumophila* by using 16S rRNA gene sequencing (Table) and subsequent sequence-based typing (*10*). Serotyping for *L. pneumophila* indicated both the clinical and environmental isolates were ST185, serogroup 6. We confirmed the presence of *L. pneumophila* inside *V. vermiformis* replicative phagosomes by transmission electron micrograph (Figure 1, panel B).

For confirmation, we performed whole-genome sequencing on clinical and environmental isolates. We extracted genomic DNA by using the NucleoSpin Tissue Kit (Macherey-Nagel, https://www.mn-net.com). We prepared libraries according to the protocol for the Nextera XT DNA Library Prep Kit (Illumina, https://www.illumina.com) and sequenced on an Illumina MiniSeq by using 2 × 150-nt reads. We deposited sequence information into BioProject (https://www.ncbi.nlm.nih.gov/bioproject) under accession no. PRJNA482644.

We trimmed sequence reads by using Trimmomatic version 0.36 (*11*) with the following parameters: Nextera clip, 2:30:10:8:true; LEADING, 20; TRAILING, 20; SLIDINGWINDOW, 4:20; MINLEN, 36. We assembled reads by using the SPAdes version 3.12 assembler in careful mode (*12*). We identified the closest related *L. pneumophila* strains for each set of contigs by using the PATRIC server (*13*) and searching for whole genome k-mer. We reordered assembled contigs by using mauve (*14*) against the closest matching reference, ATCC43290. We mapped trimmed sequence reads from both strains to the clinical isolate in the SPAdes de novo assembly by using Bowtie2 version 2.3.4.1 (https://github.com/BenLangmead/bowtie2), then processed reads with Picard (Broad Institute, https://www.broadinstitute.org) and identified single-nucleotide polymorphism insertions/deletions by using FreeBayes version 1.2.0 (https://www.geneious.com/plugins/freebayes). We filtered resulting polymorphisms with VCF.Filter version 4.2 (https://biomedical-sequencing.at/VCFFilter) by using QUAL >10 & DP >20 & QUAL/AO >10 & SAF >0 & SAR >0 & RPR >1 &

Table. Bacteria isolated from water samples by co-culture with local ameba and location of water samples in investigation of a legionellosis case, Calgary, Alberta, Canada

Ameba host and bacterium	Water sample location
Acanthamoeba sp.	
Pseudomonas stutzeri	Hospital sink
Paenibacillus terrigena	Hospital sink
Pseudacidovorax intermedius	Hospital sink
Vermamoeba vermiformis	
Acidovorax delafieldii	Home hot tub
*Legionella pneumophila**	Home hot tub

**L. pneumophila* isolate from the case-patient's home hot tub was confirmed as the same serotype and sequence type as the clinical isolate from the case-patient.

RPL >1. To rule out assembly errors or other spurious calls, we visually inspected the location of each putative polymorphism in reference assemblies of each isolate and traced back to the clinical contigs.

We constructed a phylogenetic tree by submitting assembled scaffolds to the RAST server for genome annotation (*15*). To find core conserved orthologs with default parameters, we input the resulting gene annotations into OrthoMCL (https://orthomcl.org), alongside the complete ORFeomes of 6 other *L. pneumophila* strains: Philadelphia-1 NC_002942.5, Lens NC_006369.1, Thunder Bay CP003730.1, 570-CO-H NC_016811.1, Toronto-2005 NZ_CP012019.1, and Calgary-2012 SAMN03944918. We individually aligned 2,403 identified orthologs (2,471,034 nt) across all strains by using the MUSCLE algorithm (https://www.ebi.ac.uk/Tools/msa/muscle) and concatenated orthologs into a superalignment for tree construction. We adopted RAxML version 8.2.12 (ILRI Research Computing, http://hpc.ilri.cgiar.org) with a general time-reversible nucleotide substitution model for 1,000 bootstraps to generate a maximum-likelihood phylogenetic tree.

Results of whole-genome sequencing analysis strongly suggest that clinical isolate 2017a and environmental isolate 2017b from the patient's home hot tub were of common origin. With only a few single-nucleotide polymorphism differences (Figure 2), these data indicate the hot tub was the source of the patient's infection.

Of note, we were not able to recover any legionellae from environmental samples with initial BCYE and other direct culture approaches. *L. pneumophila* serogroup 6 has not been identified in previous hot tub–associated infections, and this case might have been a result of the patient's immunocompromised status. A commercial hot tub cleaning product was subsequently used for disinfection and maintenance of the hot tub. In a 1-year follow up analysis, water samples from the hot tub were negative for amebae and any *Legionella* spp.

Conclusions

Our report demonstrates the utility of ameba co-culture and emphasizes the use of locally sourced ameba to recover the source of *L. pneumophila* from environmental samples. Our findings also suggest that investigations should include free-living ameba to indicate the presence of potentially pathogenic *Legionella* spp. and as a potential factor to minimize the need for remediation actions associated with contaminated environments.

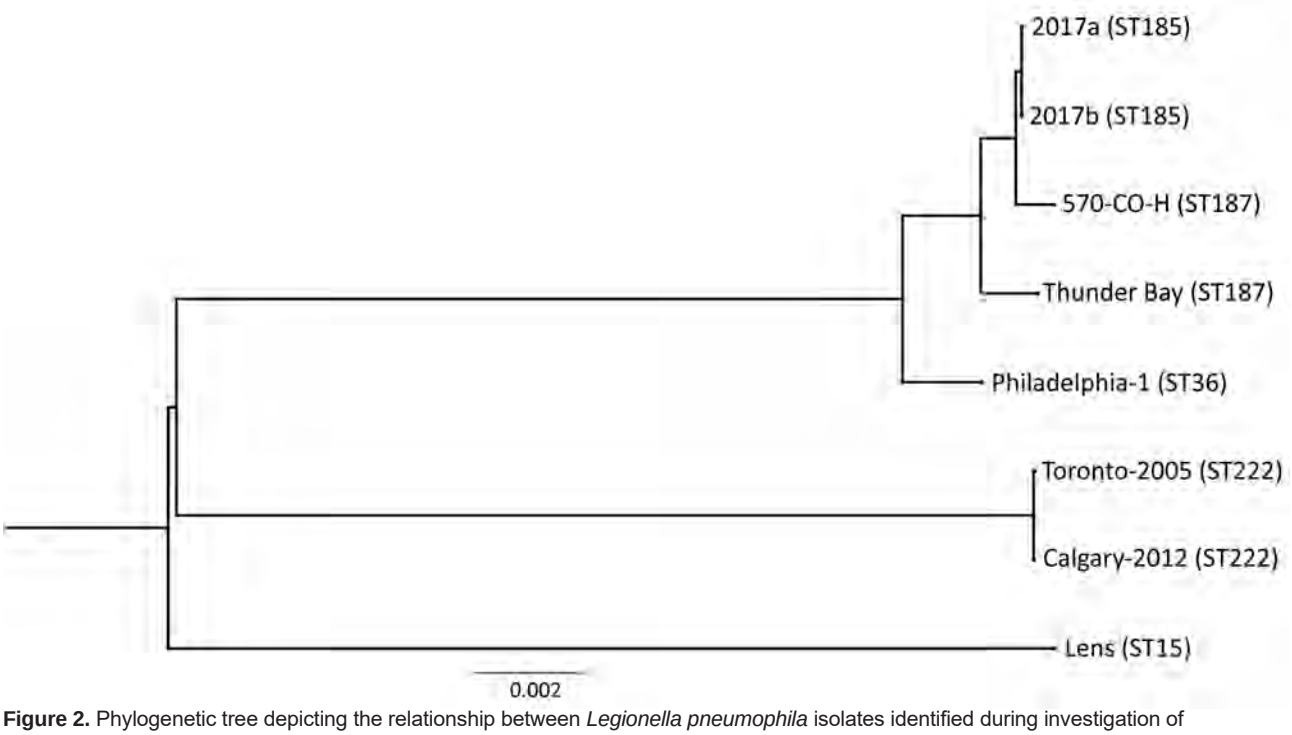

Figure 2. Phylogenetic tree depicting the relationship between *Legionella pneumophila* isolates identified during investigation of legionellosis in an immunocompromised 3-year-old girl, Calgary, Alberta, Canada, and reference sequences. *L. pneumophila* core ortholog-based maximum-likelihood phylogenetic tree shows 8 previously published genomes and sequences of the 2 isolates from this study (2017a, clinical isolate from patient; 2017b, environmental isolate from hot tub in patient's home). Tree construction was performed by using 2,403 orthologous sequences (2,471,034 nt). Each ortholog sequence was independently aligned with the MUSCLE algorithm (https://www.ebi.ac.uk/Tools/msa/muscle) and concatenated into a single superalignment, which then was subjected to 1,000 bootstrap iterations to find the maximum-likelihood phylogeny. Scale bar indicates the number of nucleotide substitutions per site. ST, sequence type.

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Consent for publication: Written informed consent was obtained from the patient's legal guardians for publication of this case report and any accompanying images.

Authors' contributions: R.D. and N.J.A. conceived and designed the study; R.D. carried out the microbiome isolation and analysis; H.M. and A.W.E. sequenced the bacterium and analyzed the data; G.J.T. collected and analyzed the clinical data; L.P.W. collected the environmental samples; R.D. and N.J.A. wrote the manuscript with all authors contributing. All authors read and approved the final manuscript.

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References

1. World Health Organization. Legionellosis. 2018 Feb 16 [cited 2018 Feb 16]. https://www.who.int/en/news-room/fact-sheets/detail/legionellosis
2. Beer KD, Gargano JW, Roberts VA, Reses HE, Hill VR, Garrison LE, et al. Outbreaks associated with environmental and undetermined water exposures—United States, 2011–2012. MMWR Morb Mortal Wkly Rep. 2015;64:849–51. https://doi.org/10.15585/mmwr.mm6431a3
3. Beauté J; The European Legionnaires' Disease Surveillance Network. Legionnaires' disease in Europe, 2011 to 2015. Euro Surveill. 2017;22. https://doi.org/10.2807/1560-7917.ES.2017.22.27.30566
4. Cunha BA, Burillo A, Bouza E. Legionnaires' disease. Lancet. 2016;387:376–85. https://doi.org/10.1016/S0140-6736(15)60078-2
5. Rowbotham TJ. Current views on the relationships between amoebae, legionellae and man. Isr J Med Sci. 1986;22:678–89.
6. Buse HY, Schoen ME, Ashbolt NJ. Legionellae in engineered systems and use of quantitative microbial risk assessment to predict exposure. Water Res. 2012;46:921–33. https://doi.org/10.1016/j.watres.2011.12.022
7. Rowbotham TJ. Isolation of *Legionella pneumophila* serogroup 1 from human feces with use of amebic cocultures. Clin Infect Dis. 1998;26:502–3. https://doi.org/10.1086/517095
8. Pagnier I, Raoult D, La Scola B. Isolation and identification of amoeba-resisting bacteria from water in human environment by using an *Acanthamoeba polyphaga* co-culture procedure. Environ Microbiol. 2008;10:1135–44. https://doi.org/10.1111/j.1462-2920.2007.01530.x
9. Dey R, Bodennec J, Mameri MO, Pernin P. Free-living freshwater amoebae differ in their susceptibility to the pathogenic bacterium *Legionella pneumophila.* FEMS Microbiol Lett. 2009;290:10–7. https://doi.org/10.1111/j.1574-6968.2008.01387.x
10. Wong S, Pabbaraju K, Burk VF, Broukhanski GC, Fox J, Louie T, et al. Use of sequence-based typing for investigation of a case of nosocomial legionellosis. J Med Microbiol. 2006;55:1707–10. https://doi.org/10.1099/jmm.0.46754-0
11. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30:2114–20. https://doi.org/10.1093/bioinformatics/btu170
12. Bankevich A, Nurk S, Antipov D, Gurevich AA, Dvorkin M, Kulikov AS, et al. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 2012;19:455–77. https://doi.org/10.1089/cmb.2012.0021
13. Wattam AR, Abraham D, Dalay O, Disz TL, Driscoll T, Gabbard JL, et al. PATRIC, the bacterial bioinformatics database and analysis resource. Nucleic Acids Res. 2014;42(D1):D581–91. https://doi.org/10.1093/nar/gkt1099
14. Darling AE, Mau B, Perna NT. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One. 2010;5:e11147. https://doi.org/10.1371/journal.pone.0011147
15. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST Server: rapid annotations using subsystems technology. BMC Genomics. 2008;9:75. https://doi.org/10.1186/1471-2164-9-75

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Human-to-Human Transmission of Influenza A(H3N2) Virus with Reduced Susceptibility to Baloxavir, Japan, February 2019

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In 2019, influenza A(H3N2) viruses carrying an I38T substitution in the polymerase acidic gene, which confers reduced susceptibility to baloxavir, were detected in Japan in an infant without baloxavir exposure and a baloxavir-treated sibling. These viruses' whole-genome sequences were identical, indicating human-to-human transmission. Influenza virus isolates should be monitored for baloxavir susceptibility.

The cap-dependent endonuclease inhibitor baloxavir marboxil is approved in Japan for the treatment of influenza virus infection in patients >12 years of age and children <12 years of age weighing ≥10 kg. In phase 2 and 3 clinical trials of baloxavir, treatment-emergent amino acid substitutions—I38T or I38F for influenza A(H1N1)pdm09 (pH1N1) virus and I38T or I38M for influenza A(H3N2) virus in the polymerase acidic (PA) protein—were detected (*1*,*2*). The frequency of infections with these viruses was higher in patients <12 years of age than in those 12–64 years of age (*3*). Furthermore, PA I38 substitutions emerged more frequently in influenza A(H3N2) viruses than in pH1N1 virus or influenza B virus (*3*).

In phase 3 trials, patients infected with mutant viruses encoding the PA I38 substitution exhibited prolonged virus shedding, and the median time to symptom alleviation was longer in baloxavir recipients infected with these viruses than those infected with viruses not harboring these substitutions (*1*,*2*). Therefore, starting in the 2017–18 influenza season, we began monitoring baloxavir susceptibility of influenza viruses nationwide (*4*). In the 2018–19 season, we found that 1.5% (5/323) of pH1N1 and 9.5% (32/337) of H3N2 viruses possessed a PA I38 substitution (Table 1). All 5 pH1N1 viruses and 28 of 32 H3N2 viruses encoding a PA I38 substitution were recovered from patients after baloxavir administration. In January 2019, we detected a mutant influenza A(H3N2) virus carrying the PA I38T substitution from a hospitalized 5-year-old child who was not treated with baloxavir (*5*). We subsequently detected 3 similar mutant H3N2 viruses from 3 baloxavir-untreated children. Two of 3 were detected in sporadic cases and the other from a family cluster. Here, we report on the family cluster.

The Study

In February 2019, we detected 2 H3N2 viruses in siblings within a family cluster (Figure). The first child (a 10-year-old) experienced symptom onset on February 5 and was treated with baloxavir 12 hours later; this child's fever resolved within a half day of baloxavir administration. The second child (an 8-month-old infant weighing <10 kg) experienced symptom onset on February 6 and received neuraminidase (NA) inhibitor oseltamivir 12 hours later; the infant's fever resolved within 2 days of oseltamivir administration.

We collected a nasal blow sample from the 10-year-old child 3 days after baloxavir administration and a nasal aspirate from the infant on the first day of oseltamivir administration. Deep sequencing analysis (*4*) of the virus isolates (A/Kanagawa/IC18144/2019 in 10-year-old and A/Kanagawa/IC18141/2019 in infant) with MiSeq (Illumina, https://www.illumina.com) revealed that the whole-genome sequences of these viruses were identical. Both viruses possessed the PA I38T substitution and did not contain wild-type 38I. No amino acid substitutions associated with reduced susceptibility to NA inhibitors were detected.

We determined the susceptibilities of this mutant virus to baloxavir acid (hydrolyzed active form; MedChemexpress, https://www.medchemexpress.com) and the 4 NA

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Table 1. Influenza viruses with I38 substitutions in polymerase acidic protein, Japan, 2018–19*

Influenza type or subtype	Total frequency	Age group, y				
		0–11	12–19	20–64	≥65	Unknown
A(H1N1)pdm09	5/323 (1.5)	4/230 (1.7)	1/35 (2.9)	0/41	0/14	0/3
A(H3N2)	32/337 (9.5)	26/215 (12.1)	5/45 (11.1)	1/54 (1.9)	0/16	0/7
B	0/36	0/21	0/7	0/6	0/1	0/1

*Values are no./total (%).

inhibitors approved for use in Japan: oseltamivir carboxylate (Sequoia Research Products, http://www.seqchem.com), peramivir (Sequoia Research Products), zanamivir (Sequoia Research Products), and laninamivir (Daiichi Sankyo, https://www.daiichisankyo.com). Because the genomic sequences of A/Kanagawa/IC18141/2019 and A/Kanagawa/IC18144/2019 were identical, we analyzed only A/Kanagawa/IC18141/2019. We determined antiviral susceptibilities by using a focus reduction assay and a fluorescent NA inhibition assay (NA-Fluor Influenza Neuraminidase Assay Kit; Applied Biosystems, https://www.thermofisher.com) (*4*) and calculated 50% inhibitory concentration (IC_{50}) values using MikroWin 2010 (Labsis, https://labsis.de). To interpret the NA inhibitor susceptibility, we applied the World Health Organization criteria of IC_{50} fold-change values compared with reference IC_{50} values (*6*). The World Health Organization criteria define influenza A virus inhibition as normal (<10-fold increase), reduced (10–100-fold increase), or highly reduced (>100-fold increase).

The mutant virus encoding the PA I38T substitution showed normal inhibition with all 4 NA inhibitors but exhibited a 186-fold higher IC_{50} value (236 nmol/L) to baloxavir compared with the median IC_{50} value of influenza A(H3N2) viruses isolated in the 2018–19 season in Japan (1.27 nmol/L; Table 2). These results indicate that the mutant virus we isolated carrying the PA I38T substitution had reduced susceptibility to baloxavir but remained susceptible to NA inhibitors (*5*,*7*).

Conclusions

During the 2018–19 influenza season in Japan, we detected 32 mutant influenza A(H3N2) viruses carrying various types of PA I38 substitutions, 4 of which were isolated from children <12 years of age without prior baloxavir exposure. Almost all mutant viruses isolated from baloxavir-treated patients possessed mixed PA I38T/I, I38M/I, I38R/I, I38T/M/I, I38T/K/I, or I38T/M/R substitutions (*5*), indicating these mutant viruses emerged under the selective pressure of baloxavir. In contrast, the 4 mutant viruses recovered from children without prior baloxavir treatment, including the virus described in this study, contained the PA I38T substitution and not a mixture including wild-type I38. These 4 children were probably infected with mutant viruses acquired from hosts previously treated with baloxavir.

Previous studies reported that oseltamivir-resistant viruses were detected in oseltamivir-treated 1–12-year-old children on day ≥4 after oseltamivir administration (*8*,*9*). During our monitoring for baloxavir-induced mutant influenza viruses, we found that, among baloxavir-treated patients, all but 1 of the mutant viruses were detected 3–6 days after baloxavir administration. One mutant virus

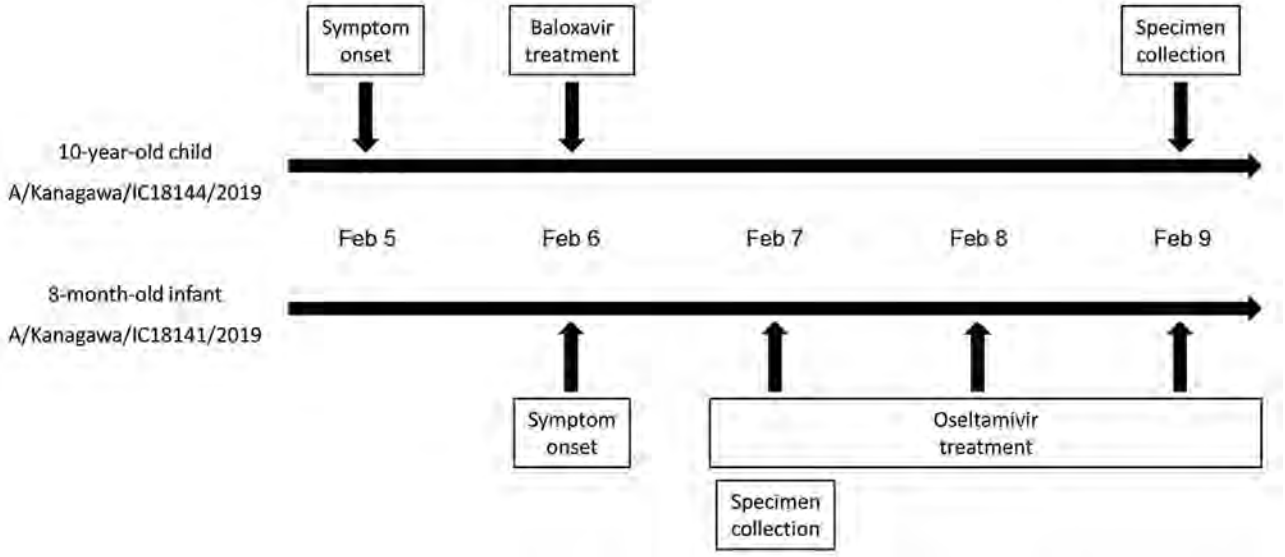

Figure. Clinical timeline of 2 siblings infected with mutant influenza A(H3N2) viruses encoding the polymerase acidic I38T substitution, Japan, February 2019. Whole-genome sequences of A/Kanagawa/IC18144/2019 (isolate no. EPI ISL 346656) and A/Kanagawa/IC18141/2019 (isolate no. EPI ISL 345215) are available from the GISAID EpiFlu database (http://www.gisaid.org).

Table 2. Susceptibility of influenza A(H3N2) virus carrying polymerase acidic I38T substitution detected in children within family cluster, February 2019, compared with 2018–19 seasonal virus, Japan*

	Median IC_{50} ± SD, nmol/L				
		NA inhibitors (WHO criteria)			
Influenza virus	Baloxavir	Oseltamivir	Peramivir	Zanamivir	Laninamivir
A/Kanagawa/IC18141/2019	236.08	0.37 (NI)	0.18 (NI)	1.01 (NI)	1.27 (NI)
A(H3N2) of 2018–19	1.27 ± 1.08†	0.37 ± 0.17‡	0.13 ± 0.03‡	0.79 ± 0.33‡	1.00 ± 0.21‡

*We determined antiviral susceptibilities using a focus reduction assay and a fluorescent NA inhibition assay (NA-Fluor Influenza Neuraminidase Assay Kit; Applied Biosystems, https://www.thermofisher.com) (*4*) and calculated IC_{50} values using MikroWin 2010 (Labsis, https://labsis.de). We expressed NA inhibitor susceptibilities using WHO criteria for influenza A virus inhibition, which define susceptibility on the basis of the -fold change in IC_{50} compared with the IC_{50} of reference isolates (*6*). WHO inhibition was defined as normal (<10-fold increase), reduced (10–100-fold increase), or highly reduced (>100-fold increase) in comparison with the median value of isolates from the same influenza season. IC_{50}, 50% inhibitory concentration; NA, neuraminidase; NI, normal inhibition; WHO, World Health Organization.
†n = 83.
‡n = 170.

was detected the day after baloxavir administration in a 2-year-old child from a family cluster, and this virus possessed a mixture of I38T/I substitutions (50% T and 50% I). This child might have been infected with a mixed population containing mutant and wild-type viruses; this incident suggested possible human-to-human transmission of the mutant influenza A(H3N2) virus encoding the PA I38T substitution.

The 8-month-old infant infected with A/Kanagawa/IC18141/2019 in this study had no exposure to baloxavir before specimen collection. The sibling of this infant, infected with A/Kanagawa/IC18144/2019, was treated with baloxavir for a half day before the infant's symptoms began. A/Kanagawa/IC18141/2019 and A/Kanagawa/IC18144/2019 viruses possessed the same genomic sequences. In Kanagawa, Japan, H3N2 virus activity was highest in February 2019, and an influenza outbreak occurred in the primary school attended by the sibling. Furthermore, during October 2018–February 2019, baloxavir was supplied to medical institutions that together served ≈5.6 million persons in Japan. These observations suggest 2 possibilities: the infant was infected by the sibling who was infected by another host harboring the virus with the PA I38T substitution, or both children were infected by another host harboring the virus with the PA I38T substitution. The median incubation period of influenza A virus is 1.4 days (*10*), and virus shedding can be detected 1 day before the onset of symptoms (*11*). Considering that the infant did not have much contact with the outside family, the infant acquiring the mutant virus from the sibling is the most likely option.

During our monitoring, 4 of 5 pH1N1 viruses and 26 of 32 H3N2 viruses with the PA I38 substitution were isolated from children <12 years of age. Our results confirm that the frequency of viruses with this mutation is higher in patients <12 years of age than those 12–64 years of age, as previously reported (*5*). Therefore, baloxavir susceptibility of influenza viruses, especially among infected children <12 years of age, should be closely monitored for public health planning purposes and for making clinical recommendations for antiviral drug use.

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References

1. Hayden FG, Sugaya N, Hirotsu N, Lee N, de Jong MD, Hurt AC, et al.; Baloxavir Marboxil Investigators Group. Baloxavir marboxil for uncomplicated influenza in adults and adolescents. N Engl J Med. 2018;379:913–23. https://doi.org/10.1056/NEJMoa1716197
2. Omoto S, Speranzini V, Hashimoto T, Noshi T, Yamaguchi H, Kawai M, et al. Characterization of influenza virus variants induced by treatment with the endonuclease inhibitor baloxavir marboxil. Sci Rep. 2018;8:9633. https://doi.org/10.1038/s41598-018-27890-4
3. Shionogi & Co. Ltd. Research and development at Shionogi. 2019 Mar 14 [cited 2019 May 27]. http://www.shionogi.co.jp/en/ir/pdf/e_p190314.pdf
4. Takashita E, Morita H, Ogawa R, Nakamura K, Fujisaki S, Shirakura M, et al. Susceptibility of influenza viruses to the novel cap-dependent endonuclease inhibitor baloxavir marboxil. Front Microbiol. 2018;9:3026. https://doi.org/10.3389/fmicb.2018.03026
5. Takashita E, Kawakami C, Ogawa R, Morita H, Fujisaki S, Shirakura M, et al. Influenza A(H3N2) virus exhibiting reduced susceptibility to baloxavir due to a polymerase acidic subunit I38T substitution detected from a hospitalised child without prior baloxavir treatment, Japan, January 2019. Euro Surveill. 2019;24. http://dx.doi.org/10.2807/1560-7917.ES.2019.24.12.1900170
6. World Health Organization. Meetings of the WHO working group on surveillance of influenza antiviral susceptibility—Geneva, November 2011 and June 2012. Wkly Epidemiol Rec. 2012;87:369–74.
7. Takashita E, Kawakami C, Morita H, Ogawa R, Fujisaki S, Shirakura M, et al.; On Behalf Of The Influenza Virus Surveillance Group Of Japan. Detection of influenza A(H3N2) viruses exhibiting reduced susceptibility to the novel cap-dependent endonuclease

inhibitor baloxavir in Japan, December 2018. Euro Surveill. 2019;24. http://dx.doi.org/10.2807/1560-7917.ES.2019.24.3.1800698
8. Kiso M, Mitamura K, Sakai-Tagawa Y, Shiraishi K, Kawakami C, Kimura K, et al. Resistant influenza A viruses in children treated with oseltamivir: descriptive study. Lancet. 2004;364:759–65. https://doi.org/10.1016/S0140-6736(04)16934-1
9. Whitley RJ, Hayden FG, Reisinger KS, Young N, Dutkowski R, Ipe D, et al. Oral oseltamivir treatment of influenza in children. Pediatr Infect Dis J. 2001;20:127–33. https://doi.org/10.1097/00006454-200102000-00002
10. Lessler J, Reich NG, Brookmeyer R, Perl TM, Nelson KE, Cummings DAT. Incubation periods of acute respiratory viral infections: a systematic review. Lancet Infect Dis. 2009;9:291–300. https://doi.org/10.1016/S1473-3099(09)70069-6
11. Lau LL, Cowling BJ, Fang VJ, Chan KH, Lau EH, Lipsitch M, et al. Viral shedding and clinical illness in naturally acquired influenza virus infections. J Infect Dis. 2010;201:1509–16. https://doi.org/10.1086/652241

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Orolabial Lymphogranuloma Venereum, Michigan, USA

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Orolabial lymphogranuloma venereum was diagnosed for a man in Michigan, USA, who had sex with men, some infected with HIV. High index of suspicion for lymphogranuloma venereum led to accurate diagnosis, successful therapy, and description of an L2b variant with a unique genetic mutation.

Lymphogranuloma venereum (LGV) is typically a genital ulcer disease with inguinal adenopathy, caused by L serovars of *Chlamydia trachomatis*. Most commonly today, the infection causes proctocolitis, particularly in men who have sex with men (MSM) with HIV infection. We describe a case of LGV at an unusual site (orolabial) with submandibular adenopathy in a man with a history of having sex with men with advanced HIV infection.

The Study

In February 2019, a 25-year-old man with a history of having sex with men with advanced HIV infection sought care at the Wayne State University Physician Group Infectious Diseases Clinic in Detroit, Michigan, USA, for 2 large ulcers over his lower lip. He had noticed the ulcers 2 weeks earlier, associated with pain and purulent discharge, along with a rapidly enlarging, painful swelling over his upper left neck. He reported no history of fever or chills. He also reported having had unprotected insertive and receptive oral and anal sex with >30 male partners during the previous year. He reported that he found anonymous sexual contacts by using "sex apps" and did not trade sex for drugs or money. In 2007, the patient had received a diagnosis of HIV infection and was taking antiretroviral drugs intermittently. His medical history included syphilis, gonorrhea, and genital chlamydia infections.

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Physical examination revealed that the patient was afebrile with stable vital signs. His lower lip was markedly swollen and tender, with 2 large, purulent ulcers: 1 deep ulcer on the central lower lip and 1 over the left side near the angle of his mouth. Also, left-sided submandibular adenopathy was present as a large (≈10 cm diameter), tender, nonfluctuant swelling, which was neither warm nor erythematous. His oropharynx, buccal mucosa, and tongue appeared normal. Results of the rest of the examination, including the external genitalia, groin, and perianal region, were unremarkable.

Diagnostic testing of the lip ulcer included nucleic acid amplification testing (NAAT) for herpes simplex viruses (HSVs) 1 and 2 and *Chlamydia* (Roche LightCycler real-time PCR for HSVs 1 and 2, https://diagnostics.roche.com; BD Viper XTR technology for GC/CT NAAT, https://www.bd.com). We performed NAAT on pharyngeal samples to test for *C. trachomatis* and *Neisseria gonorrhoeae*. Additional diagnostics included rapid plasma reagin testing, serologic testing for treponema, HIV viral load measurement, and CD4+ lymphocyte count. Valacyclovir was empirically prescribed; however, the patient did not fill this prescription.

C. trachomatis was identified in samples from the lip and throat; NAAT results for HSVs and *N. gonorrheae* were negative. The patient's HIV load was 300,000 copies/mL, and CD4+ lymphocyte count was 73/μL. Oral doxycycline (100 mg 2×/d for 3 weeks) was prescribed, and the lip ulcers and cervical adenopathy resolved over the next month. Concomitantly, appropriate antiretroviral therapy was initiated.

Swab samples of the lip ulcers were submitted to the San Francisco Public Health Department, where diagnosis of LGV was confirmed by use of a laboratory-developed, Clinical Laboratory Improvement Amendments–approved test (*1*). The lip ulcer samples then underwent LGV genotyping by sequencing the entire *ompA* gene at the Centers for Disease Control and Prevention (CDC). LGV genotyping confirmed *C. trachomatis* serovar L2b on the basis of sequence variations compared with serovar L2 (*2*). Furthermore, the *ompA* gene, which encodes the chlamydial major outer membrane protein (MOMP), contained a 5′ deletion of 1–82 nt in constant domain (CD) 1 and a 3′ deletion of 904–1,185 nt, spanning CD4, variable domain (VD) 4, and CD5.

Table. Reported cases of oropharyngeal LGV worldwide*

Year	Country	Specimen site	Genotype†	Prevalence of CT+ oropharyngeal samples	Reference
1974	Thailand	Oral ulcer	LGV	Single patient	(*3*)
2011	United Kingdom	Throat	LGV	1/51 (2%)	(*4*)
2012	Finland	Pharynx	L2b	1/56 (2%)	(*5*)
2013	United Kingdom	Oral ulcer	LGV	4/4 (100%)	(*6*)
2015	Netherlands	Pharynx	LGV	2/23 (8.7%)	(*7*)
2018	France	Oropharynx	L2, L2b	6/62 (9.7%)	(*8*)
2019	Spain	Tongue ulcer	LGV	Single patient	(*9*)

*CT+, *Chlamydia trachomatis*–positive; LGV, lymphogranuloma venereum.
†L2 and L2b variant genotypes were determined by sequence variations in *ompA* (*2*). LGV genotypes were determined according to the methods described in each study, either anti-LGV complement fixation test or LGV reverse transcription PCR.

Conclusions

The prompt diagnosis of LGV infection involving the lip with resultant submandibular adenopathy in this patient was based on a high index of clinical suspicion and specialized laboratory testing. Additional testing at CDC confirmed *C. trachomatis* serovar L2b.

Rare cases of oral/oropharyngeal LGV have been described (Table) (*3*–*9*). For patients who engage in high-risk sexual behavior, with anal–oral contact, LGV is a possible cause of orolabial/oropharyngeal infection. The substantial cervical lymphadenopathy in this patient was reminiscent of inguinal bubo associated with genital LGV, which may serve as a diagnostic clue. Moreover, clinical manifestations in the ongoing epidemic of LGV among MSM show a shift from the classical inguinal form (inguinal adenopathy with penile lesion) to the anorectal form. Our report of orolabial LGV adds to the possibility of gastrointestinal tract infection in this epidemic, perhaps explaining the discrepancy in the ratio of anorectal LGV to inguinal LGV cases (*10*).

In the past several years, reports of LGV have been increasing in western Europe and the United States, primarily among MSM (*11*). An outbreak of 38 cases of LGV during August 2015–April 2016 encountered at the Wayne State University Physician Group Infectious Diseases Clinic in Detroit was recently reported and occurred among MSM with HIV infection (*12*). Among the 38 cases, 21 (55%) were confirmed by the CDC laboratory–developed LGV NAAT (*2*) on the basis of 19 positive rectal swab specimens and 2 positive swab samples from penile lesions; treatment with doxycycline (100 mg 2×/d for 21 days) was successful for all patients. Although the L2b serovar was correlated with a proctitis outbreak among MSM in Amsterdam in 2000, no cases of LGV at the orolabial site were detected in this outbreak or a 1980s outbreak in San Francisco, California, USA (*13*).

The identification of 5′ and 3′ deletions in *ompA* indicate a novel subtype of serovar L2b, further highlighting the rarity of this patient's case. Chlamydial MOMP functions as a porin with VD1, VD2, and VD4 at the surface of the chlamydial elementary body directed toward the external environment and host cells. VD4 encodes subspecies-specific neutralizing epitopes (*14*,*15*). It remains unclear how or whether the deletions in CD1, CD4, VD4, and CD5 characterized in this case affect pathogenicity and tissue tropism. Identification of rare clinical manifestations such as this warrant further genetic investigation to provide more information about the molecular mechanisms behind the pathogenesis and transmission of chlamydia.

A widely available Food and Drug Administration–approved molecular test for diagnosis of LGV would be highly useful, especially at the point of care. In its absence, a probable LGV case can be supported by *C. trachomatis* NAAT positivity of lesion specimens. However, extragenital specimens (e.g., from a lip lesion) are currently not approved by the Food and Drug Administration as a specimen type for commercial *C. trachomatis* NAAT, which poses a challenge for laboratories because they must perform validations for such specimens for their Clinical Laboratory Improvement Amendments certification. Similar obstacles apply to laboratory-developed LGV-specific testing. As a result, the availability of specific diagnostic testing is scarce. At present, to ensure prompt resolution of symptoms, prevention of complications, and treatment of the sex partner, all suspected cases should be presumptively treated while awaiting diagnostic evaluation.

In summary, this case indicates that LGV infection should be considered for patients, especially MSM, with orolabial lesions and cervical adenopathy. Successful treatment of the patient reported here was based on a *C. trachomatis*–positive NAAT result, and LGV specialized testing served as a supplement for full investigation of this unusual case.

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References

1. Morré SA, Spaargaren J, Fennema JS, de Vries HJ. Molecular diagnosis of lymphogranuloma venereum: PCR-based restriction fragment length polymorphism and real-time PCR. J Clin Microbiol. 2005;43:5412Y5413.

2. Lan J, Ossewaarde JM, Walboomers JM, Meijer CJ, van den Brule AJ. Improved PCR sensitivity for direct genotyping of *Chlamydia trachomatis* serovars by using a nested PCR. J Clin Microbiol. 1994;32:528–30.
3. Andrada MT, Dhar JK, Wilde H. Oral lymphogranuloma venereum and cervical lymphadenopathy. Case report. Mil Med. 1974;139:99–101. https://doi.org/10.1093/milmed/139.2.99
4. Elgalib A, Alexander S, Tong CYW, White JA. Seven days of doxycycline is an effective treatment for asymptomatic rectal *Chlamydia trachomatis* infection. Int J STD AIDS. 2011;22:474–7. https://doi.org/10.1258/ijsa.2011.011134
5. Korhonen S, Hiltunen-Back E, Puolakkainen M. Genotyping of *Chlamydia trachomatis* in rectal and pharyngeal specimens: identification of LGV genotypes in Finland. Sex Transm Infect. 2012;88:465–9. https://doi.org/10.1136/sextrans-2011-050458
6. Dosekun O, Edmonds S, Stockwell S, French P, White JA. Lymphogranuloma venereum detected from the pharynx in four London men who have sex with men. Int J STD AIDS. 2013;24:495–6. https://doi.org/10.1177/0956462412472830
7. van Rooijen MS, van der Loeff MF, Morré SA, van Dam AP, Speksnijder AG, de Vries HJ. Spontaneous pharyngeal *Chlamydia trachomatis* RNA clearance. A cross-sectional study followed by a cohort study of untreated STI clinic patients in Amsterdam, The Netherlands. Sex Transm Infect. 2015;91:157–64. https://doi.org/10.1136/sextrans-2014-051633
8. Desclaux A, Touati A, Neau D, Laurier-Nadalié C, Bébéar C, de Barbeyrac B, et al. Extra-rectal lymphogranuloma venereum in France: a clinical and molecular study. Sex Transm Infect. 2018;94:3–8. https://doi.org/10.1136/sextrans-2017-053126
9. Riera-Monroig J, Fuertes de Vega I. Lymphogranuloma venereum presenting as an ulcer on the tongue. Sex Transm Infect. 2019;95:169–70. https://doi.org/10.1136/sextrans-2018-053787
10. de Vries HJ. The enigma of lymphogranuloma venereum spread in men who have sex with men: does ano-oral transmission plays a role? Sex Transm Dis. 2016;43:420–2. https://doi.org/10.1097/OLQ.0000000000000466
11. Kapoor S. Re-emergence of lymphogranuloma venereum. J Eur Acad Dermatol Venereol. 2008;22:409–16. https://doi.org/10.1111/j.1468-3083.2008.02573.x
12. de Voux A, Kent JB, Macomber K, Krzanowski K, Jackson D, Starr T, et al. Notes from the Field: Cluster of lymphogranuloma venereum cases among men who have sex with men—Michigan, August 2015–April 2016. MMWR Morb Mortal Wkly Rep. 2016;65:920–1. https://doi.org/10.15585/mmwr.mm6534a6
13. Spaargaren J, Schachter J, Moncada J, de Vries HJ, Fennema HS, Peña AS, et al. Slow epidemic of lymphogranuloma venereum L2b strain. Emerg Infect Dis. 2005;11:1787–8. https://doi.org/10.3201/eid1111.050821
14. Olsen AW, Follmann F, Erneholm K, Rosenkrands I, Andersen P. Protection against *Chlamydia trachomatis* infection and upper genital tract pathological changes by vaccine-promoted neutralizing antibodies directed to the VD4 of the major outer membrane protein. J Infect Dis. 2015;212:978–89. https://doi.org/10.1093/infdis/jiv137
15. Baehr W, Zhang YX, Joseph T, Su H, Nano FE, Everett KD, et al. Mapping antigenic domains expressed by *Chlamydia trachomatis* major outer membrane protein genes. Proc Natl Acad Sci U S A. 1988;85:4000–4. https://doi.org/10.1073/pnas.85.11.4000

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Preventing Sexual Transmission of Zika Virus Infection during Pregnancy, Puerto Rico, USA, 2016[1]

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We examined condom use throughout pregnancy during the Zika outbreak in Puerto Rico during 2016. Overall, <25% of women reported consistent condom use during pregnancy. However, healthcare provider counseling was associated with a 3-fold increase in consistent use, reinforcing the value of provider counseling in Zika prevention efforts.

Zika virus infection during pregnancy can cause brain abnormalities, microcephaly, and other birth defects in exposed offspring (*1*,*2*). Although transmission of Zika virus primarily occurs through the bite of an infected mosquito, it can also be transmitted by having intercourse with an infected partner (*3*,*4*). In 2016, the Centers for Disease Control and Prevention (CDC) released guidance for prevention of sexual transmission of Zika virus for pregnant women and couples planning to conceive (*3*–*5*). In areas where Zika virus transmission was active, pregnant women and their male partners were advised to consistently and correctly use condoms when having intercourse or to abstain from intercourse during pregnancy to reduce the risk for sexual transmission of Zika virus (*3*–*5*). Corresponding with CDC guidance to healthcare providers (*3*–*5*), the American College of Obstetricians and Gynecologists and the Society for Maternal–Fetal Medicine released interim guidance outlining the need to provide counseling about recommended prevention measures to women and their partners who were at risk for exposure to Zika virus infection (*6*).

The Study

In 2016, the Puerto Rico Department of Health and CDC partnered to conduct the Pregnancy Risk Assessment Monitoring System–Zika Postpartum Emergency Response Study, a hospital-based survey that collected data from women after delivery and before hospital discharge about their prenatal experiences and behaviors related to detection and prevention of Zika virus infection during pregnancy (*7*). The island-wide study was implemented during August 28–December 3, 2016. Hospitals reporting ≥100 births during 2015 were eligible to participate. A total of 36 hospitals were eligible and agreed to participate, representing 98% of live births in Puerto Rico.

Women with a recent live birth who were residents of Puerto Rico, had delivered their infant in a participating hospital, and were able to complete the survey in Spanish or English were eligible to participate. To select the study sample, we randomly sampled delivery dates (clusters) within each hospital. All eligible women who delivered their infant on one of the randomly selected delivery dates were invited to participate. Hospital delivery logs were used to identify women for sampling. Sampled women were approached by study staff (24 hours after vaginal deliveries and 36 hours after cesarean deliveries). Overall, of 2,933 women eligible to participate, 2,364 (80.6%) completed surveys.

For women who were sexually active during pregnancy, we assessed the prevalence of condom use during pregnancy, overall and by select maternal characteristics. We constructed 3 separate multivariable logistic regression models to examine

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[1]Preliminary results from this study were presented at the 2018 American Public Health Association Annual Meeting in San Diego, California, USA, November 10–14, 2018.

factors associated with receiving prenatal provider counseling on condom use for Zika virus infection prevention; any condom use during pregnancy; and consistent condom use during pregnancy. Each model was further adjusted for maternal characteristics, infant birth month (August–September 2016 vs. October–December 2016), and geographic region.

Of 2,229 respondents included in the analysis, most were 20–34 years of age (79.7%), had more than a high school education (68.9%), were unmarried (68.5%), and participated in the Special Supplemental Nutrition Program for Women, Infants, and Children (WIC) during pregnancy (88.3%). Most (80.6%) women reported being sexually active during pregnancy (Figure).

Overall, most (86.8%) women reported receiving counseling to use condoms during pregnancy to prevent Zika virus infection. The prevalence of receiving counseling during pregnancy on condom use was significantly higher for mothers ≤19 years of age (89.7%; adjusted prevalence ratio [aPR] 1.10, 95% CI 1.03–1.18) and those 20–34 years of age (87.3%; aPR 1.07, 95% CI 1.01–1.13) compared with women ≥35 years of age (81.5%; p<0.01, adjusted linear trend) (Table 1). Receiving counseling

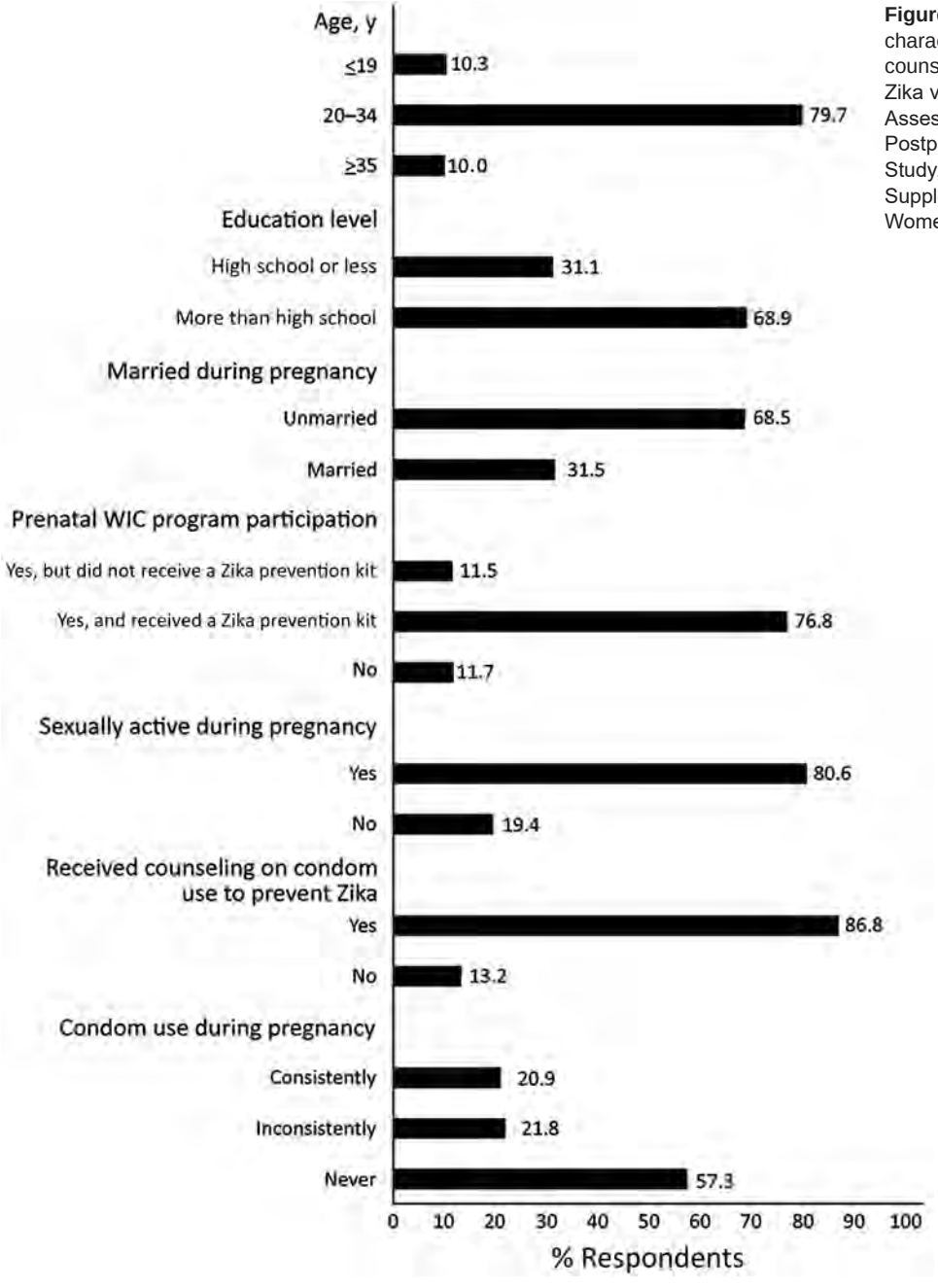

Figure. Distribution of maternal characteristics and receipt of counseling on condom use to prevent Zika virus infection, Pregnancy Risk Assessment Monitoring System–Zika Postpartum Emergency Response Study, Puerto Rico, 2016. WIC, Special Supplemental Nutrition Program for Women, Infants, and Children

Table 1. Adjusted prevalence estimates and ratios of receipt of provider counseling on condom use during pregnancy to prevent Zika virus infection by maternal characteristics, Pregnancy Risk Assessment Monitoring System–Zika Postpartum Emergency Response Study, Puerto Rico, USA, 2016*

Characteristic	Received counseling on condom use to prevent Zika, n = 2,229†		
	% Respondents (95% CI)‡	Crude PR (95% CI)	Adjusted PR (95% CI)§
Age, y			
≤19	89.7 (85.6–92.7)	1.17 (1.09–1.26)	1.10 (1.03–1.18)
20–34	87.3 (85.7–88.7)	1.13 (1.06–1.21)	1.07 (1.01–1.13)
≥35	81.5 (77.0–85.3)	Referent¶	Referent¶
Education level			
High school or less	86.3 (83.5–88.6)	1.02 (0.99–1.05)	0.99 (0.96–1.03)
More than high school	87.1 (85.4–88.5)	Referent	Referent
Marital status during pregnancy			
Unmarried	86.2 (84.4–87.8)	1.03 (0.99–1.07)	0.98 (0.95–1.01)
Married	88.0 (85.5–90.1)	Referent	Referent
Prenatal WIC program participation			
Yes, did not receive a Zika prevention kit	80.2 (75.4–84.3)	1.13 (1.02–1.24)	1.14 (1.02–1.26)
Yes, received a Zika prevention kit	90.3 (88.9–91.5)	1.27 (1.16–1.38)	1.28 (1.17–1.40)
No	70.6 (64.0–76.5)	Referent	Referent
Sexually active during pregnancy			
Yes	87.0 (85.5–88.3)	Referent	Referent
No	86.3 (83.1–89.0)	1.00 (0.96–1.03)	0.99 (0.96–1.03)

*PR, prevalence ratio; WIC, Special Supplemental Nutrition Program for Women, Infants, and Children.
†Unweighted sample size.
‡Adjusted weighted percentage.
§All prevalence and prevalence ratio estimates were adjusted for maternal age and education, marital status, prenatal WIC participation, sexual activity during pregnancy, infant birth month, and region.
¶Adjusted p value (<0.01) for linear trend based on maternal age.

during pregnancy on condom use was also higher for women who participated in the WIC program, both those who received a Zika Prevention Kit (containing condoms, repellent, bed nets, and larvicide) from WIC (90.3%; aPR 1.28, 95% CI 1.17–1.40) and those who did not receive a kit (80.2%; aPR 1.14, 95% CI 1.02–1.26) compared with those who did not participate in a WIC program (70.6%).

For women who were sexually active during pregnancy, 20.9% used condoms consistently, 21.8% inconsistently, and 57.3% never (Figure). Multivariable analyses (Table 2) showed that the prevalence of any condom use during pregnancy was higher for women with a high school diploma or less (46.8% vs. 41.1%; aPR 1.14, 95% CI 1.02–1.27), those who were WIC program participants and received a Zika Prevention Kit (44.5% vs. 32.3%; aPR 1.38, 95% CI 1.12–1.70), and those who reported prepregnancy condom use (64.7% vs. 40.5%; aPR 1.60, 95% CI 1.42–1.80) compared with their counterparts (Table 2). Receiving healthcare provider counseling during pregnancy regarding the need for condom use was strongly associated with any condom use during pregnancy. Counseled women were >2 times as likely to report any condom use during pregnancy (45.9% vs. 20.4%; aPR 2.25, 95% CI 1.73–2.91) than were noncounseled women (Table 2).

Similar to the prevalence of any condom use, the prevalence of consistent condom use during pregnancy was higher for less educated women (25.2% vs. 19.2%; aPR 1.31, 95% CI 1.07–1.60) and those who reported prepregnancy condom use (34.7% vs. 19.5%; aPR 1.78, 95% CI 1.44–2.21). Women who were counseled on condom use were 3 times as likely to report consistent condom use during pregnancy than were noncounseled women (22.8% vs. 7.4%; aPR 3.07, 95% CI 1.97–4.79) (Table 2).

Conclusions

Although <25% of women reported consistently using condoms during pregnancy, counseling by prenatal care providers was associated with marked increases in any condom use and consistent condom use. Healthcare providers, including doctors, nurses, and other providers in various settings (e.g., prenatal care visits, WIC program visits), can play an important role in prevention of sexual transmission of Zika virus infection by counseling pregnant patients on the importance of consistent and correct condom use. These findings can be used to target and further refine Zika virus prevention messaging and interventions and can apply more broadly to the prevention of other sexually transmitted infections during pregnancy, such as syphilis and genital herpes that, if left untreated, can increase the risk for adverse maternal and infant outcomes (*8*,*9*).

This study also shows how more traditional surveillance systems focused on maternal and child health can successfully be adapted to rapidly collect information from pregnant women during public health emergencies. Interviewing women after delivery and before hospital discharge, although labor- and cost-intensive, can be implemented rapidly with high response rates during urgent situations, such as the Zika outbreak. This type of design might be appropriate for other public health emergencies that affect the health of pregnant women and newborns when the emergency is geographically limited.

Table 2. Adjusted prevalence estimates and ratios of self-reported condom use during pregnancy by maternal characteristics and receipt of provider counseling on condom use during pregnancy, Pregnancy Risk Assessment Monitoring System–Zika Postpartum Emergency Response Study, Puerto Rico, USA, 2016*

	Total, n = 1,794†					
	Any condom use			Consistent condom use		
Characteristic	% Respondents (95% CI)‡	Crude PR (95% CI)	Adjusted PR (95% CI)§	% Respondents (95% CI)‡	Crude PR (95% CI)	Adjusted PR (95% CI)§
Age, y						
≤19	51.5 (43.9–59.0)	1.62 (1.29–2.04)	1.22 (0.96–1.54)	24.5 (18.3–31.9)	1.68 (1.12–2.53)	1.13 (0.73–1.75)
20–34	41.8 (39.3–44.3)	1.16 (0.95–1.40)	0.99 (0.82–1.18)	20.4 (18.4–22.5)	1.12 (0.80–1.57)	0.94 (0.67–1.31)
≥35	42.4 (35.3–49.8)	Referent¶	Referent	21.7 (15.8–29.1)	Referent#	Referent
Education level						
High school or less	46.8 (42.4–51.4)	1.24 (1.12–1.38)	1.14 (1.02–1.27)	25.2 (21.5–29.2)	1.40 (1.17–1.69)	1.31 (1.07–1.60)
More than high school	41.1 (38.6–43.7)	Referent	Referent	19.2 (17.1–21.5)	Referent	Referent
Marital status during pregnancy						
Unmarried	41.4 (38.8–44.1)	1.03 (0.92–1.15)	0.91 (0.82–1.02)	20.4 (18.4–22.7)	1.05 (0.88–1.26)	0.93 (0.78–1.10)
Married	45.3 (41.4–49.3)	Referent	Referent	22.0 (18.9–25.3)	Referent	Referent
Prenatal WIC program participation						
Yes, did not receive a Zika prevention kit	41.6 (34.9–48.6)	1.32 (1.02–1.71)	1.29 (0.99–1.68)	18.7 (14.1–24.3)	1.08 (0.72–1.61)	0.96 (0.64–1.43)
Yes, received a Zika prevention kit	44.5 (41.9–47.1)	1.52 (1.23–1.87)	1.38 (1.12–1.70)	21.4 (19.3–23.7)	1.36 (1.00–1.86)	1.10 (0.80–1.51)
No	32.3 (26.2–39.0)	Referent	Referent	19.5 (14.4–26.0)	Referent	Referent
Prepregnancy condom use						
Yes	64.7 (57.5–71.2)	1.64 (1.45–1.85)	1.60 (1.42–1.80)	34.7 (28.2–41.9)	1.84 (1.48–2.28)	1.78 (1.44–2.21)
No**	40.5 (38.2–42.8)	Referent	Referent	19.5 (17.7–21.4)	Referent	Referent
Received counseling on condom use to prevent Zika						
Yes	45.9 (43.4–48.4)	2.57 (1.99–3.32)	2.25 (1.73–2.91)	22.8 (20.9–25.0)	3.28 (2.12–5.09)	3.07 (1.97–4.79)
No	20.4 (15.7–26.0)	Referent	Referent	7.4 (4.8–11.4)	Referent	Referent

*PR, prevalence ratio; WIC, Special Supplemental Nutrition Program for Women, Infants, and Children.
†Unweighted sample size.
‡Adjusted weighted percentage.
§All prevalence and prevalence ratio estimates were adjusted for maternal age, education, marital status, prenatal WIC participation, receipt of provider counseling, infant birth month, and region.
¶Adjusted p value (<0.01) for linear trend based on maternal age.
#Adjusted p value (<0.05) for linear trend based on maternal age.
**Included women who reported they were not doing anything to prevent pregnancy and women who were using a contraceptive method other than condoms to prevent pregnancy.

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References

1. Rasmussen SA, Jamieson DJ, Honein MA, Petersen LR. Zika virus and birth defects: reviewing the evidence for causality. N Engl J Med. 2016;374:1981–7. https://doi.org/10.1056/NEJMsr1604338
2. Centers for Disease Control and Prevention. Congenital Zika Syndrome and other birth defects; 2018 [cited 2019 Jan 2]. https://www.cdc.gov/pregnancy/zika/testing-follow-up/zika-syndrome-birth-defects.html
3. Petersen EE, Meaney-Delman D, Neblett-Fanfair R, Havers F, Oduyebo T, Hills SL, et al. Update: interim guidance for preconception counseling and prevention of sexual

transmission of Zika virus for persons with possible Zika virus exposure—United States, September 2016. MMWR Morb Mortal Wkly Rep. 2016;65:1077–81. https://doi.org/10.15585/mmwr.mm6539e1
4. Polen KD, Gilboa SM, Hills S, Oduyebo T, Kohl KS, Brooks JT, et al. Update: interim guidance for preconception counseling and prevention of sexual transmission of Zika virus for men with possible Zika virus exposure—United States, August 2018. MMWR Morb Mortal Wkly Rep. 2018;67:868–71. https://doi.org/10.15585/mmwr.mm6731e2
5. Oster AM, Russell K, Stryker JE, Friedman A, Kachur RE, Petersen EE, et al. Update: interim guidance for prevention of sexual transmission of Zika virus—United States, 2016. MMWR Morb Mortal Wkly Rep. 2016;65:323–5. https://doi.org/10.15585/mmwr.mm6512e3
6. The American College of Obstetricians and Gynecologists Women's Health Care Physicians, Society for Maternal-Fetal Medicine. Practice advisory interim guidance for care of obstetric patients during a Zika virus outbreak; 2017 [cited 2019 Aug 23]. https://www.acog.org/Clinical-Guidance-and-Publications/Committee-Opinions/Immunization-Infectious-Disease-and-Public-Health-Preparedness-Expert-Work-Group/Management-of-Patients-in-the-Context-of-Zika-Virus
7. Puerto Rico Department of Health. Puerto Rico Pregnancy Risk Assessment Monitoring System–Zika Postpartum Emergency Response, PRAMS-ZPER 2.0 Protocol; 2017 [cited 2019 Aug 23]. https://www.cdc.gov/prams/special-projects/zika/docs/pdf/english/PRAMS_ZPER-2.0_Protocol_FINAL_508tagged.pdf
8. Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines; 2015 [cited 2019 Mar 18]. https://www.cdc.gov/std/tg2015/default.htm
9. Centers for Disease Control and Prevention. Sexually transmitted disease surveillance; 2016 [cited 2019 Jan 2]. https://www.cdc.gov/std/stats16/CDC_2016_STDS_Report-for-508WebSep21_2017_1644.pdf

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Drug-Susceptible and Multidrug-Resistant *Mycobacterium tuberculosis* in a Single Patient

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A patient who had initial infection with mixed strains of drug-susceptible and multidrug-resistant tuberculosis was presumed to have acquired drug resistance before confirmation that sequential strains were genotypically distinct. Transmitted infection with mixed strains is likely underappreciated; identifying these infections requires spoligotyping and whole-genome sequencing.

Multidrug-resistant (MDR) tuberculosis (TB), defined as infection with *Mycobacterium tuberculosis* that is resistant to isoniazid and rifampin, can be transmitted and manifest as a primary infection without a patient having received those medications or can be acquired by the patient during drug therapy. A person may be initially infected with ≥1 *M. tuberculosis* strain with different patterns of drug resistance (*1–4*). We present such a case, which is likely uncommon but underappreciated; identification requires spoligotyping and whole-genome sequencing of sequential strains.

A 28-year-old man came to a hospital in Virginia, 1 year after immigrating from the Philippines, with a 4-week history of fevers, night sweats, weight loss, voice change, and cervical lymphadenopathy. A computed tomography scan showed no lung parenchymal or pleural abnormality. Cervical lymph node biopsy showed caseating granulomas with acid-fast bacilli (AFB). Sputum smears demonstrated 4+ AFB. Rapid nucleic acid amplification testing by commercial line probe assay confirmed *M. tuberculosis* complex from both sites without *rpoB*, *katG*, or *inhA* mutation. Test results for HIV were positive; HIV viral load was 521,800 copies/mL and CD4 count 7 cells/mm^3. Diagnoses were made of TB lymphadenitis and presumed laryngeal TB. We started the patient on antiretroviral drugs after initiating isoniazid, rifampin, ethambutol, and pyrazinamide. Phenotypic testing ultimately showed susceptibility to isoniazid, rifampin, ethambutol, and pyrazinamide. The patient achieved sputum culture conversion to negative before beginning his eighth week of treatment and transitioned to a continuation phase of isoniazid and rifampin 5 days/week.

Twenty weeks into treatment, the patient's sputum AFB culture obtained 12 weeks after initiation was reported as positive. Given this relapse, we conducted therapeutic drug monitoring for isoniazid and rifampin. Estimated peak serum drug concentrations were 2.07 (range 3–5) μg/mL for isoniazid and 5.98 (range 8–24) μg/mL for rifampin. Molecular sequencing of the week 12 *M. tuberculosis* isolate at the Centers for Disease Control and Prevention (*5*) found mutations in *rpoB* (GACCAG>GAG; Asp516Gln517Glu) and *inhA* (C-15T), but the subsequently tested pretreatment sputum isolate was confirmed negative for mutations in the resistance-determining regions of *rpoB* (rifampin), *katG* and *inhA* (isoniazid), and *pncA* (pyrazinamide). Both isolates had mutations in the *embB* gene Leu355Leu (silent) and Glu-378Ala (reported as unlikely to cause resistance alone and, more commonly, a marker of Indo-Oceanic strain lineage) (*6*). We switched the patient's drug regimen to levofloxacin, linezolid, capreomycin, para-aminosalicylate, ethambutol, and pyrazinamide. Phenotypic susceptibility tests later confirmed MDR TB: 25% resistance to isoniazid at 1.0 μg/mL and 100% resistance to rifampin at 1.0 μg/mL. The patient continued with this MDR TB regimen for 15 months after culture conversion to negative. He has remained healthy.

We clinically interpreted this scenario as one of acquired drug resistance, likely contributed by the subtarget concentration of anti-TB drugs. However, analyses of mycobacterial interspersed repetitive unit–variable-number tandem-repeat typing, along with insertion sequence 6110 restriction fragment length polymorphism analyses of the pretreatment drug-susceptible strain and the week 12 MDR strain, showed distinct spoligotypes. Whole-genome sequencing differed by >100 single nucleotide polymorphisms, supporting that these strains were genotypically distinct. Upon further questioning, the patient related that before immigrating he lived in a small apartment in Manila where friends and family would frequently lodge before seeking treatment at the city's referral hospital.

Prior treatment with isoniazid and rifampin is a major risk factor for MDR TB. Comprehensive epidemiologic studies and improved access to whole-genome sequencing have revealed that primary MDR TB transmitted to the patient can be common (*7*). Our case adds to the literature on transmitted MDR TB dynamics and shows how initial infections with mixed strains may be an underreported cause of treatment failure. The clonal diversity from a *M. tuberculosis* sample is clearly attenuated following conventional culture techniques on solid agar (*8*). Although heteroresistance at drug-resistant loci of *M. tuberculosis* can be detected at low levels with newer next-generation sequencing

Table. Characteristics of cases of treatment failure resulting from concurrent infection with mixed strains of drug-resistant *Mycobacterium tuberculosis**

Patient age, y/sex	Immune status	Site(s) of infection	Time to recurrence	Platform for strain identification	Patient country of origin	Country of diagnosis	Reference
24/M	Immunocompetent	Lymph node, gastric aspirate	100 d	Mixed-linker fingerprint PCR	Nepal	Germany	(*1*)
23/M	HIV negative	Pulmonary	150 d	Spoligotyping and MIRU	Somalia	USA	(*4*)
24/M	HIV negative	Pulmonary, gastric aspirate	90 d	IS6110 RFLP and spoligotyping	Kazakhstan	Germany	(*2*)
62/M	HIV negative	Pulmonary	120 d	MIRU-VNTR	Portugal	UK	(*3*)
28/M	HIV positive	Lymph node, trachea	84 d	Spoligotyping and WGS	Philippines	USA	This study

*IS, insertion sequence; MIRU-VNTR, mycobacterial interspersed repetitive unit–variable number tandem repeat; RFLP, restriction fragment length polymorphism; WGS, whole genome sequencing.

techniques from cultured growth, these analyses cannot distinguish infection with multiple strains from heteroresistance among subpopulations of the same strain (*9*).

As with the previous cases (*1–4*) of mixed-strain infection, the patient we report did not have prior history of TB treatment and showed initial improvement but then had a recrudescence of symptoms and culture reversion 115 days into first-line therapy (range 90–150 days in previously reported cases) (Table). Applying whole-genome sequencing to additional samples collected from our patient and meticulous isolation of different colonies of cultured growth might have detected heteroresistance, and consequent alteration of the initial treatment regimen could have prevented the recrudescence of disease. However, the detection of lower-level genetic heteroresistance has not been rigorously studied for its effect on populations of persons initiating TB treatment and will undoubtedly vary based on the drug-specific locus that is heteroresistant and the quantitative level of detection. Well-curated prospective cohorts that contribute sequencing data may refine our understanding of this effect but will require nuanced bioinformatics *(10)*.

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References

1. Theisen A, Reichel C, Rüsch-Gerdes S, Haas WH, Rockstroh JK, Spengler U, et al. Mixed-strain infection with a drug-sensitive and multidrug-resistant strain of *Mycobacterium tuberculosis*. Lancet. 1995;345:1512–3. https://doi.org/10.1016/S0140-6736(95)91073-5
2. Niemann S, Richter E, Rüsch-Gerdes S, Schlaak M, Greinert U. Double infection with a resistant and a multidrug-resistant strain of *Mycobacterium tuberculosis*. Emerg Infect Dis. 2000;6:548–51. https://doi.org/10.3201/eid0605.000518
3. Hingley-Wilson SM, Casey R, Connell D, Bremang S, Evans JT, Hawkey PM, et al. Undetected multidrug-resistant tuberculosis amplified by first-line therapy in mixed infection. Emerg Infect Dis. 2013;19:1138–41. https://doi.org/10.3201/eid1907.130313
4. Mendez MP, Landon ME, McCloud MK, Davidson P, Christensen PJ. Co-infection with pansensitive and multidrug-resistant strains of *Mycobacterium tuberculosis*. Emerg Infect Dis. 2009;15:578–80. https://doi.org/10.3201/eid1504.080592
5. Yakrus MA, Driscoll J, Lentz AJ, Sikes D, Hartline D, Metchock B, et al. Concordance between molecular and phenotypic testing of *Mycobacterium tuberculosis* complex isolates for resistance to rifampin and isoniazid in the United States. J Clin Microbiol. 2014;52:1932–7. https://doi.org/10.1128/JCM.00417-14
6. Campbell PJ, Morlock GP, Sikes RD, Dalton TL, Metchock B, Starks AM, et al. Molecular detection of mutations associated with first- and second-line drug resistance compared with conventional drug susceptibility testing of *Mycobacterium tuberculosis*. Antimicrob Agents Chemother. 2011;55:2032–41. https://doi.org/10.1128/AAC.01550-10
7. Shah NS, Auld SC, Brust JCM, Mathema B, Ismail N, Moodley P, et al. Transmission of extensively drug-resistant tuberculosis in South Africa. N Engl J Med. 2017;376:243–53. https://doi.org/10.1056/NEJMoa1604544
8. Martín A, Herranz M, Ruiz Serrano MJ, Bouza E, García de Viedma D. The clonal composition of *Mycobacterium tuberculosis* in clinical specimens could be modified by culture. Tuberculosis (Edinb). 2010;90:201–7. https://doi.org/10.1016/j.tube.2010.03.012
9. Operario DJ, Koeppel AF, Turner SD, Bao Y, Pholwat S, Banu S, et al. Prevalence and extent of heteroresistance by next generation sequencing of multidrug-resistant tuberculosis. PLoS One. 2017;12:e0176522. https://doi.org/10.1371/journal.pone.0176522
10. McNerney R, Clark TG, Campino S, Rodrigues C, Dolinger D, Smith L, et al. Removing the bottleneck in whole genome sequencing of *Mycobacterium tuberculosis* for rapid drug resistance analysis: a call to action. Int J Infect Dis. 2017;56:130–5. https://doi.org/10.1016/j.ijid.2016.11.422

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Mutation and Diversity of Diphtheria Toxin in *Corynebacterium ulcerans*

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Corynebacterium ulcerans infection is emerging in humans. We conducted phylogenetic analyses of *C. ulcerans* and *C. diptheriae*, which revealed diverse diphtheria toxin in *C. ulcerans*. Diphtheria toxin diversification could decrease effectiveness of diphtheria toxoid vaccine and diphtheria antitoxin for preventing and treating illnesses caused by this bacterium.

Corynebacterium ulcerans is a rod-shaped, aerobic, gram-positive bacterium closely related to *C. diphtheria*. Some strains of *C. ulcerans* can produce diphtheria toxin, which causes respiratory diphtheria in humans and animals. Reports of human infections with *C. ulcerans* have increased during the past 20 years, and *C. ulcerans* is a recognized emerging human pathogen (*1*). Humans can contract toxin-producing *C. ulcerans* from companion animals (*2*,*3*). Human death can occur if appropriate treatment is delayed (*4*). Non–toxin-producing *C. diphtheriae* and *C. ulcerans* can convert to toxin-producing strains through a process of lysogeny with diphtheria toxin gene–carrying corynebacteriophages (*5*–*7*). Although increased coverage of the diphtheria toxoid vaccine has reduced the frequency of *C. diphtheriae* infections, reports of *C. ulcerans* infections in humans are increasing.

A report evaluating the differences in the amino acid sequences of the diphtheria toxins in *C. diphtheriae* and *C. ulcerans* used only limited data, comparing 1 strain of *C. diphtheriae* against 2 strains of *C. ulcerans* (*8*), leaving the differences among the toxins of these 2 species unclear. Others have conducted bacterial genome analyses and deposited several genomic sequences of *C. diphtheriae* and *C. ulcerans* strains into a public database. We collected amino acid sequences of the diphtheria toxin and the nucleic acid sequences of the 16S rRNA gene of 6 *C. diphtheriae* strains and 6 *C. ulcerans* strains from the National Center for Biotechnology Information genome database (https://www.ncbi.nlm.nih.gov/genome). Then, we performed phylogenetic analyses by using MEGA 7.0 (https://www.megasoftware.net).

We found that the 16S rRNA gene sequences divided into separate *C. diphtheriae* and *C. ulcerans* strains with some sequence variability among the strains in each species (Figure, panel A). The amino acid sequences of the toxins also divided into separate clades for each species. However, we noted that *C. diphtheriae* strains were identical, but *C. ulcerans* strains were diverse (Figure, panel B), suggesting that *C. ulcerans* tends to acquire mutations more frequently than *C. diphtheriae.* Two possible explanations for this phenomenon are that *C. ulcerans* is maintained by various animals, increasing its diversity compared with *C. diphtheria*, which is believed to infect only humans; or that

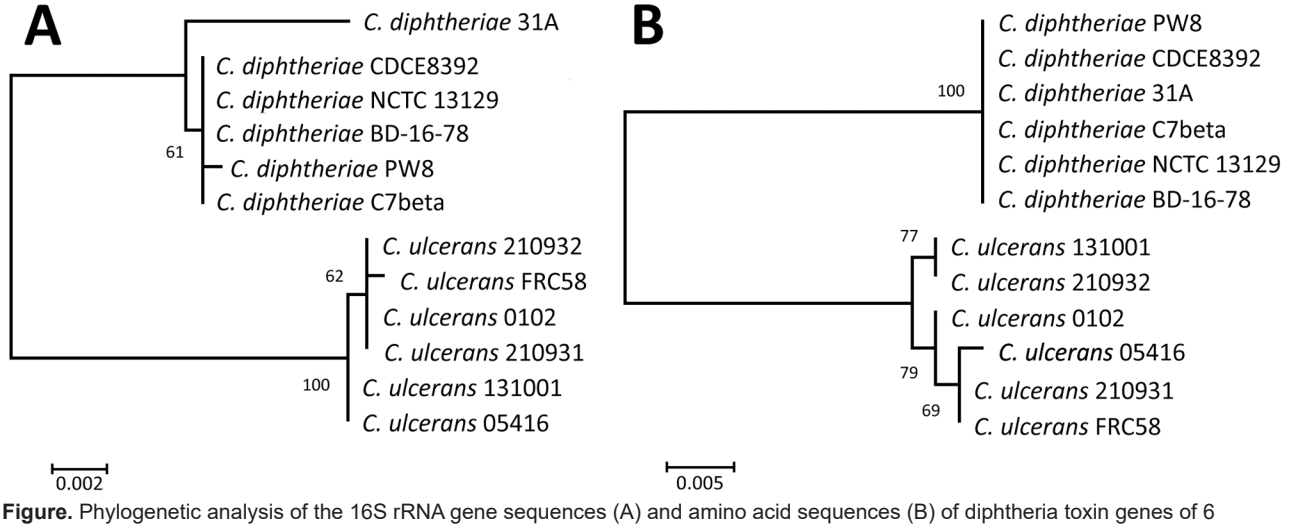

Figure. Phylogenetic analysis of the 16S rRNA gene sequences (A) and amino acid sequences (B) of diphtheria toxin genes of 6 *Coynebacterium ulcerans* strains and 6 *C. ulcerans* strains. All strains had the diphtheria toxin gene; whole-genome analysis data are available from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/genome). We generated phylogenetic trees by using the maximum-likelihood method in MEGA 7.0 (https://www.megasoftware.net). 16S rRNA gene sequences were analyzed by the Hasegawa-Kishino-Yano model with 1,000 bootstrap replications; amino acid sequences were analyzed by the Whelan and Goldman model with 100 bootstrap replications. Scale bars indicate substitutions per site.

C. ulcerans has a phage-independent pathway to acquire the diphtheria toxin–encoding gene, as reported (*9*).

Most severe human cases of disease caused by toxigenic *C. ulcerans* have occurred in unvaccinated or inadequately vaccinated persons. However, a fatal case was reported in a person who received a diphtheria vaccination booster ≈10 years before disease onset (*10*). Diversification of the *C. ulcerans* diphtheria toxin gene is of note because accumulation of these gene mutations potentially could lead to decreased effectiveness of the diphtheria toxoid vaccine for prevention and diphtheria antitoxin for treatment of toxigenic *C. ulcerans* disease.

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References

1. Hacker E, Antunes CA, Mattos-Guaraldi AL, Burkovski A, Tauch A. *Corynebacterium ulcerans*, an emerging human pathogen. Future Microbiol. 2016;11:1191–208. https://doi.org/10.2217/fmb-2016-0085
2. Katsukawa C, Komiya T, Umeda K, Goto M, Yanai T, Takahashi M, et al. Toxigenic *Corynebacterium ulcerans* isolated from a hunting dog and its diphtheria toxin antibody titer. Microbiol Immunol. 2016;60:177–86. https://doi.org/10.1111/1348-0421.12364
3. Katsukawa C, Komiya T, Yamagishi H, Ishii A, Nishino S, Nagahama S, et al. Prevalence of *Corynebacterium ulcerans* in dogs in Osaka, Japan. J Med Microbiol. 2012;61:266–73. https://doi.org/10.1099/jmm.0.034868-0
4. Otsuji K, Fukuda K, Endo T, Shimizu S, Harayama N, Ogawa M, et al. The first fatal case of *Corynebacterium ulcerans* infection in Japan. JMM Case Rep. 2017;4:e005106. https://doi.org/10.1099/jmmcr.0.005106
5. Freeman VJ. Studies on the virulence of bacteriophage-infected strains of *Corynebacterium diphtheriae.* J Bacteriol. 1951;61:675–88.
6. Sekizuka T, Yamamoto A, Komiya T, Kenri T, Takeuchi F, Shibayama K, et al. *Corynebacterium ulcerans* 0102 carries the gene encoding diphtheria toxin on a prophage different from the *C. diphtheriae* NCTC 13129 prophage. BMC Microbiol. 2012;12:72. https://doi.org/10.1186/1471-2180-12-72
7. Sangal V, Hoskinsson PA. Corynephages: infections of the infectors. In: Burkovski A, editor. Diphtheria and its etiological agents. Dordrecht (the Netherlands): Springer; 2014. p. 67–82.
8. Sing A, Hogardt M, Bierschenk S, Heesemann J. Detection of differences in the nucleotide and amino acid sequences of diphtheria toxin from *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* causing extrapharyngeal infections. J Clin Microbiol. 2003;41:4848–51. https://doi.org/10.1128/JCM.41.10.4848-4851.2003
9. Meinel DM, Margos G, Konrad R, Krebs S, Blum H, Sing A. Next generation sequencing analysis of nine *Corynebacterium ulcerans* isolates reveals zoonotic transmission and a novel putative diphtheria toxin-encoding pathogenicity island. Genome Med. 2014;6:113. https://doi.org/10.1186/s13073-014-0113-3
10. Vandentorren S, Guiso N, Badell E, Boisrenoult P, Micaelo M, Troché G, et al. Toxigenic *Corynebacterium ulcerans* in a fatal human case and her feline contacts, France, March 2014. Euro Surveill. 2014;19:20910. https://doi.org/10.2807/1560-7917.ES2014.19.38.20910

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Ophthalmomyiasis Caused by *Chrysomya bezziana* after Periocular Carcinoma

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We treated a homeless man in Iran with a history of squamous cell carcinoma who had ophthalmomyiasis caused by *Chrysomya bezziana* parasites. This case highlights a much-neglected condition and describes measures to prevent it.

Ophthalmomyiasis is principally manifested as orbital myiasis, ophthalmomyiasis external, and ophthalmomyiasis interna (*1*,*2*). *Chrysomya bezziana* (screwworm) has been implicated in cancer-associated myiasis of the skin, larynx, face, and breast (*3*–*5*). Ophthalmomyiasis is uncommon but becomes significant in debilitated and compromised patients.

In December 2017, a 75-year-old homeless man sought care at the University Hospital of Nikookari Eye Center (Tabriz, northwestern Iran). His medical history included a surgery for left-side periocular squamous cell carcinoma ≈15 years earlier. After 2 years, the patient observed recurrence of the squamous cell carcinoma, but he did not seek further evaluation and treatment. He reported loss of sight since 2015 because of the tumor extension into the orbit and globe of his eye. He had intermittent pain.

On examination, his systemic findings were unremarkable. Orbital and periocular examination revealed extensive

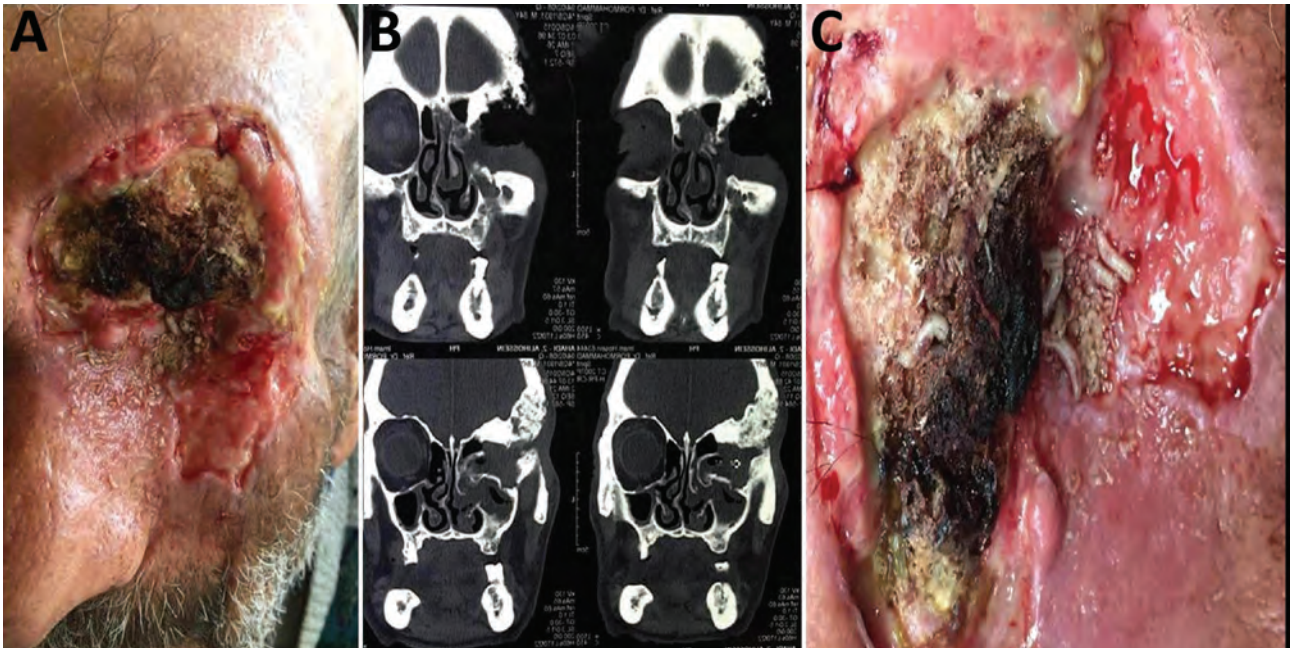

Figure. A 75-year-old man with ophthalmomyiasis after periocular squamous cell carcinoma, Iran. A) Extensive tissue necrosis and the extension of the eyelids, eyebrow, and orbit. B) Computed tomography scan showing huge invasion and necrosis of all cavities of the orbit. C) Live screwworm larvae (*Chrysomya bezziana*) inside the necrotic tissue of the orbit.

tissue necrosis and extension to the eyelids, eyebrow, and orbit (Figure, panel A). The globe seemed to be totally invaded and necrotized by the tumor. A computed tomography scan of the area showed huge invasion and necrosis of all cavities of the orbit, including the globe and bone absorption of the superotemporal area of orbit; we further suspected extension of the tumor to the maxillary and ethmoidal sinuses (Figure, panel B). We also found different sizes of live larvae inside the necrotic tissue of orbit and periorbital skin (Figure, panel C; Video, https://wwwnc.cdc.gov/EID/article/25/11/18-1706-V1.htm).

Our sequencing and phylogenetic analyses of cytochrome b gene showed that isolated larvae (GenBank accession no. MN158142) were confirmed as *C. bezziana* with 100% identity (query coverage: 100%) isolated from livestock in Oman. We planned to admit the patient for further management, but he denied the admission and accepted only irrigation of the site with normal saline. After irrigation that removed only the superficial larvae, he left the hospital and never returned for further treatment.

This case highlights a much-neglected squamous cell carcinoma of the periorbital region with orbital invasion necrosis of globe and orbital soft tissue and massive and extensive larvae infestation caused by the *C. bezziana* screwworm. To prevent myiasis cases such as the one we describe, healthcare providers should emphasize general cleanliness of surroundings, maintenance of good personal hygiene, provision of basic sanitation, and health education.

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We thank the Nikookari Eye Center for helping us identify the *C. bezziana* parasite and providing photographs of the same.

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References

1. Khataminia G, Aghajanzadeh R, Vazirianzadeh B, Rahdar M. Orbital myiasis. J Ophthalmic Vis Res. 2011;6:199–203.
2. Sigauke E, Beebe WE, Gander RM, Cavuoti D, Southern PM. Case report: ophthalmomyiasis externa in Dallas County, Texas. Am J Trop Med Hyg. 2003;68:46–7. https://doi.org/10.4269/ajtmh.2003.68.46
3. Hawayek LH, Mutasim DF. Myiasis in a giant squamous cell carcinoma. J Am Acad Dermatol. 2006;54:740–1. https://doi.org/10.1016/j.jaad.2005.07.012
4. Rubio C, Ladrón de Guevara C, Martín MA, Campos L, Quesada A, Casado M. Cutaneous myiasis over tumor-lesions: presentation of three cases [in Spanish]. Actas Dermosifiliogr. 2006;97:39–42. https://doi.org/10.1016/S0001-7310(06)73346-4
5. Kwong A, Yiu WK, Chow LW, Wong S. *Chrysomyabezziana*: a rare infestation of the breast. Breast J. 2007;13:297–301. https://doi.org/10.1111/j.1524-4741.2007.00426.x

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Dengue Fever in the Darfur Area, Western Sudan

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We report an outbreak of dengue in Darfur, western Sudan, during September 2014–April 2015. Dengue virus–specific PCR testing of 50 samples from nonmalaria febrile illness case-patients confirmed 35 dengue cases. We detected 7 cases of dengue shock syndrome and 24 cases of dengue hemorrhagic fever.

Dengue is a mosquito-transmitted arboviral disease caused by 4 closely related dengue virus serotypes (DENV1–4); the primary vector of DENV is *Aedes aegypti* mosquitoes (*1*). Dengue infection has different clinical manifestations of disease, ranging from a self-limiting illness to the fatal severe forms of dengue hemorrhagic fever or dengue shock syndrome (*1*).

Dengue is a rapidly growing global public health problem, and cases have been identified in ≥128 countries (*2*,*3*). Several factors might contribute to dengue transmission, including human population growth, density, and movement, and international travel and trade (*4*,*5*), as well as scarcity and poor storage of water and global climate change. Secondary infection with different virus serotypes and sex and young age of patients seem to be associated with development of severe disease (*1*).

The Darfur region of Sudan is composed of 5 states covering an area of 493,180 km^2 of desert and semidesert. This region has a population of 7.5 million persons living in a humanitarian crisis since 2003. We report dengue fever in this region of western Sudan.

On September 16, 2014, a large number of case-patients with nonmalarial febrile illness came to outpatient clinics in AlFashir, the capital of North Darfur State. An outbreak investigation team was assembled and deployed to the area by the Federal Ministry of Health. Using active surveillance, this team identified 155 suspected cases of hemorrhagic fever in various localities within the state through April 12, 2015. The suspected case-patients had fever (152/155), bleeding (140/155), headache (73/155), joint pain (52/155), and neurologic signs (9/155). Most case-patients came from AlFashir, the initial location of the outbreak. The outbreak peaked during October 2014 with 77 (49.7%) suspected cases (Figure). Most (52%) of the suspected case-patients were <20 years of age (52%) (age range 18 months–74 years), and the female:male ratio was 1:1.5.

Clinical manifestations of the suspected case-patients suggested involvement of hemorrhagic fevers. Considering the history of similar epidemics of fevers in Sudan and our limited resources, we tested samples for yellow fever virus, Rift Valley fever virus, and DENV (*6*,*7*). We tested blood samples from suspected case-patients for these infections by using IgM-specific ELISAs, and all positive results were confirmed by use of a disease-specific PCR in the Central National Public Health Laboratory in Khartoum (*7*).

We obtained only 50 blood samples from 155 suspected case-patients because of patient or family refusal to participate in this study. A total of 35 (70.0%) samples were positive for DENV-1 or DENV-3 serotypes. Eight deaths occurred among the DENV-positive persons (mortality rate 5.2%) during the investigation period. Four of the fatal cases were in children <14 years of age, 2 in adult men, and 2 in adult women. Clinical examination of the confirmed infected persons identified 7 cases of dengue shock syndrome and 24 cases of dengue hemorrhagic fever.

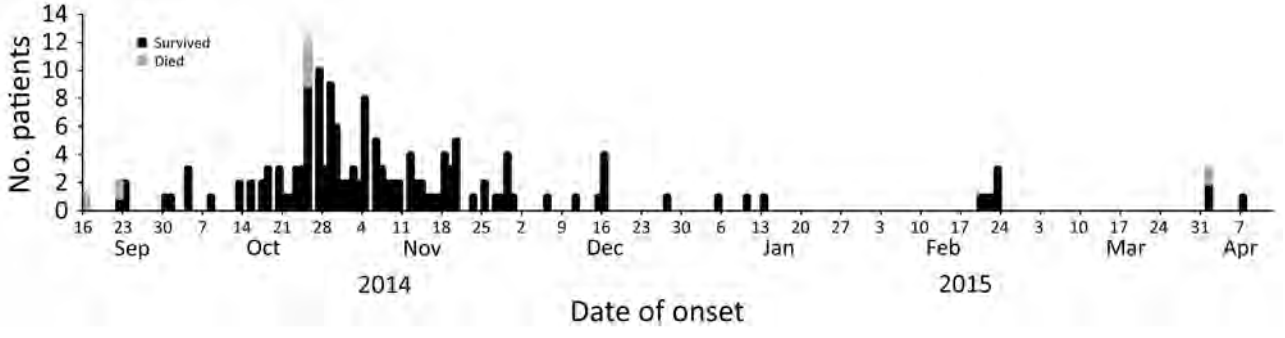

Figure. Number of dengue case-patients per week, Darfur area, western Sudan, September 16, 2014–April 7, 2015.

We report emergence of dengue in the Greater Darfur area of Sudan. Dengue is a major public health issue in this country, but had been confined to the eastern region of the country and the Red Sea coastal and subcoastal states (*4*). Frequent outbreaks have been reported to the World Health Organization Regional Office for the Eastern Mediterranean (*6*). Greater Darfur, a region affected by a civil war, has had massive population displacement, resulting in most persons living in densely populated refugee camps with limited access to education and health services. Lack of water supply necessitated its storage in human-made containers (*8*), which favored breeding of *Ae. aegypti* mosquitoes and increased human–mosquito contact.

The severity of these infections could be because our surveillance selected only the most severe cases, enhanced by the poor healthcare-seeking behavior of the local population, who came to health clinics only when the disease was severe (*7*). The actual prevalence of dengue could be much higher than that detected because our surveillance system likely detected only the most severe cases (*7*). A wider and better surveillance system is urgently needed to detect nonsevere cases and determine the actual prevalence of the disease and population at risk.

In addition, Gayer et al. suggested that socioeconomic and environmental changes associated with the civil war in Sudan made communities vulnerable to the emergence of infectious diseases (*8*). The geopolitical and security issues surrounding the refugee camps suggest that dengue was imported into the area through members of United Nations Peacekeeping Forces from dengue-endemic areas rather than being introduced from East Sudan, as has been observed in Australia (*8*–*10*).

Our study had some limitations. First, we observed weak healthcare-seeking behavior, which resulted in insufficient blood samples for diagnosis. Second, because of limited resources, we could not investigate co-infections with DENV-1 and DENV-3. Third, our survey was conducted in healthcare clinics where only severe case-patients were seen. We recommend improvement of surveillance and development of an early warning system to reduce future impacts of such epidemics. We also highlight the need to improve health and living conditions of persons living in humanitarian crisis settings.

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References

1. Simmons CP, Farrar JJ, Nguyen V, Wills B. Dengue. N Engl J Med. 2012;366:1423–32. https://doi.org/10.1056/NEJMra1110265
2. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7. https://doi.org/10.1038/nature12060
3. Brady OJ, Gething PW, Bhatt S, Messina JP, Brownstein JS, Hoen AG, et al. Refining the global spatial limits of dengue virus transmission by evidence-based consensus. PLoS Negl Trop Dis. 2012;6:e1760. https://doi.org/10.1371/journal.pntd.0001760
4. Soghaier MA, Himatt S, Osman KE, Okoued SI, Seidahmed OE, Beatty ME, et al. Cross-sectional community-based study of the socio-demographic factors associated with the prevalence of dengue in the eastern part of Sudan in 2011. BMC Public Health. 2015;15:558. https://doi.org/10.1186/s12889-015-1913-0
5. Wilder-Smith A, Gubler DJ. Geographic expansion of dengue: the impact of international travel. Med Clin North Am. 2008;92: 1377–90, x. https://doi.org/10.1016/j.mcna.2008.07.002
6. Endy TP, Anderson KB, Nisalak A, Yoon I-K, Green S, Rothman AL, et al. Determinants of inapparent and symptomatic dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS Negl Trop Dis. 2011;5:e975. https://doi.org/10.1371/journal.pntd.0000975
7. Ahmed A, Elduma A, Magboul B, Higazi T, Ali Y. The first outbreak of dengue fever in greater Darfur, Western Sudan. Trop Med Infect Dis. 2019;4:43. https://doi.org/10.3390/tropicalmed4010043
8. Gayer M, Legros D, Formenty P, Connolly MA. Conflict and emerging infectious diseases. Emerg Infect Dis. 2007;13:1625–31. https://doi.org/10.3201/eid1311.061093
9. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proc Natl Acad Sci U S A. 2015;112:14688–93. https://doi.org/10.1073/pnas.1508114112
10. Kitchener S, Leggat PA, Brennan L, McCall B. Importation of dengue by soldiers returning from East Timor to north Queensland, Australia. J Travel Med. 2002;9:180–3. https://doi.org/10.2310/7060.2002.24234

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Severe Fever with Thrombocytopenia Syndrome Virus RNA in Semen, Japan

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Severe fever with thrombocytopenia syndrome virus (SFTSV) can be transmitted between humans. We describe a case of severe fever with thrombocytopenia syndrome in which SFTSV RNA was detected in semen after its disappearance from serum. Our findings indicate possible sexual transmission of this emerging virus.

Severe fever with thrombocytopenia syndrome (SFTS) is a life-threatening emerging infectious disease caused by severe fever with thrombocytopenia syndrome virus (SFTSV), a tickborne virus (genus *Banyang virus*, family *Phenuiviridae*). Recently, the person-to-person transmission of SFTSV has been described (*1*,*2*), and the most common risk factor of the transmission is direct blood exposure (*2*). However, SFTSV RNA has been detected in nonblood samples, such as throat, urine, and fecal specimens, especially in fatal cases (*3*). Asymptomatic infections through personal contact without blood exposure have also been reported (*1*). We describe a case in which viral RNA was detected in semen after viral RNA clearance from blood.

During May 2018, a previously healthy 50-year-old man hunted boar in the Goto Islands in western Japan. Eight days after hunting, he experienced high fever, myalgia, and diarrhea. He did not have hematuria or bloody diarrhea. Disturbance of consciousness occurred 6 days after symptom onset; on that day, he visited a local hospital and was referred to and admitted to Nagasaki University Hospital (Nagasaki, Japan). Body temperature was 39.0°C, and he was disoriented; Glasgow coma scale score was 9. He had no jaundice, signs of meningeal irritation, or apparent tick bites. Laboratory tests at admission had the following results: leukocytes 2.4×10^3 cells/μL; platelets 35×10^3/μL; serum creatine 3.04 mg/dL; aspartate aminotransferase 508 U/L; lactate dehydrogenase 1,404 U/L; and creatine kinase 15,449 U/L.

Because of the patient's low platelet count and other suggestive signs and symptoms, we suspected SFTS. Serum SFTSV RNA level was 2.03×10^8 copies/mL by real-time reverse transcription PCR (RT-PCR) analysis (Appendix, https://wwwnc.cdc.gov/EID/article/25/11/1061-App1.pdf). We confirmed diagnosis of SFTS on the basis of these results; however, we did not detect viral RNA in a urine sample. We conducted RT-PCR tests of semen and urine using procedures developed for serum; all RT-PCR tests were performed in the Department of Virology, Institute of Tropical Medicine, Nagasaki University, Nagasaki.

We considered this case severe, with multiple poor prognosis factors, such as disturbance of consciousness, laboratory data, and high viral load in serum (*4*). We performed palliative therapy, including continuous hemodiafiltration, mechanical ventilation, and central venous nutrition. In addition, we treated the patient with recombinant human soluble thrombomodulin for disseminated intravascular coagulation (380 U/kg/d for 6 d) and granulocyte colony-stimulating factor (filgrastim) for neutropenia (300 μg on the third hospital day). We also administered intravenous immunoglobulin (5,000 mg/d for 3 d), because it has been reported effective for SFTS (*5*), and the patient received platelet transfusions for severe thrombocytopenia.

We observed restoration of platelet count 10 days after symptom onset. Other abnormal laboratory findings recovered 7–13 days after symptom onset. The viral load in serum began to decrease from day 8 after onset and became negative on day 30 after onset. Although the patient's general status was gradually improved and laboratory tests recovered to almost normal levels by day 30, we detected

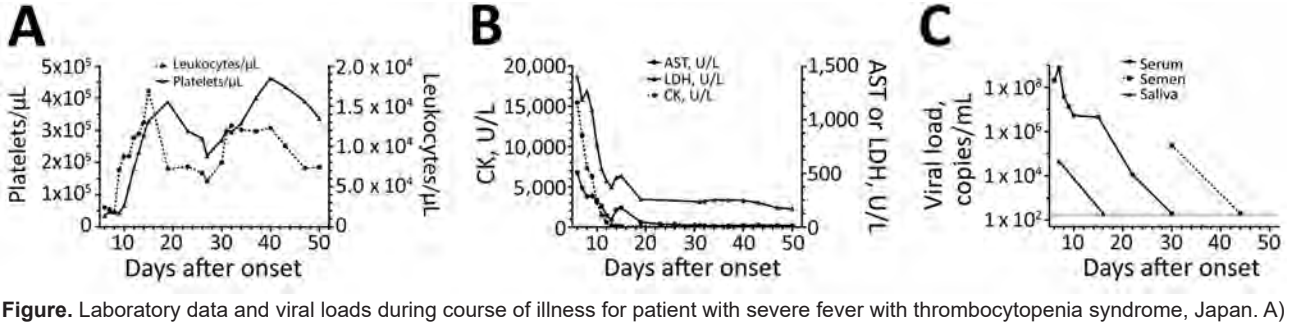

Figure. Laboratory data and viral loads during course of illness for patient with severe fever with thrombocytopenia syndrome, Japan. A) Leukocyte and platelet counts; B) AST, LDH, and CK levels; C) viral loads in serum, semen, and saliva. Dashed line in panel C indicates detection threshold (2×10^2 copies/mL). AST, aspartate aminotransferase; CK, creatine kinase; LDH, lactate dehydrogenase.

SFTSV RNA at 2.4×10^5 copies/mL in his semen that day. On day 44, we could no longer detect semen SFTSV RNA, and he was discharged on day 51 after onset (Figure 1).

In this study, SFTSV RNA was detected in semen, and SFTSV persisted longer in semen than in serum. It is well known that some viruses, such as Zika virus and Ebola virus, can be sexually transmitted; these viruses have been detected in semen for a prolonged period after symptom onset (*6*,*7*). Thus, we considered the potential risk for sexual transmission of SFTSV.

Compared with that of Zika and Ebola viruses, the clinical significance of potential sexual transmission of SFTSV is unknown. However, this possibility should be taken into consideration in sexually active patients with SFTSV. Our findings suggest the need for further studies of the genital fluid of SFTS patients, women as well as men, and counseling regarding sexual behavior for these patients.

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References

1. Huang D, Jiang Y, Liu X, Wang B, Shi J, Su Z, et al. A cluster of symptomatic and asymptomatic infections of severe fever with thrombocytopenia syndrome caused by person-to-person transmission. Am J Trop Med Hyg. 2017;97:396–402. https://doi.org/10.4269/ajtmh.17-0059
2. Chen H, Hu K, Zou J, Xiao J. A cluster of cases of human-to-human transmission caused by severe fever with thrombocytopenia syndrome bunyavirus. Int J Infect Dis. 2013;17:e206–8. https://doi.org/10.1016/j.ijid.2012.11.006
3. Zhang YZ, He YW, Dai YA, Xiong Y, Zheng H, Zhou DJ, et al. Hemorrhagic fever caused by a novel bunyavirus in China: pathogenesis and correlates of fatal outcome. Clin Infect Dis. 2012;54:527–33. https://doi.org/10.1093/cid/cir804
4. Gai ZT, Zhang Y, Liang MF, Jin C, Zhang S, Zhu CB, et al. Clinical progress and risk factors for death in severe fever with thrombocytopenia syndrome patients. J Infect Dis. 2012;206:1095–102. https://doi.org/10.1093/infdis/jis472
5. Kim UJ, Kim DM, Ahn JH, Kang SJ, Jang HC, Park KH, et al. Successful treatment of rapidly progressing severe fever with thrombocytopenia syndrome with neurological complications using intravenous immunoglobulin and corticosteroid. Antivir Ther. 2016;21:637–40. https://doi.org/10.3851/IMP3036
6. Fischer WA, Brown J, Wohl DA, Loftis AJ, Tozay S, Reeves E, et al. Ebola virus ribonucleic acid detection in semen more than two years after resolution of acute Ebola virus infection. Open Forum Infect Dis. 2017;4:ofx155. https://doi.org/10.1093/ofid/ofx155
7. Medina FA, Torres G, Acevedo J, Fonseca S, Casiano L, De León-Rodríguez CM, et al. Duration of the presence of infectious Zika virus in semen and serum. J Infect Dis. 2019;219:31–40.

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Canine Distemper Virus in Asiatic Lions of Gujarat State, India

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In September 2018, an epizootic infection caused by canine distemper virus emerged in an Asiatic lion population in India. We detected the virus in samples from 68 lions and 6 leopards by reverse transcription PCR. Whole-genome sequencing analysis demonstrated the virus strain is similar to the proposed India-1/Asia-5 strain.

Canine distemper virus (CDV; genus *Morbillivirus*) causes highly contagious disease in a wide range of carnivores. Epizootic disease in lions in a wildlife sanctuary in California, USA, in 1992 and Serengeti National Park, Tanzania, in 1994 underlined the potential of CDV to cause fatality in wild felids (*1*,*2*). The disease often manifests as respiratory and gastrointestinal signs that progress to neurologic disease (*2*).

A single isolated population of Asiatic lions (*Panthera leo persica*) resides in the Gir forests of Gujarat State, India, the last natural habitat for this species. Conservation efforts brought this lion population back from the brink of extinction and increased their numbers (*3*).

During 2 weeks in September 2018, the unusual death of 28 lions of all age groups was reported from Gir Wildlife Sanctuary. A detailed investigation revealed 18 additional lions exhibited dullness, dehydration, lacrimation, cough, diarrhea, and seizures. Necropsy of 2 carcasses showed edema and purulent exudates in the lungs. Histopathology of lungs from both lions showed mononuclear cell infiltration with mild thickening of interalveolar septa.

The Indian Council of Medical Research, National Institute of Virology (Pune, India), received ocular,

nasal, and rectal swab specimens packed in a viral transport medium and blood samples from 229 wild and 87 captive lions and visceral organs, including lung, liver, heart, and kidney, from 3 dead lions for virologic investigation. Of the 229 wild lions, 20 showed clinical signs, including dullness, lacrimation, cough, diarrhea, and seizures; 2 of the 87 captive lions showed lacrimation and respiratory distress.

We extracted RNA by using Magmax Total RNA Isolation Kit (ThermoFisher Scientific, https://www.thermofisher.com) and processed samples for heminested PCR for CDV and nested PCR for *Paramyxovirus*, as described previously (*4*). We obtained 287 bp by heminested reverse transcription PCR (RT-PCR) and 500 bp by *Paramyxovirus* nested RT-PCR. We detected CDV from ≥1 sample from 68 (21.3%) lions, including 56 (24.5%) wild and 12 (13.8%) captive lions. All 22 of the lions with clinical signs were PCR-positive for CDV. Among the samples tested, 18/90 (20%) blood, 26/131 (19.8%) rectal, 28/131 (21.4%) nasal, and 10/132 (7.5%) ocular specimens, as well as the visceral organs from the 3 dead lions, were CDV positive.

We performed RNA library preparation and quantification for next-generation sequencing by using methods described previously (*5*) and analyzed reads by using CLC Genomics Workbench version 11.0.1 (QIAGEN, https://www.qiagen.com). We retrieved the near-complete genome (15 kb) of CDV from 11 lion samples by using a combination of de novo assembly and reference mapping. We calculated percent nucleotide differences by using the p-distance method in MEGA version 7.0 (*6*). We performed similarity and divergence calculations for the hemagglutinin (H) gene and generated a neighbor-joining tree for the complete genome and H gene region. We performed bootstrap replication of 1,000 cycles to assess the robustness of the tree.

Phylogenetic analysis showed that sequences from this outbreak clustered with East-African CDV strains (Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/25/11/19-0120-Techapp1.pdf). The complete genome of CDV among the positive samples displayed 100% similarity, except for the spleen and lung of 1 lion, which were 99.9% similar. We noted a 3.4% nucleotide difference between the complete genome of Gir CDV and the East-African strain (Table 1).

Phylogenetic analysis of the CDV sequences of Gir lions against available partial CDV sequences of H gene previously collected from tigers and a dog from India demonstrated a distinct cluster for sequences from India (Appendix Figure 2). Bhatt et al. (*7*) hypothesized a novel CDV strain, India-1/Asia-5, among domestic dogs in India. Our analysis of sequences from the CDV outbreak in Gir lions strengthens that hypothesis. We observed 95.8%–96.8% nucleotide similarity for the H gene region (Table 1) of CDV sequences from the Gir outbreak with the proposed India-1/Asia-5 strain. We noted minimal nucleotide similarity between

Table. Nucleotide similarity of the hemagglutinin gene and complete genome of canine distemper virus sequences to those from samples collected from an Asiatic lion, India, 2018*

GenBank accession no.	Source	Geographic location	Year	Lineage	H gene	Complete genome
MK037459	Asiatic lion	Gir National Park, Gujarat State, India	2018		100.0	100.0
MK037460					100.0	100.0
MK037461					100.0	100.0
MK037462					100.0	99.9
MK037463					100.0	99.9
MK037464					100.0	99.9
MK037465					100.0	100.0
MK037466					100.0	100.0
MK037467					100.0	100.0
MK037468					100.0	100.0
KU578257.1	Dog	Africa	1994	East African	96.1	96.6
MH496778.1	Dog	Thailand	2014	Asia 4	94.5	94.1
KJ466106.1	Raccoon dog	China	2012	Asia 1	94.0	94.1
AY649446.1	Racoon	USA	2001	America 2	94.9	94.9
AY466011.2	Racoon	USA	1998	America 1	93.0	94.0
AY386316.1	Dog	Germany	NA	Europe	95.9	94.8
AB475099.1	Dog	Japan	NA	Asia 2	93.4	94.1
KC479141.1	Dog	India	2012		97.1	NA
KC479140.1	Tiger	India	2012		96.9	NA
KC479139.1	Tiger	India	2012		97.1	NA
KC479138.1	Red panda	India	2013		97.4	NA
LC011103.1	Dog	India	2012		97.4	NA
Vaccine strains						
AF378705.1 CDV strain Onderstepoort					92.4	92.1
EU726268.1 CDV strain CDV3					92.6	92.0
Z35493.1CDV Convac					92.0	NA

*Reference sample is GenBank accession no. MK037469. NA, not available. GenBank accession nos. MK037459–68 represent sequence data from this study.

Gir CDV outbreak strains and Asia 3 strains and maximum similarity with Rockborne-like strains. Whole-genome sequence analysis showed that Gir CDV strains had ≈8% nucleotide difference from the 2 known vaccine-derived strains of America 1 genotypes (Table 1).

After we confirmed CDV in Asiatic lions, samples were collected from other wild animals in Gir Wildlife Sanctuary. We detected CDV in 6/52 (12%) samples from *Panthera pardus* leopards but not in samples from *Viverricula indica* civets, *Panthera tigris* tigers, *Canis lupus pallipes* wolves, or *Caracal caracal* cats.

Before 2019, few instances of CDV were reported in lions, tigers, red pandas, and leopards from zoos and forests in India. However, canine distemper is prevalent among dogs in India (*8*), and the free-ranging dog population often poses a threat of CDV transmission to wildlife (*9*). Other wildlife species also could play a role in maintenance and transmission of CDV (*9*). Vaccination is an option; attenuated Onderstepoort strain was used successfully in captive African lions in Maasai Mara National Reserve in Kenya, and a live attenuated canine vaccine was used in a vaccine trial in tigers (*10*). Reintegration of the existing lion population from the Gir region to different sanctuaries can ensure the protection and conservation of the species.

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References

1. Appel MJ, Yates RA, Foley GL, Bernstein JJ, Santinelli S, Spelman LH, et al. Canine distemper epizootic in lions, tigers, and leopards in North America. J Vet Diagn Invest. 1994;6:277–88. https://doi.org/10.1177/104063879400600301
2. Roelke-Parker ME, Munson L, Packer C, Kock R, Cleaveland S, Carpenter M, et al. A canine distemper virus epidemic in Serengeti lions (*Panthera leo*). Nature. 1996;379:441–5. https://doi.org/10.1038/379441a0
3. Bauer H, Packer C, Funston P., Henschel P, Nowell K. *Panthera leo*. the IUCN red list of threatened species 2016: e.T15951A115130419. http://dx.doi.org/10.2305/IUCN.UK.2016-3.RLTS.T15951A107265605.en
4. Frisk AL, König M, Moritz A, Baumgärtner W. Detection of canine distemper virus nucleoprotein RNA by reverse transcription-PCR using serum, whole blood, and cerebrospinal fluid from dogs with distemper. J Clin Microbiol. 1999;37:3634–43.
5. Yadav PD, Nyayanit DA, Shete AM, Jain S, Majumdar TP, Chaubal GY, et al. Complete genome sequencing of Kaisodi virus isolated from ticks in India belonging to *Phlebovirus* genus, family *Phenuiviridae*. Ticks Tick Borne Dis. 2019;10:23–33. https://doi.org/10.1016/j.ttbdis.2018.08.012
6. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4. https://doi.org/10.1093/molbev/msw054
7. Bhatt M, Rajak KK, Chakravarti S, Yadav AK, Kumar A, Gupta V, et al. Phylogenetic analysis of haemagglutinin gene deciphering a new genetically distinct lineage of canine distemper virus circulating among domestic dogs in India. Transbound Emerg Dis. 2019;66:1252–67. https://doi.org/10.1111/tbed.13142
8. Ashmi JM, Thangavelu A. Senthilkumar TMA, Manimaran K. Molecular characterization of canine distemper virus from Tamil Nadu, India. Indian J Anim Sci. 2017;87:1062–7.
9. Viana M, Cleaveland S, Matthiopoulos J, Halliday J, Packer C, Craft ME, et al. Dynamics of a morbillivirus at the domestic-wildlife interface: canine distemper virus in domestic dogs and lions. Proc Natl Acad Sci U S A. 2015;112:1464–9. https://doi.org/10.1073/pnas.1411623112
10. Sadler RA, Ramsay E, McAloose D, Rush R, Wilkes RP. Evaluation of two canine distemper virus vaccines in captive tigers (*Panthera Tigris*). J Zoo Wildl Med. 2016;47:558–63. https://doi.org/10.1638/2015-0223.1

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Routine Culture–Resistant *Mycobacterium tuberculosis* Rescue and Shell-Vial Assay, France

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We used shell-vial assay with a medium that buffered rifampin to isolate routine culture–resistant *Mycobacterium tuberculosis* bacteria from cerebrospinal fluid and rifampin-containing intervertebral disc and vertebral corpus of a patient in treatment for Pott's disease and disseminated tuberculosis. Whole-genome sequencing confirmed *M. tuberculosis* lineage 4 (Euro-American) strain.

Culturing *Mycobacterium tuberculosis* from clinical specimens confirms the viability of mycobacteria and enables drug susceptibility testing (*1*). Routinely used culture protocols may fail to isolate *M. tuberculosis* from vertebral biopsy specimens in 17%–50% of cases (*2*) and from cerebrospinal fluid (CSF) specimens in >80% of cases (*3*). However, the shell-vial culture assay (*4*) has demonstrated a high sensitivity for the isolation of mycobacteria, representing an alternative method for growing *M. tuberculosis* (*5*,*6*).

We report a case of disseminated tuberculosis (TB) documented by culturing *M. tuberculosis* strain P7739 from a patient who was previously treated with antituberculous drugs. We used the shell-vial assay, even though strain P7739 resisted standard cell-free culture techniques. The Ethics Committee of Institut Hospitalier Universitaire Méditerranée Infection (Marseille, France) approved this study (no. 2016-024, October 19, 2016).

A 47-year-old man who was HIV negative and had no previous history of TB received a diagnosis of Pott's disease with systemic tuberculosis on the basis of clinical symptoms of spondylodiscitis, myelitis, meningitis, and pulmonary miliary infection. In January 2017, the patient suffered lumbar pain; a computer tomodensitometry scan showed corporeal bone defects in the L1 vertebra. Ten months later, magnetic resonance imaging revealed T12–L1 vertebral spondylodiscitis with a paravertebral abscess in the right iliopsoas muscle. We performed 2 biopsies of the vertebral corpus of T12 and L1 and 1 biopsy of the intervertebral disc; we also collected CSF and sputum. These clinical specimens remained negative for *M. tuberculosis* using microscopic examination after Ziehl-Neelsen staining, real-time PCR (GenExpert, https://www.cepheid.com), culture in liquid medium BBL Mycobacteria Growth Indicator Tube (Becton Dickinson, https://www.bd.com), and in solid culture media including Coletsos medium (bioMérieux, https://www.biomerieux.com). Thirteen days later, the neurologic condition of the patient deteriorated with meningeal syndrome. Examination by magnetic resonance imaging showed a triventricular hydrocephalus and transependymal periventricular resorption, which led to an emergency external ventricular bypass. We strongly suspected TB, so we introduced TB treatment (900 mg rifampin, 300 mg isoniazid, 1,800 mg pyrazinamide, and 1,200 mg ethambutol daily). We performed a second round of clinical sampling from bronchoalveolar fluid, T12 vertebral corpus and T12–L1 intervertebral discs, and CSF 9 days after the initiation of TB treatment. Microscopic examination after Ziehl-Neelsen staining remained negative, as did results from the GenExpert assay, except for the detection of a rifampin-susceptible *M. tuberculosis* complex mycobacterium in 3 vertebral biopsy specimens and the intervertebral disc specimen. Culturing in MGIT tubes and on Coletsos remained negative after 8 weeks of incubation.

We inoculated 5 samples (1 CSF, 3 bone biopsy, 1 intervertebral disc) on human embryonic lung (HEL) fibroblasts (HEL 299 ATCC CCL-137; American Type Culture Collection, https://www.lgcstandards-atcc.org) using the shell-vial assay incorporating negative controls, as described previously (*6*). After 17–28 days of incubation, negative control vials remained sterile, whereas these 5 inoculated cell cultures grew Ziehl-Neelsen–positive mycobacteria (Figure). Seven-day subculture on Coletsos medium yielded colonies identified as *M. tuberculosis* strain P7739 by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (*7*). In vitro susceptibility assays indicated susceptibility to rifampin, ethambutol, chloramphenicol, clofazimine, and trimethoprim/sulfamethoxazole and resistance to minocycline and pyrazinamide (*8*). The genome sequence of *M. tuberculosis* strain P7739 (1 scaffold with 4,392,478 bp and 64.8% guanine-cytosine content) mapped more closely with *M. tuberculosis* Erdman (GenBank accession no. NC_020559.1) using CLC Genomics version 7 (https://www.qiagenbioinformatics.com). Annotation using Prokka version 1.12 https://github.com/tseemann/prokka) yielded 4,171 protein-coding genes, 2 repeat regions, and 55 RNA genes (51 tRNA, 3 rRNA, and 1 tmRNA). Single-nucleotide polymorphism (SNP) comparison with different lineages at the core genome level using TB profiler

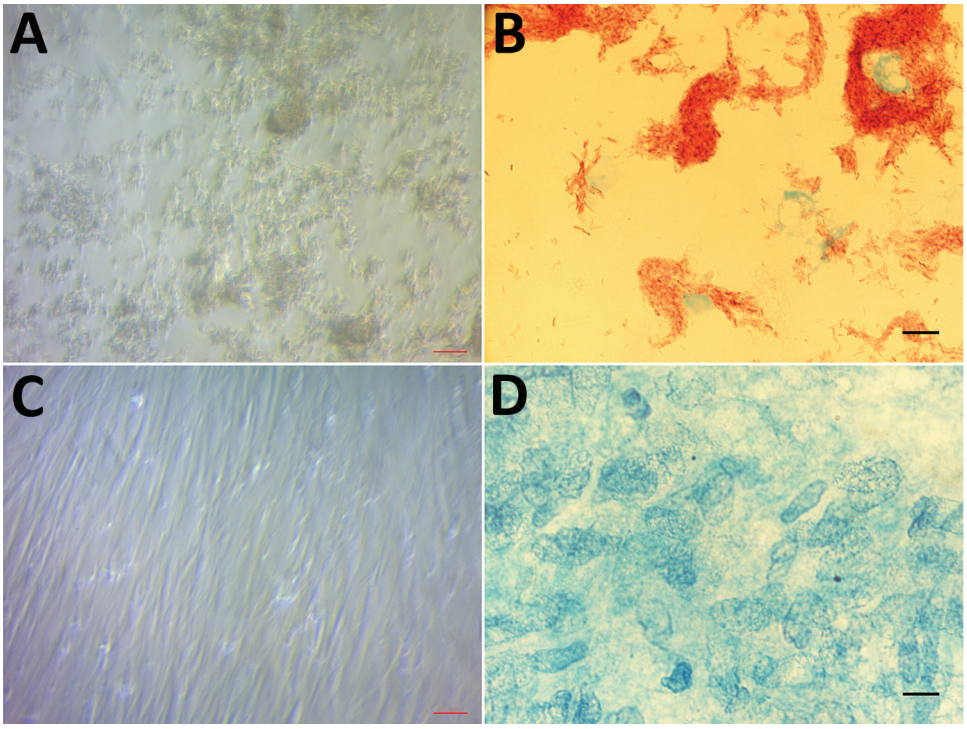

Figure. Light microscopic examination of human embryonic lung cells inoculated for 28 days from a clinical sample from a 47-year-old man with Pott's disease and systemic tuberculosis, France. A) Cytopathic effect consisting of cell lysis caused by growing *Mycobaterium tuberculosis*. Original magnification ×200. B) *M. tuberculosis* mycobacteria observed after Ziehl-Neelsen staining. Original magnification ×1,000, by oil immersion. C) Absence of any cytopathic effect in negative control cell culture. Original magnification ×200. D) Absence of any mycobacteria in the negative control cell culture after Ziehl-Neelsen staining. Original magnification ×1,000 by oil immersion.

(http://tbdr.lshtm.ac.uk) indicated that strain P7739 (GenBank accession no. CAABOY010000000) was related to *M. tuberculosis* lineage 4 and did not encode for antimicrobial resistance–associated mutations.

We isolated *M. tuberculosis* strain P7739 from this patient using the shell-vial assay 9 days after initiation of TB treatment. Liquid chromatography mass spectrometry (LC/MS) (Acquity I-Class Vion-IMS Q-Tof, Waters, https://www.waters.com) detected 0.45 μg rifampin/g in the intervertebral disc specimen and 0.04 μg rifampin/g in the 2 vertebral bone biopsy specimens; 1 exhibited anti-TB activity. Further dosage with rifampin at 0, 7, and 14 days postincubation with HEL revealed that 80% of the amount of free rifampin was lost at day 14 (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/11/19-0431-App1.pdf).

Gouriet et al. reported that 11.5% of *Mycobacterium* sp. isolates are cultured only in a cell culture assay (*6*). We cultured *M. tuberculosis* using the shell-vial assay after incubating for 17–28 days, in accordance with previous studies (*5*). Our findings confirm the use of the shell-vial assay in diagnosis of tuberculosis in patients in whom TB is suspected and whose specimens do not grow on conventional media, especially after initiating TB treatment in patients before sampling. Our observations suggest that cell culture medium buffers the anti-TB activity of clinical specimens, thus enabling growth of *M. tuberculosis* mycobacteria that are no longer exposed to anti-TB activity.

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References

1. Asmar S, Drancourt M. Rapid culture-based diagnosis of pulmonary tuberculosis in developed and developing countries. Front Microbiol. 2015 [cited 2018 Oct 10]. https://www.frontiersin.org/articles/10.3389/fmicb.2015.01184/full
2. Colmenero JD, Ruiz-Mesa JD, Sanjuan-Jimenez R, Sobrino B, Morata P. Establishing the diagnosis of tuberculous vertebral osteomyelitis. Eur Spine J. 2013;22(Suppl 4):579–86. https://doi.org/10.1007/s00586-012-2348-2
3. Modi M, Sharma K, Prabhakar S, Goyal MK, Takkar A, Sharma N, et al. Clinical and radiological predictors of outcome in tubercular meningitis: a prospective study of 209 patients. Clin Neurol Neurosurg. 2017;161:29–34. https://doi.org/10.1016/j.clineuro.2017.08.006
4. Marrero M, Raoult D. Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever rickettsia in blood culture. Am J Trop Med Hyg. 1989;40:197–9. https://doi.org/10.4269/ajtmh.1989.40.197
5. Fournier P-E, Drancourt M, Lepidi H, Gevaudan M-J, Raoult D. Isolation of mycobacteria from clinical samples using the

centrifugation-shell vial technique. Eur J Clin Microbiol Infect Dis. 2000;19:69–70. https://doi.org/10.1007/s100960050015
6. Gouriet F, Fenollar F, Patrice J-Y, Drancourt M, Raoult D. Use of shell-vial cell culture assay for isolation of bacteria from clinical specimens: 13 years of experience. J Clin Microbiol. 2005;43:4993–5002. https://doi.org/10.1128/JCM.43.10.4993-5002.2005
7. Zingue D, Flaudrops C, Drancourt M. Direct matrix-assisted laser desorption ionisation time-of-flight mass spectrometry identification of mycobacteria from colonies. Eur J Clin Microbiol Infect Dis. 2016;35:1983–7. https://doi.org/10.1007/s10096-016-2750-5
8. Bouzid F, Astier H, Osman DA, Javelle E, Hassan MO, Simon F, et al. Extended spectrum of antibiotic susceptibility for tuberculosis, Djibouti. Int J Antimicrob Agents. 2018;51:235–8. https://doi.org/10.1016/j.ijantimicag.2017.07.007

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Molecular Epidemiology of Hantaviruses in the Czech Republic

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During 2008–2018, we collected samples from rodents and patients throughout the Czech Republic and characterized hantavirus isolates. We detected Dobrava-Belgrade and Puumala orthohantaviruses in patients and Dobrava-Belgrade, Tula, and Seewis orthohantaviruses in rodents. Increased knowledge of eco-epidemiology of hantaviruses will improve awareness among physicians and better outcomes of patients.

The most prevalent hantaviruses in Europe are Tula virus, Puumala virus (PUUV), and Dobrava-Belgrade virus (DOBV), all orthohantaviruses; PUUV and DOBV cause hemorrhagic fever with renal syndrome (*1*). Four DOBV genotypes of different virulences in humans are known: the nonpathogenic Saaremaa; Kurkino, which causes mostly mild disease; and Dobrava and Sochi, which are both highly pathogenic (*2*).

Tula virus is the most frequently detected hantavirus in rodents in the Czech Republic, followed by PUUV in Moravia and DOBV in South Bohemia. The seroprevalence of hantaviruses in humans in the Czech Republic is 1%–1.4% (*3*). In 2009, one case of DOBV infection in a hospitalized patient was reported in the Czech Republic (*4*), and in 2011, two more occurred at the Czech Republic–Slovakia border (*5*); in 2017, a fatal DOBV case was reported (*6*). All 4 of these cases were classified as Dobrava genotype by PCR and sequencing. Overall, 82 hantavirus infections were reported in humans in the Czech Republic during 2008–2017 (*7*). In this study, we aimed to determine the location of DOBV reservoirs in the Czech Republic and molecularly characterize positive samples.

During 2010–2017, we collected 1,551 wild rodents from different locations of the Czech Republic: 618 yellow-necked mice (*Apodemus flavicollis*), 37 wood mice (*A. sylvaticus*), 222 striped field mice (*A. agrarius*), 445 bank voles (*Myodes glareolus*), 40 common voles (*Microtus arvalis*), and 189 field voles (*Microtus agrestis*). We trapped all rodents as specified by the Animal Protection Act No. 246/1992 of the Czech Republic. Moreover, we obtained 61 clinical samples acquired from patients with hantavirus infections during 2008–2018; these hantavirus diagnoses were based on serologic testing (detection of hantavirus-specific IgG and IgM) and clinical and laboratory findings (fever, renal dysfunction, thrombocytopenia).

We tested human serum samples by Anti-Hantavirus Pool 1 "Eurasia" ELISA (IgG and IgM) and confirmed previous hantavirus results by immunoblot EUROLINE Anti-Hanta Profile 1 (IgG and IgM) (both Euroimmun, https://www.euroimmun.com). We isolated RNA from rodent lung samples and human serum, plasma, and whole blood samples using QIAamp Viral RNA Kit (QIAGEN, https://www.qiagen.com) and QIAzol (QIAGEN) or TRIzol (Invitrogen, https://www.thermofisher.com). We screened rodent and human RNA samples for hantavirus RNA using a reverse transcription PCR that amplified a 390-bp fragment of the large (L) segment (*8*). We tested L segment–positive samples with additional PCRs targeting regions of the medium (M) and small (S) segments (*4*). We analyzed hantavirus sequences using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), aligned with BioEdit (*9*), and built phylogenetic trees using MEGA 7.0 (https://megasoftware.net).

Of 1,551 rodent samples, 43 (2.77%) were PCR positive for hantavirus. These 43 animals were from 3 sampled localities: Celadna in the Beskydy Mountain region (in 2010, n = 9), Petrovice u Karviné (in 2014, n = 33), and Velká Stolová Mountain (in 2014, n = 1). From 9 hantavirus-positive mice collected in 2010, we

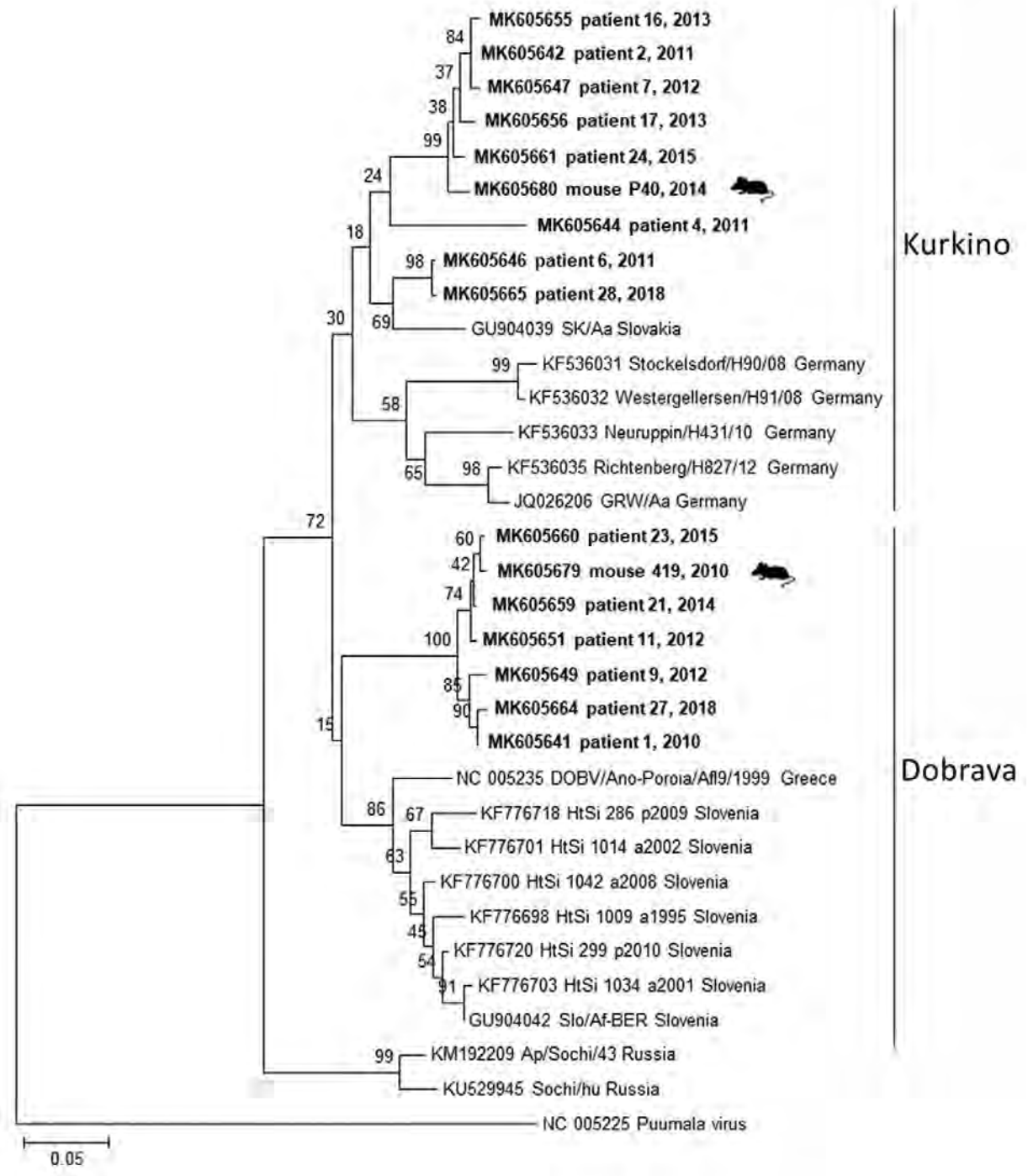

Figure. Phylogenetic tree constructed with partial 195-bp fragments of the DOBV large segment from humans and mice, Czech Republic, 2010–2018. Sequences from this study (bold) were compared with available sequences from the GenBank database; patient numbers are provided, and mouse samples are labeled. Samples with sequences identical to another sample were excluded for simplification purposes. Sequences were aligned with BioEdit (*9*), and the phylogenetic tree was prepared by using MEGA 7.0 (https://megasoftware.net) and the neighbor-joining method. Analyses were performed with the Jukes-Cantor model by using a gamma distribution with 5 rate categories and a bootstrap value of 1,000. The genotype clusters are labeled. Scale bar indicates nucleotide substitutions per site. The Puumala virus sequence was used as an outgroup. DOBV, Dobrava-Belgrade virus.

recovered 6 sequences: 2 identical sequences of DOBV Dobrava in 2 *A. flavicollis* mice (GenBank accession no. MK605679) and 4 sequences (2 identical) of Seewis virus in 3 *A. flavicollis* mice and 1 *A. sylvaticus* mouse (GenBank accession nos. MK605682–4). The Seewis virus sequences clustered with sequences derived from shrews from the Beskydy Mountain region (GenBank accession nos. JQ425316, JQ425337, JQ425340; data not shown). From 33 positive *A. agrarius* mice trapped in Petrovice u Karviné, we recovered 10 Kurkino genotype sequences with 99.1%–100% similarity (GenBank accession nos. MK605680–1). We detected Tula virus (GenBank accession no. MK605685) in 1 field vole trapped at Velká Stolová Mountain.

Of 61 seropositive patients, 32 were PCR positive for DOBV and 3 PCR positive for PUUV (acquired outside of the Czech Republic). We recovered partial L segment sequences from 28 of 32 DOBV-positive patients (GenBank accession nos. MK605641–65). Because of low quality, we manually shortened these sequences to 195 bp. We obtained partial (264-bp) M segment sequences from 6 patients (GenBank accession nos. MK605666–71) and partial (531-bp) S segment sequences from 7 patients (GenBank accession nos. MK605672–8). We constructed phylogenetic trees to compare the virus sequences from humans and rodents. Analysis of the L segment revealed that samples clustered into 2 separate groups by DOBV genotype (Dobrava or Kurkino), and virus sequences from the same areas (regardless of human or rodent origin) clustered into the same clades (Figure). Clustering by DOBV genotype was also observed in phylogenetic trees constructed with the M and S segments (data not shown).

Most Dobrava-positive patients were from the mountain regions Jeseniky (northwest Silesia) and Beskydy (south Silesia), whereas Kurkino cases occurred in the lowlands between these 2 mountain regions (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/11/19-0449-App1.pdf). The only confirmed fatal DOBV case in the Czech Republic was in a patient living in Kladno District, Central Bohemia region (*6*). The geographic distribution of DOBV genotypes seems to be linked with the distribution of *Apodemus* spp. mice (*10*). The higher number of hantavirus cases in Silesia might be caused by an increased prevalence of DOBV in rodents or could be the result of an increased awareness of DOBV among local physicians.

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References

1. Olsson GE, Leirs H, Henttonen H. Hantaviruses and their hosts in Europe: reservoirs here and there, but not everywhere? Vector Borne Zoonotic Dis. 2010;10:549–61. https://doi.org/10.1089/vbz.2009.0138
2. Klempa B, Avsic-Zupanc T, Clement J, Dzagurova TK, Henttonen H, Heyman P, et al. Complex evolution and epidemiology of Dobrava-Belgrade hantavirus: definition of genotypes and their characteristics. Arch Virol. 2013;158:521–9. https://doi.org/10.1007/s00705-012-1514-5
3. Pejcoch M, Kríz B. Hantaviruses in the Czech Republic. Emerg Infect Dis. 2003;9:756–7. https://doi.org/10.3201/eid0906.020772
4. Papa A, Zelená H, Barnetová D, Petrousová L. Genetic detection of Dobrava/Belgrade virus in a Czech patient with haemorrhagic fever with renal syndrome. Clin Microbiol Infect. 2010;16:1187–90. https://doi.org/10.1111/j.1469-0691.2009.03075.x
5. Zelena H, Zvolankova V, Zuchnicka J, Liszkova K, Papa A. Hantavirus infection during a stay in a mountain hut in Northern Slovakia. J Med Virol. 2011;83:496–500. https://doi.org/10.1002/jmv.21984
6. Zelená H, Rumlerová M, Kodras K, Beroušková P, Mrázek J, Smetana J. Hantavirus causing fatal haemorrhagic fever in the Czech Republic [in Czech]. Epidemiol Mikrobiol Imunol. 2017;66:149–52.
7. Státní Zdravotní Ústav. Cases of selected infectious diseases in the Czech Republic, January–July 2019. 2019 [cited 2019 Sep 10]. http://szu.cz/publikace/data/2019/vyskyt-vybranych-hlasenych-infekci-v-ceske-republice-leden-1?lang=1
8. Klempa B, Fichet-Calvet E, Lecompte E, Auste B, Aniskin V, Meisel H, et al. Hantavirus in African wood mouse, Guinea. Emerg Infect Dis. 2006;12:838–40. https://doi.org/10.3201/eid1205.051487
9. Hall TA. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp Ser. 1999;41:95–8.
10. Sibold C, Ulrich R, Labuda M, Lundkvist Å, Martens H, Schütt M, et al. Dobrava hantavirus causes hemorrhagic fever with renal syndrome in central Europe and is carried by two different *Apodemus* mice species. J Med Virol. 2001;63:158–67. https://doi.org/10.1002/1096-9071(20000201)63:2<158::AID-JMV1011>3.0.CO;2-%23

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Tamdy Virus in Ixodid Ticks Infesting Bactrian Camels, Xinjiang, China, 2018

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We isolated Tamdy virus (TAMV; strain XJ01/TAMV/China/2018) from *Hyalomma asiaticum* ticks infesting Bactrian camels in Xinjiang, China, in 2018. The genome of the strain showed high nucleotide similarity with previously described TAMV strains from Asia. Our study highlights the potential threat of TAMV to public health in China.

Tamdy virus (TAMV) was first isolated from the tick species *Hyalomma asiaticum asiaticum* collected from sheep in the Tamdinsky district of the Bukhara region, Uzbekistan, in 1971 (*1*). Subsequently, large-scale surveillance of TAMV from Ixodidae ticks using newborn mice successfully isolated 47 TAMV strains from various tick species from Armenia, Kazakhstan, Kyrgyzstan, Turkmenistan, and Uzbekistan, highlighting both its widespread distribution and its ability to infect mammals (*2*). Recently, TAMV was identified in Turkey from *Hyalomma* spp. ticks collected from *Meriones tristrami* gerbils in the Middle East (*3*). Sequence comparison and phylogenetic analyses of full-length TAMV genomes from different subspecies of *H. asiaticum* ticks taxonomically classified it in the genus *Nairovirus*, family Bunyaviridae (*4*). TAMV was also detected in 1973 from a febrile patient in Kyrgyzstan (*5*).

In May 2018, fourteen ticks attached to 2-humped Bactrian camels (*Camelus bactrianus*) were collected from a camel farm in Xinjiang, China. We extracted total RNA of the ticks using the E.Z.N.A. Total RNA Kit (Omega Bio-tek, https://www.omegabiotek.com). We used a transcriptomics approach to investigate the viruses harbored by the ticks and used the BGI mRNA Library Preparation protocol according to MGIEasy mRNA Library Prep Kit (BGI, https://www.bgi.com) to construct the RNA sequencing libraries for each tick. We conducted paired-end (100-bp) sequencing of each RNA library on the BGISEQ-500RS platform (BGI). We obtained 493,090,699 raw reads and then adaptor and quality trimmed them with the Fastp program (*6*), resulting in a total of 492,344,756 clean reads. These reads were de novo assembled using Trinity (*7*) with default settings. We compared the assembled contigs using BLASTn (http://blast.ncbi.nlm.nih.gov/blast.cg) against the nucleotide database downloaded from GenBank, with an E-value cutoff set at 1×10^{-5}.

We identified contigs annotated as the large (L), medium (M), and small (S) gene segments of TAMV (family Nairoviridae, genus *Orthonairovirus*) in 1 tick (pool 10). To confirm the assembled viral contigs, we mapped reads back to the full-length genome of the TAMV strain LEIV-1308Uz (GenBank accession nos. KP792726–8, corresponding to the L, M, and S gene segments) as reference with Bowtie2 (*8*) and inspected using Geneious version 11.1.5 (Biomatters, Ltd., https://www.geneious.com). After removing repetitive reads, we mapped 34,172 reads to the L gene segment (depth 252 $\pm$ SD 120), 60,184 reads to the M gene segment (depth: 1186 $\pm$ 418), and 8,724 reads to the S gene segment (depth 392 $\pm$ 138). The virus genome obtained comprised L segment (encoding the RNA-dependent RNA polymerase [RdRp]), 12,215 bp; M segment (encoding the glycoprotein precursor), 4,565 bp; and S segment (encoding the nucleocapsid), 2,005 bp.

We then cultured the grinding fluid supernatant corresponding to pool 10 in Vero cells in Dulbecco Modified Eagle medium. We observed apparent cytopathic effects, such as higher cell refractive index, cell shrinkage, size reduction, rounding, and shedding, in infected Vero cells at 11 days postinfection (Figure, panel A). The virus strain was named XJ01/TAMV/China/2018 (hereafter XJ01). After 2 passages, we performed further transcriptome sequencing of the first- and second-generation virus suspension from cell cultures. We assembled the complete genome sequences of XJ01 again, as described, and found that the TAMV genomes from cell cultures were identical to those from the original sample.

To confirm the genome sequence of XJ01, we designed 14 paired primers for Sanger sequencing (Appendix Table, https://wwwnc.cdc.gov/EID/article/25/11/19-0512-App1.pdf). The consensus gene sequences of Sanger sequencing of the amplified products were consistent with those from transcriptome sequencing and were deposited in GenBank (accession nos. MK757580–2). Sequence comparison revealed that XJ01 was highly similar to 3 previously described TAMV strains from Asia; sequence identities were

[1]These authors contributed equally to this article.

94.8%–94.9% for the L segment, 93.5%–94.7% for the M segment, and 95.4%–96.8% for the S segment.

Phylogenetic analysis of representative strains of the family Nairoviridae using RAxML (*9*) revealed that the 4 TAMV strains clustered with high bootstrap support and fell within the *Orthonairovirus* genus in the RdRp tree (Figure, panel B). In addition, they were closely related to several other orthonairoviruses from ticks, including Burana virus,

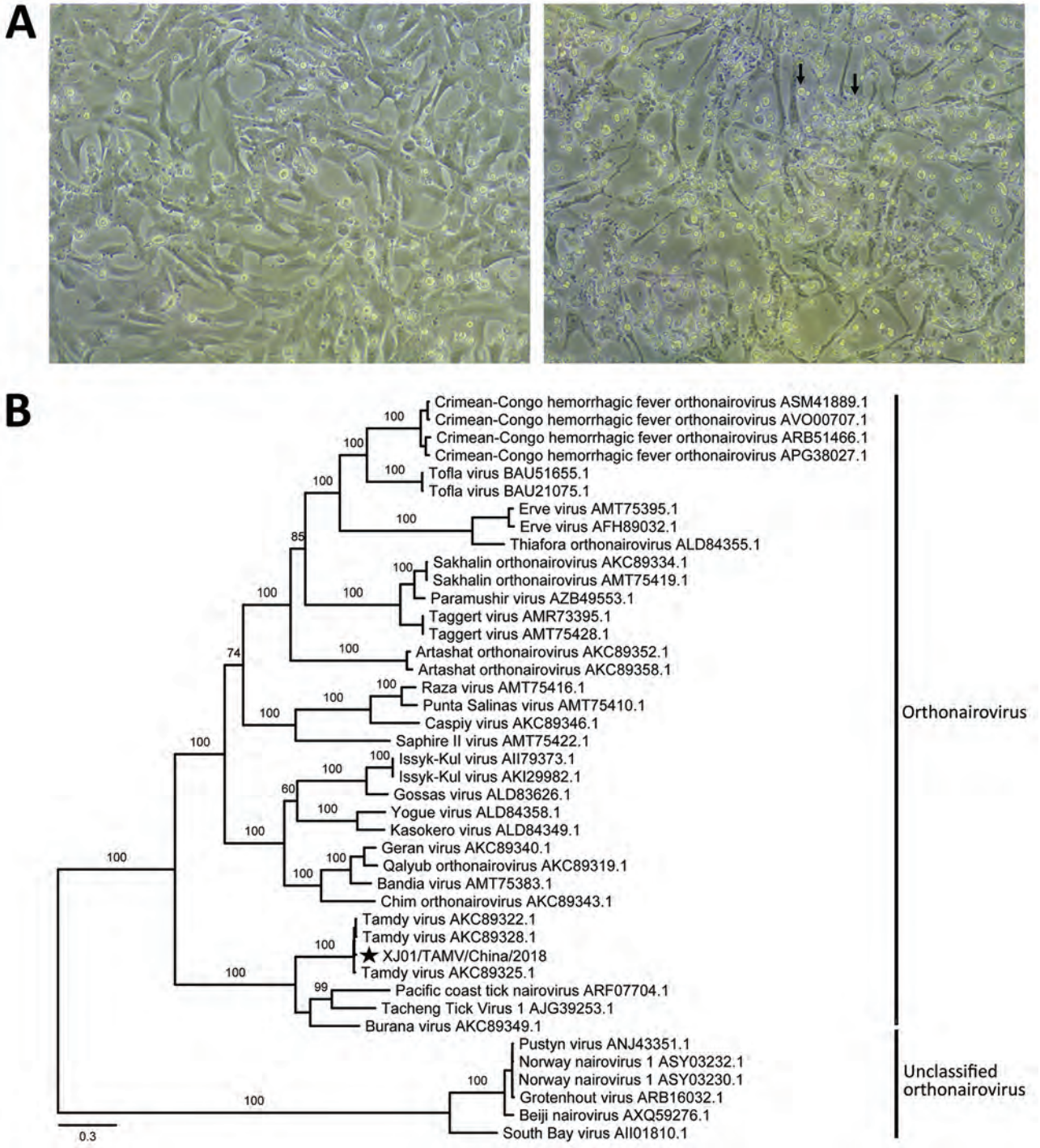

Figure. Identification of the Tamdy virus (TAMV) strain XJ01/TAMV/China/2018 from *Hyalomma asiaticum* ticks infesting Bactrian camels in Xinjiang, China, 2018, by cell culture and phylogenetic analysis. A) Light micrographs of cytopathic effects caused by TAMV infection at 11 days postinfection. Left, normal Vero cells as control; right, infected Vero cells with apparent cytopathic effects (black arrows). Original magnification ×100. B) Phylogenetic analysis of the RNA-dependent RNA polymerase protein sequences of TAMV and representative viruses in the family Nairoviridae. Scale bar indicates nucleotide substitutions per site. Star indicates strain from this study.

Tacheng tick virus 1, and Pacific coast tick nairovirus in all the RdRp, glycoprotein precursor, and nucleocapsid trees and formed a small cluster in the *Orthonairovirus* genus (Figure, panel B; Appendix Figures 1, 2).

We also obtained the cytochrome c oxidase gene sequence of the tick and deposited it in GenBank (accession no. MK757583). A BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) revealed that the top hit was from *H. asiaticum* (GenBank accession no. KX882103.1) with sequence identity of 99%; this species is a widely distributed tick in China, especially in northwestern China (*10*).

In summary, we identified a TAMV strain from Ixodid ticks collected in Xinjiang, China, that poses a threat to public health in Xinjiang and even globally. Because of the ability of TAMV to infect mammals including humans, the lack of effective antiviral drugs and prophylactic vaccines, and the widespread distribution of its major host in China, extensive TAMV surveillance is urgently needed.

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References

1. Lvov DK, Sidorova GA, Gromashevsky VL, Kurbanov M, Skvoztsova LM, Gofman YP, et al. Virus "Tamdy"—a new arbovirus, isolated in the Uzbee S.S.R. and Turkmen S.S.R. from ticks *Hyalomma asiaticum asiaticum* Schulee et Schlottke, 1929, and *Hyalomma plumbeum plumbeum* Panzer, 1796. Arch Virol. 1976;51:15–21. PubMed https://doi.org/10.1007/BF01317830
2. L'vov DK, Sidorova GA, Gromashevskiĭ VL, Skvortsova TM, Aristova VA. Isolation of Tamdy virus (*Bunyaviridae*) pathogenic for man from natural sources in Central Asia, Kazakhstan and Transcaucasia [in Russian]. Vopr Virusol. 1984;29:487–90.
3. Brinkmann A, Dinçer E, Polat C, Hekimoğlu O, Hacıoğlu S, Földes K, et al. A metagenomic survey identifies Tamdy orthonairovirus as well as divergent phlebo-, rhabdo-, chu- and flavi-like viruses in Anatolia, Turkey. Ticks Tick Borne Dis. 2018;9:1173–83. https://doi.org/10.1016/j.ttbdis.2018.04.017
4. L'vov DK, Al'khovskiĭ SV, Shchelkanov MI, Shchetinin AM, Aristova VA, Gitel'man AK, et al. Taxonomy of previously unclassified Tamdy virus (TAMV) (*Bunyaviridae, Nairovirus*) isolated from the *Hyalomma asiaticum asiaticum* Schülce et Schlottke, 1929 (*Ixodidae, Hyalomminae*) in the Middle East and transcaucasia [in Russian]. Vopr Virusol. 2014;59:15–22.
5. Karas FR, Vargina SG, Steblyanko SN, Kolpakov VN, Seropolko AA. Ecology of Tamdy virus in Kyrgystan. Proceedings of X Symposium. Ecology of viruses. Baku (Azerbaijan): Ministry of Health of Azerbaijan Republic of USSR; 1976. p. 87–88.
6. Chen S, Zhou Y, Chen Y, Gu J. fastp: an ultra-fast all-in-one FASTQ preprocessor. Bioinformatics. 2018;34:i884–90. https://doi.org/10.1093/bioinformatics/bty560
7. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat Biotechnol. 2011;29:644–52. https://doi.org/10.1038/nbt.1883
8. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods. 2012;9:357–9. PubMed https://doi.org/10.1038/nmeth.1923
9. Stamatakis A, Ludwig T, Meier H. RAxML-III: a fast program for maximum likelihood–based inference of large phylogenetic trees. Bioinformatics. 2005;21:456–63. PubMed https://doi.org/10.1093/bioinformatics/bti191
10. Sheng J, Jiang M, Yang M, Bo X, Zhao S, Zhang Y, et al. Tick distribution in border regions of northwestern China. Ticks Tick Borne Dis. 2019;10:665–9.

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Introduction of Avian Influenza A(H6N5) Virus into Asia from North America by Wild Birds

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An avian influenza A(H6N5) virus with all 8 segments of North American origin was isolated from wild bird feces in South Korea. Phylogenetic analysis suggests that this virus may have been introduced into Asia by wild birds, highlighting the role of wild birds in the dispersal of these viruses.

Avian influenza viruses (AIVs) have evolved into phylogenetically independent lineages as a result of separated distribution and migration of wild waterfowl, which are the natural reservoir of the viruses, because of natural geographic barriers. However, the ecologic separation of the migrating hosts seems incomplete, as some species of wild waterfowl (e.g., Northern pintails) migrate across the Bering Strait and serve as an intercontinental bridge for AIV between Eurasia and North America (*1*). Evidence for intercontinental exchange of AIV has been reported more frequently along continental margins where migratory flyways overlap, such as western Alaska (*2*); previous studies have described the exchange of gene segments or dispersal of complete virus between the 2 continents through migratory bird movements (*2*–*4*). We report detection of a fully North American AIV A(H6N5) subtype from South Korea during 2017.

During September 2017–March 2018, a total of 4,403 fresh fecal samples were collected in wild bird habitats of South Korea as part of an annual surveillance program for AIV. The sampling was focused on waterfowl at freshwater habitats such as rivers, streams, and reservoirs. We screened the samples for influenza A virus by real-time reverse transcription PCR (rRT-PCR) targeting the matrix gene and by embryonated chicken egg inoculation. A total of 131 samples tested positive for the matrix gene rRT-PCR; 58 AIV subtypes were isolated, including 1 H1N1, 6 H1N2, 2 H1N3, 1 H3N8, 4 H5N2, 3 H5N3, 40 highly pathogenic H5N6 (*5*), and 1 H6N5. We sequenced full-length genomes of the isolates using next-generation sequencing, as described previously (*5*). We deposited the nucleotide sequences of A/Mandarin duck/Korea/K17-1638-5/2017(H6N5) virus (hereafter 1638-5/2017 virus) in GenBank (accession nos. MK830100–7).

The Gimpo area (37°43′37″N, 126°39′54″E), from where the H6N5 virus was isolated, is one of the major wintering sites of such migratory birds as the greater white-fronted goose (*Anser albifrons*), bean goose (*Anser fabalis*), mallard (*Anas platyrhynchos*), and Eastern spot-billed duck (*Anas poecilorhyncha*) (Appendix 1 Figure 1, http://wwwnc.cdc.gov/EID/article/25/11/19-0604-App1.pdf). We identified the host of influenza A virus–positive feces using a DNA barcoding technique (*5*). We conducted comparative phylogenetic analysis of the 1638-5/2017 virus to trace its origin. For each segment, we aligned sequences with most of the closest full-length related sequences obtained from BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and used

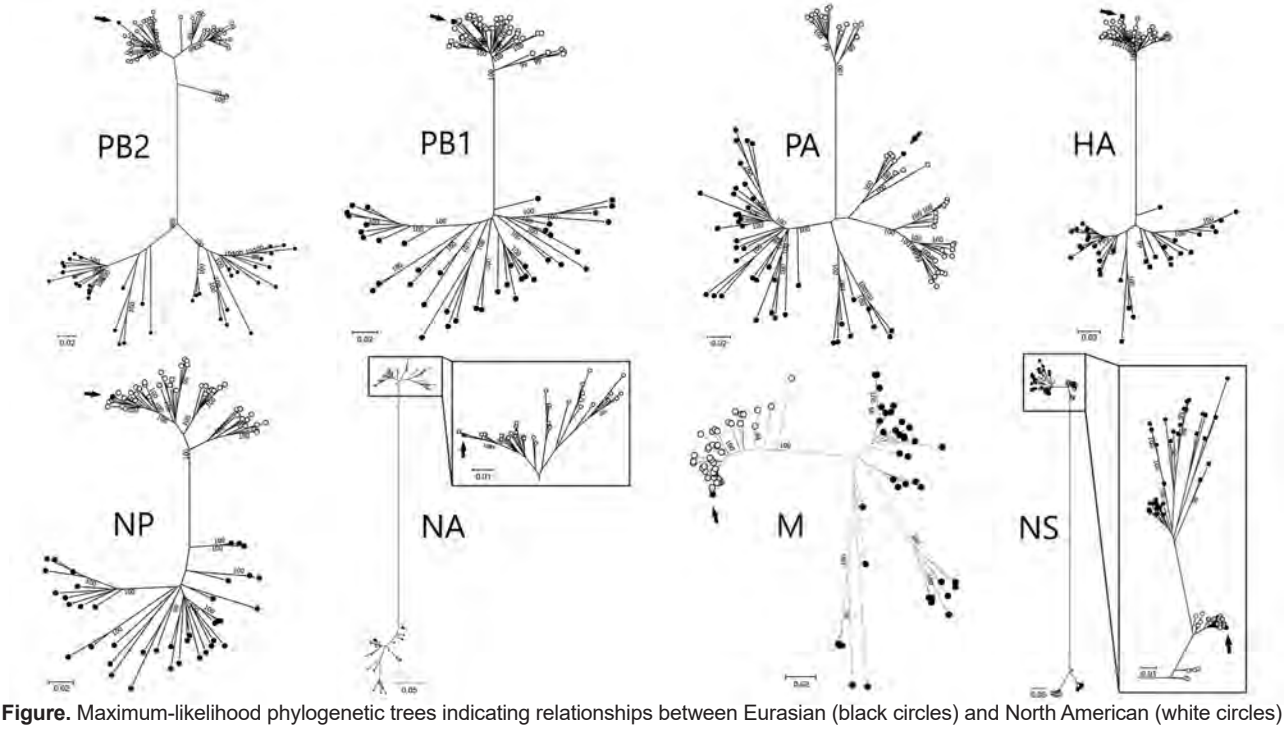

Figure. Maximum-likelihood phylogenetic trees indicating relationships between Eurasian (black circles) and North American (white circles) lineages of avian influenza A viruses. Black arrows indicate genome sequences of A/Mandarin duck/Korea/K17-1638–5/2017(H6N5) virus isolated in this study. Bootstrap values >99% are shown. Maximum-likelihood phylogenetic trees with complete strain names are shown in Appendix 1 Figure 2 (http://wwwnc.cdc.gov/EID/article/25/11/19-0604-App1.pdf). Scale bars indicate nucleotide substitutions per site. HA, hemagglutinin gene; M, matrix gene; NA, neuraminidase gene; NP, nucleoprotein gene; NS, nonstructural gene; PA, polymerase acidic gene; PB, polymerase basic gene.

these sequences for phylogenetic analysis. We constructed maximum-likelihood phylogenetic trees using the general time-reversible plus gamma substitution model with 1,000 bootstrap replications in RAxML version 8 (*6*).

Search results in the BLAST database indicated that all 8 viral gene segments of the 1638-5/2017 virus shared >98% nucleotide identity with North American wild bird AIV collected during 2015–2017 (Appendix 1 Table). Only genetic sequences of North American ancestry virus were in the sequencing data from 1638-5/2017 virus, suggesting the absence of contamination of viral genes from Eurasian-lineage virus during the sampling and virus isolation. Consistent with these findings, maximum-likelihood phylogenetic analysis indicated that all 8 gene segments clustered together with the sequences of AIV isolated from North American wild birds during 2016–2017, rather than those of Eurasian isolates (Figure; Appendix 1 Figure 2; Appendix 2, http://wwwnc.cdc.gov/EID/article/25/11/19-0604-App2.xlsx). These results strongly suggest the introduction of AIV of North American ancestry into Eurasia by wild birds.

The high nucleotide similarity of each gene segment with North American AIV identified during 2015–2017 suggests that the genome constellation of the 1638-5/2017 virus had been recently dispersed from North America to Eurasia. Mandarin ducks (*Aix galericulata*) are found mainly in the Far East and Southeast Asia, within the East Asian–Australasian flyway, and are not recognized as making regular movements between Eurasia and North America. The 1638-5/2017 virus might have been introduced from North America into Eurasia by wild birds migrating between Eurasia and North America and then been transmitted to Mandarin ducks.

Repeated reports of intercontinental transmission of AIV, particularly detections of fully Eurasian AIV, including H8N4 (*4*) and H9N2 (*7*); H16N3 (*8*) and highly pathogenic H5Nx (*3*) in North America; and the fully North American H6N5 virus in South Korea (this study) suggest that dispersal of AIV between Eurasia and North America is bidirectional and might not be exceedingly rare (*2*,*7*). Bahl et al. reported that the multiple introductions of Eurasian H6 AIV resulted in the establishment of viral sublineages in the North American AIV gene pool and changed the evolutionary dynamics of AIV in wild birds in North America (*9*). In addition, the North American N8 subtype gene has been established in wild bird populations migrating through the East Asian–Australasian flyway and was identified from a chicken farm and live bird market in South Korea (*10*). Enhanced influenza surveillance along migratory flyways and complete genome sequencing of identified viruses would be essential for better understanding of the intercontinental migrations of AIV and for early detection of the introduction of novel strains.

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References

1. Olsen B, Munster VJ, Wallensten A, Waldenström J, Osterhaus AD, Fouchier RA. Global patterns of influenza A virus in wild birds. Science. 2006;312:384–8. https://doi.org/10.1126/science.1122438
2. Ramey AM, Pearce JM, Flint PL, Ip HS, Derksen DV, Franson JC, et al. Intercontinental reassortment and genomic variation of low pathogenic avian influenza viruses isolated from northern pintails (*Anas acuta*) in Alaska: examining the evidence through space and time. Virology. 2010;401:179–89. https://doi.org/10.1016/j.virol.2010.02.006
3. Lee DH, Torchetti MK, Winker K, Ip HS, Song CS, Swayne DE. Intercontinental spread of Asian-origin H5N8 to North America through Beringia by migratory birds. J Virol. 2015;89:6521–4. https://doi.org/10.1128/JVI.00728-15
4. Ramey AM, Reeves AB, Donnelly T, Poulson RL, Stallknecht DE. Introduction of Eurasian-origin influenza A(H8N4) virus into North America by migratory birds. Emerg Infect Dis. 2018;24:1950–3. https://doi.org/10.3201/eid2410.180447
5. Kwon JH, Jeong S, Lee DH, Swayne DE, Kim YJ, Lee SH, et al. New reassortant clade 2.3.4.4b avian influenza A(H5N6) virus in wild birds, South Korea, 2017–18. Emerg Infect Dis. 2018;24:1953–5. https://doi.org/10.3201/eid2410.180561
6. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3. https://doi.org/10.1093/bioinformatics/btu033
7. Ramey AM, Reeves AB, Sonsthagen SA, TeSlaa JL, Nashold S, Donnelly T, et al. Dispersal of H9N2 influenza A viruses between East Asia and North America by wild birds. Virology. 2015;482:79–83. https://doi.org/10.1016/j.virol.2015.03.028
8. Huang Y, Wille M, Benkaroun J, Munro H, Bond AL, Fifield DA, et al. Perpetuation and reassortment of gull influenza A viruses in Atlantic North America. Virology. 2014;456-457:353–63. https://doi.org/10.1016/j.virol.2014.04.009
9. Bahl J, Vijaykrishna D, Holmes EC, Smith GJ, Guan Y. Gene flow and competitive exclusion of avian influenza A virus in natural reservoir hosts. Virology. 2009;390:289–97. https://doi.org/10.1016/j.virol.2009.05.002
10. Lee DH, Lee HJ, Lee YN, Park JK, Lim TH, Kim MS, et al. Evidence of intercontinental transfer of North American lineage avian influenza virus into Korea. Infect Genet Evol. 2011;11:232–6. https://doi.org/10.1016/j.meegid.2010.09.012

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Human Case of *Ehrlichia chaffeensis* Infection, Taiwan

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In 2018, an immunosuppressed woman in southern Taiwan had onset of fever, chills, myalgia, malaise, thrombocytopenia, lymphocytopenia, and elevated hepatic transaminases. Investigation revealed infection with *Ehrlichia chaffeensis*. This autochthonous case of human monocytotropic ehrlichiosis was confirmed by PCR, DNA sequencing, and seroconversion.

Human monocytic ehrlichiosis (HME) is an acute, febrile, tickborne disease caused by the bacterium *Ehrlichia chaffeensis*. HME was first reported in the United States in 1986 (*1*), and >1,000 ehrlichiosis cases have been reported annually since 2012 (https://www.cdc.gov/ehrlichiosis/stats/index.html). In Asia, however, only a limited number of HME cases have been reported in 3 countries (Thailand, South Korea, and China) (*2*–*4*).

Clinical manifestations of HME range from mild febrile illness to severe multiple organ failure. The most common symptoms of HME are fever, headache, myalgia, malaise, nausea, vomiting, diarrhea, and abdominal pain (*5*–*7*), which are difficult to differentiate from the symptoms of other febrile infectious diseases. Therefore, HME must be confirmed by laboratory diagnosis.

Although HME has not been documented in Taiwan, serologic evidence of *Ehrlichia* spp. has been detected in small mammals, such as *Rattus norvegicus*, *R. losea*, and *Bandicota indica* rats that are found around international and local harbors (*8*). In addition, *Haemaphysalis flava* ticks infected with *Ehrlichia* spp. have been collected from pale thrush birds (*Turdus pallidus*) and identified in Taiwan (*9*). We report an autochthonous human case of *E. chaffeensis* infection in Taiwan.

In mid-July 2018, a 66-year-old woman living in the Namaxia District of Kaohsiung City in southern Taiwan was admitted to Kaohsiung Chang Gung Memorial Hospital with a 5-day history of intermittent fever (39.8°C), chills, myalgias, malaise, mild dyspnea, and diffuse abdominal pain. The patient had underlying hypertension, type 2 diabetes mellitus, alcoholic fatty liver, and gastroesophageal reflux disease. Laboratory examinations at admission showed that the patient had thrombocytopenia; lymphocytopenia; elevated levels of C-reactive protein, aspartate aminotransferase, alanine aminotransferase, and creatinine; and an increased number of polymorphonuclear leukocytes (Table). Whole blood counts were within reference ranges, and no leukocytopenia was observed. A chest radiograph showed mild infiltration over the bilateral lower lung fields. Laboratory tests for dengue, influenza A and B, hepatitis A, hepatitis B, and hepatitis C viruses were all

Table. Laboratory and diagnostic findings for a human case of *Ehrlichia chaffeensis* infection, Taiwan*

Laboratory or diagnostic finding	Patient value or result	Reference value or method
Leukocytes	5,800/μL	3.9–10.6 × 10^3/μL
Red blood cell	4,950,000/μL	3.9–5.4 × 10^6/μL
Hemoglobin	14.3 g/dL	12–16 g/dL
Platelets	27,000/μL	150–400 × 10^3/μL
Segment	82.7%	42%–74%
Lymphocyte	13%	25%–56%
Creatinine	1.16 mg/dL	0.44–1.03 mg/dL
Aspartate aminotransferase	97 U/L	0–37 U/L
Alanine aminotransferase	71 U/L	0–40 U/L
C-reactive protein	131.2 mg/L	<5 mg/L
Dengue virus	Negative	Rapid test, ELISA, PCR
Influenza virus	Negative	Antigen
HAV/HBV/HCV	Negative	HAV IgM (ECLIA), HBV HBsAg (ECLIA), HCV Anti-HCV (ECLIA)
Leptospira interrogans	Negative	MAT or isolation
Coxiella burnetii	Negative	PCR or IFA
Orientia tsutsugamushi	Negative	PCR or IFA
Rickettsia typhi/R. prowazekii	Negative	PCR or IFA
R. rickettsii	Negative	IFA
R. conorii	Negative	IFA
Anaplasma phagocytophilum	Negative	PCR or IFA
Ehrlichia chaffeensis	Positive	PCR or IFA

*Diagnostic methods based on guidelines on standard operating procedure for laboratory diagnosis provided by Taiwan Centers for Disease Control (https://www.cdc.gov.tw). ECLIA: electrochemiluminescence immunoassay; HAV, hepatitis A virus, HBsAG, hepatitis B surface antigen; HBV, hepatitis B virus, HCV, hepatitis C virus; IFA, immunofluorescence assay; MAT, microscopic agglutination test.

negative. She was admitted under the impression of atypical infection and thrombocytopenia.

The patient is a coffee farmer who lives in a rural region in Kaohsiung. Although she claimed not to have received arthropod or animal bites, small mammals and birds had often been seen around her workplace and house. Therefore, arthropodborne rickettsial diseases were suspected, and oral doxycycline (100 mg every 12 h) for 4 days and intravenous ceftriaxone (1 g every 12 h) for 7 days were prescribed as empirical therapy on the patient's first day at the hospital. Because ehrlichial infection had not been confirmed during hospitalization, the patient was discharged with a prescription (500 mg cefadroxil monohydrate every 12 h) to be taken for 5 days because of suspicion of atypical bacterial infection.

Blood specimens collected from the patient on day 6 (acute-phase specimens) and day 20 (convalescent-phase specimens) after illness onset were sent to the Taiwan Centers for Disease Control (Taipei, Taiwan) for laboratory diagnosis of zoonotic diseases. DNA extracted from acute-phase blood specimens using the QIAamp DNA blood Mini Kit (QIAGEN GmbH, https://www.qiagen.com) was used to detect *Ehrlichia chaffeensis* infection using a primer set targeting ehrlichial 16S rRNA gene (forward primer: AGCGGCTATCTGGTTCGA; reverse primer: CATGCTCCACCGCTTGTG) and an *E. chaffeensis*–specific primer set targeting the nitrogen assimilation regulatory protein (*ntrX*) gene (forward primer: TGCCGGTAGATATAGTATCGA; reverse primer: ATTTGCGATGAAGTGCGG) by QuantiNova SYBR green real-time PCR (QIAGEN). The PCR products of 16S rRNA (182 bp; GenBank accession no. MN088851) and the *ntrX* gene sequence (153 bp; GenBank accession no. MN096569) were determined and analyzed. The sequence was 100% homologous with the sequences of *E. chaffeensis* strains, including the Arkansas, Jax, Saint Vincent, West Paces, Wakulla, Osceola, Liberty, and Heartland strains. The PCR results were negative for *Coxiella burnetii*, *Orientia tsutsugamushi*, typhus group rickettsiae, spotted fever group rickettsiae, and *Anaplasma phagocytophilum* (Appendix Table, https://wwwnc.cdc.gov/EID/article/25/11/19-0665-App1.pdf). Paired (acute- and convalescent-phase) serum samples were used to detect antibodies against *E. chaffeensis* by using indirect immunofluorescence assay according to the manufacturer's recommendation (Focus Diagnostics, https://www.focusdx.com). IgG against *E. chaffeensis* showed seroconversion (titers ranging from <1:16 to 1:256) of the paired serum samples. IgG against *Coxiella burnetii*, *Orientia tsutsugamushi*, typhus group rickettsiae, spotted fever group rickettsiae, and *Anaplasma phagocytophilum* were all negative. The results of the microscopic agglutination test and the isolation of *Leptospira* spp. were also negative.

The presence of an HME case highlights the need for further studies of the prevalence, geographic distribution, and control of this disease in Taiwan. Human monocytic ehrlichiosis patients with immunosuppressive conditions, such as diabetes, might have a higher risk for hospitalization and life-threatening complications (*10*). In this case, the suspicion of rickettsial infection was based on the patient's potential exposure to arthropodborne pathogens at her workplace and home, and the patient responded quickly to doxycycline treatment. Physician awareness of HME and early diagnosis and treatment are essential to improve disease outcomes.

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References

1. Dawson JE, Anderson BE, Fishbein DB, Sanchez JL, Goldsmith CS, Wilson KH, et al. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. J Clin Microbiol. 1991;29:2741–5.
2. Heppner DG, Wongsrichanalai C, Walsh DS, McDaniel P, Eamsila C, Hanson B, et al. Human ehrlichiosis in Thailand. Lancet. 1997;350:785–6. https://doi.org/10.1016/S0140-6736(05)62571-8
3. Heo EJ, Park JH, Koo JR, Park MS, Park MY, Dumler JS, et al. Serologic and molecular detection of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila* (human granulocytic ehrlichiosis agent) in Korean patients. J Clin Microbiol. 2002;40:3082–5. https://doi.org/10.1128/JCM.40.8.3082-3085.2002
4. Zhang L, Shan A, Mathew B, Yin J, Fu X, Zhang J, et al. Rickettsial seroepidemiology among farm workers, Tianjin, People's Republic of China. Emerg Infect Dis. 2008;14:938–40. https://doi.org/10.3201/eid1406.071502
5. Paddock CD, Childs JE. *Ehrlichia chaffeensis*: a prototypical emerging pathogen. Clin Microbiol Rev. 2003;16:37–64. https://doi.org/10.1128/CMR.16.1.37-64.2003
6. Stone JH, Dierberg K, Aram G, Dumler JS. Human monocytic ehrlichiosis. JAMA. 2004;292:2263–70. https://doi.org/10.1001/jama.292.18.2263
7. Biggs HM, Behravesh CB, Bradley KK, Dahlgren FS, Drexler NA, Dumler JS, et al. Diagnosis and management of tickborne rickettsial diseases: Rocky Mountain spotted fever and other spotted fever group rickettsioses, ehrlichioses, and anaplasmosis—United States. MMWR Recomm Rep. 2016;65:1–44. https://doi.org/10.15585/mmwr.rr6502a1
8. Tsai KH, Chang SF, Yen TY, Shih WL, Chen WJ, Wang HC, et al. Prevalence of antibodies against *Ehrlichia* spp. and *Orientia tsutsugamushi* in small mammals around harbors in Taiwan. Parasit Vectors. 2016;9:45. https://doi.org/10.1186/s13071-016-1318-7

9. Kuo CC, Lin YF, Yao CT, Shih HC, Chung LH, Liao HC, et al. Tick-borne pathogens in ticks collected from birds in Taiwan. Parasit Vectors. 2017;10:587. https://doi.org/10.1186/s13071-017-2535-4
10. Nichols Heitman K, Dahlgren FS, Drexler NA, Massung RF, Behravesh CB. Increasing incidence of ehrlichiosis in the United States: a summary of national surveillance of *Ehrlichia chaffeensis* and *Ehrlichia ewingii* infections in the United States, 2008–2012. Am J Trop Med Hyg. 2016;94:52–60. https://doi.org/10.4269/ajtmh.15-0540

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Psittacosis Outbreak among Workers at Chicken Slaughter Plants, Virginia and Georgia, USA, 2018

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During August–October, 2018, an outbreak of severe respiratory illness was reported among poultry slaughter plant workers in Virginia and Georgia, USA. A multiorganizational team investigated the cause and extent of illness, determined that the illness was psittacosis, and evaluated and recommended controls for health hazards in the workplace to prevent additional cases.

Psittacosis results from inhalation of aerosolized droppings or respiratory secretions of birds infected with *Chlamydia psittaci*. During 2008–2017, a total of 60 cases of psittacosis, a nationally notifiable disease in the United States, were reported to the National Notifiable Diseases Surveillance System (https://wwwn.cdc.gov/nndss).

The most common source of psittacosis in the United States is believed to be pet psittacine birds (e.g., parrots, cockatoos). The most recent large poultry-associated outbreaks in the United States were reported 3 decades ago and were linked to turkeys (*1*,*2*). *C. psittaci* prevalence in poultry in the United States is unknown, although it has been recently identified in turkeys in the United States (*3*) and turkeys and chickens overseas (*4*,*5*). Poultry can be infected but show no overt signs of illness (*6*).

During August 31–September 4, 2018, the Virginia Department of Health (VDH) received reports of 10 persons, all workers at the same chicken slaughter plant, hospitalized with fever, headache, cough, and radiographic evidence of pneumonia. Lower respiratory tract specimens (2 bronchoalveolar lavage and 1 sputum) from 3 hospitalized workers were positive for *C. psittaci* by real-time PCR targeting the *C. psittaci* locus tag CPSIT_RS01985 (*7*), performed at the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). The Virginia plant suspended operations on September 8.

On September 12, the Georgia Department of Public Health (GDPH) was notified that 3 employees of a Georgia chicken slaughter plant owned by the same company were hospitalized with pneumonia. *C. psittaci* was detected in sputum samples from all 3 patients. The Georgia plant suspended operations on September 15.

After plant closures, VDH and GDPH staff inspected the respective plants, which both slaughter only chickens, and collected environmental samples to test for *C. psittaci*. Staff collected samples from areas where workers were close to or directly handled live chickens or carcasses. Environmental samples were tested for chlamydial species by using real-time PCR, followed by high-resolution melt analysis (*8*), at the University of Georgia Infectious Disease Laboratory (Athens, GA, USA).

The company held employee meetings in each state and invited VDH and GDPH representatives to provide outbreak information and conduct active case finding. VDH and GDPH initiated investigations of cases and potential risk factors. A case was defined as illness in a worker employed during August 1–September 7, 2018, at the

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Virginia plant, or during August 13–September 28, 2018, at the Georgia plant, who had either physician-diagnosed pneumonia, or fever or chills with ≥2 symptoms of headache, cough, or muscle aches. A confirmed case required PCR detection of *C. psittaci* in a clinical specimen.

At the Virginia plant, 50 cases (including 5 confirmed) were identified; 30 cases (including 8 confirmed) were identified at the Georgia plant. PCR cycle threshold values for the 13 confirmed cases ranged from 26 to 37. Using sequencing of the outer membrane protein A (*ompA*) gene, we identified genotype D of *C. psittaci* in patient specimens; this genotype is most often found in poultry (*4*,*5*). Cases occurred during August 3–September 8 in Virginia and August 17–October 22 in Georgia.

We provide detailed characteristics for all patients (ill workers) (Table). A total of 58% of patients were men (age range 19–58 years). Bird evisceration was the most common job duty or title (reported by 53% of ill workers), consistent with previous psittacosis outbreaks (*1*,*2*,*9*,*10*). Twenty-nine workers were hospitalized (3 in intensive care) and had stays from 1 to 37 days. No deaths were reported.

C. psittaci was not detected in any of the environmental samples from the Virginia (n = 62) and Georgia (n = 46) plants. After extensive cleaning with sanitizers, including quaternary ammonia, chlorine solutions, and chlorine dioxide foam (all registered by the US Environmental Protection Agency as effective against *C. psittaci*), the Virginia and Georgia plants reopened on September 18 and 19, respectively. Georgia cases that occurred after the plant reopened were attributed to longer incubation periods. The incubation period for psittacosis is typically 1–4 weeks (*6*), but illness onset >30 days after exposure was reported in the 2 most recent poultry-associated outbreaks in the United States (*1*,*2*).

At the request of the US Department of Agriculture Food Safety and Inspection Service, the National Institute for Occupational Safety and Health conducted a health hazard evaluation of the Virginia plant on September 19–20. Recommendations to the plant included repositioning cooling fans, ensuring evisceration tools were working properly, and changes to other work practices to reduce bacterial contamination and aerosolization. A health hazard evaluation was not requested at the Georgia plant, but company management reported implementing or evaluating options to implement all applicable recommendations at the plants.

Clinicians evaluating poultry slaughter plant workers with febrile respiratory illness should consider psittacosis as a possible diagnosis. In the absence of a more likely diagnosis, clinicians should contact state health authorities to discuss whether *C. psittaci* testing should be requested through CDC, which has the only laboratory in the United States in which PCR testing for human specimens is currently available.

About the Authors

Dr. Shaw is a CDC Epidemic Intelligence Service officer at the Virginia Department of Health, Richmond, VA. Her primary research interest is applying data to improve public health knowledge and practice.

Table. Characteristics of chicken slaughter plant workers tested during outbreak of psittacosis, Virginia and Georgia, United States, 2018

Characteristic	No. (%) workers		
	Confirmed, n = 13	Other, n = 67	All, n = 80
Sex			
M	10 (77)	36 (54)	46 (58)
F	3 (23)	31 (46)	34 (42)
Symptoms			
Fever	13 (100)	57 (85)	70 (88)
Cough	11 (85)	52 (78)	63 (79)
Muscle aches	10 (77)	47 (70)	57 (71)
Headache	9 (69)	59 (88)	68 (85)
Chills	9 (69)	51 (76)	60 (75)
Gastrointestinal*	6 (75)	13 (59)	19 (63)
Clinical			
Radiologically confirmed pneumonia	13 (100)	30 (45)	43 (54)
Hospitalized	11 (85)	18 (27)	29 (36)
Intensive care	3 (23)	0	3 (4)
Job duties†			
Evisceration	8 (62)	34 (51)	42 (53)
Live-bird handling	3 (23)	6 (9)	9 (11)
Cleaning	2 (15)	5 (7)	7 (9)
Packing or shipping	1 (8)	13 (19)	14 (18)
Inspection or quality assurance	1 (8)	6 (9)	7 (9)
Other‡	0	5 (7)	5 (6)

*Data for this symptom were not collected in Virginia; numbers reflect only Georgia numerators and denominators (confirmed, n = 8, other, n = 22; total, n = 30).
†In Virginia, 1 patient did not report job duty; in Georgia, 2 patients with confirmed illness and 3 other patients reported multiple job duties.
‡Includes maintenance and human resources (plant floor–based).

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References

1. Centers for Disease Control. Psittacosis at a turkey processing plant—North Carolina, 1989. MMWR Morb Mortal Wkly Rep. 1990;39:460–1, 467–9.
2. Hedberg K, White KE, Forfang JC, Korlath JA, Friendshuh KA, Hedberg CW, et al. An outbreak of psittacosis in Minnesota turkey industry workers: implications for modes of transmission and control. Am J Epidemiol. 1989;130:569–77. https://doi.org/10.1093/oxfordjournals.aje.a115371
3. Shivaprasad HL, Carnaccini S, Bland M, Aaziz R, Moeller R, Laroucau K. An unusual outbreak of chlamydiosis in commercial turkeys involving the nasal glands. Avian Dis. 2015;59:315–22. https://doi.org/10.1637/11006-123014-Reg
4. Dickx V, Geens T, Deschuyffeleer T, Tyberghien L, Harkinezhad T, Beeckman DS, et al. *Chlamydophila psittaci* zoonotic risk assessment in a chicken and turkey slaughterhouse. J Clin Microbiol. 2010;48:3244–50. https://doi.org/10.1128/JCM.00698-10
5. Lagae S, Kalmar I, Laroucau K, Vorimore F, Vanrompay D. Emerging *Chlamydia psittaci* infections in chickens and examination of transmission to humans. J Med Microbiol. 2014;63:399–407. https://doi.org/10.1099/jmm.0.064675-0
6. Heymann D, editor. Control of communicable diseases manual, 20th ed. Washington: American Public Health Association; 2015.
7. Wolff BJ, Morrison SS, Winchell JM. Development of a multiplex TaqMan real-time PCR assay for the detection of *Chlamydia psittaci* and *Chlamydia pneumoniae* in human clinical specimens. Diagn Microbiol Infect Dis. 2018;90:167–70. https://doi.org/10.1016/j.diagmicrobio.2017.11.014
8. Mitchell SL, Wolff BJ, Thacker WL, Ciembor PG, Gregory CR, Everett KD, et al. Genotyping of *Chlamydophila psittaci* by real-time PCR and high-resolution melt analysis. J Clin Microbiol. 2009;47:175–81. https://doi.org/10.1128/JCM.01851-08
9. Rindge ME, Jungherr EL, Scruggs JH. Serologic evidence of occupational psittacosis in poultry-plant workers. N Engl J Med. 1959;260:1214–8. https://doi.org/10.1056/NEJM195906112602404
10. Laroucau K, Aaziz R, Meurice L, Servas V, Chossat I, Royer H, et al. Outbreak of psittacosis in a group of women exposed to *Chlamydia psittaci*–infected chickens. Euro Surveill. 2015;20:21155. https://doi.org/10.2807/1560-7917.ES2015.20.24.21155

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Effectiveness of Immune Checkpoint Inhibitors in Transplant Recipients with Progressive Multifocal Leukoencephalopathy

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Antibodies against PD1 have been used to treat progressive multifocal leukoencephalopathy (PML), a rare brain disease caused by JC virus. We used these antibodies (nivolumab) to treat PML in 3 kidney transplant recipients. All died within 8 weeks of diagnosis. Hence, nivolumab did not improve PML outcome after solid organ transplantation.

The role of T-cell exhaustion in the development of progressive multifocal leukoencephalopathy (PML), a rare brain disease caused by JC virus, has prompted clinicians to use immune checkpoint inhibitor molecules to treat JC virus–infected patients. Recently, Cortese et al. (*1*) used antibodies against PD1 to treat PML in 8 patients (6 with a history of blood disorders and 2 with HIV infection). They noted improvement or stabilization of symptoms for 5 patients but no benefit for the others.

Since 2017, we have treated PML in 3 kidney transplant recipients with a definitive diagnosis, according to the American Academy of Neurology (https://www.aan.com) consensus, made 5 (range 2–17) years after transplantation. We have compiled clinical and radiologic findings for these patients (Appendix Figures 1–3, https://wwwnc.cdc.gov/EID/article/25/11/19-0705-App1.pdf). Since transplantation, the patients had been receiving mycophenolic acid and steroids with either belatacept (n = 1) or tacrolimus (n = 2). At PML diagnosis, immunosuppressants were immediately withdrawn, and nivolumab (antibodies against PD1) was given at a dose of 3 mg/kg every 15 days (2 injections for 2 patients and 3 injections for 1) (Table). For the patient who had received belatacept, we performed 3 apheresis sessions to remove the drug before nivolumab initiation. All patients died within the first 8 weeks after PML diagnosis because of rapid progression of neurologic symptoms.

Table. Characteristics of 3 patients with PML who received nivolumab, France, 2017*

Patient characteristics	Total lymphocytes; CD4+; CD8+, n/mm^3	Clinical course	Additional therapy	JCV in CSF, log_{10} copies/mL	Loss of kidney function
Patient 1: age 81 y; received transplant 5 y before PML diagnosis; received treatment with Tac, MPA, prednisone	B: 300; 76; 56/LFU: 1,000; 602; 250†	Rapid progression of neurologic disorders despite 2 injections of nivolumab; death from progression of PML 6 wk after diagnosis	Mirtazapine 15 mg/d	B: 3.5/LFU: NA	No
Patient 2: age 77 y; received transplant 2 y before PML diagnosis; received treatment with belatacept, MPA, and prednisone	B: 377; 162; 106/LFU: 444; 117; 210‡	Rapid progression of neurologic disorders despite 3 injections of nivolumab; death from progression of PML 6 wk after diagnosis	Mirtazapine 15 mg/d; γ interferon therapy (100 μg) added 1 day after second and third injections	B: 2.9/LFU: 5	Yes
Patient 3: age 67 y; received transplant 17 y before PML diagnosis; received treatment with Tac, MPA, prednisone	B: 487; 287; 67/LFU: 2,076; 1,183; 477§	Rapid neurologic degradation despite 2 injections of nivolumab; death from progression of PML 4 wk after diagnosis	Mirtazapine 15 mg/d	B: 2.9/LFU: NA	No

*B, baseline; CSF, cerebrospinal fluid; JCV, JC virus; LFU, last follow-up; MPA, mycophenolic acid; NA, not available, PML, progressive multifocal leukoencephalopathy; Tac, tacrolimus.
†LFU for patient 1 was 1 wk after the second injection of nivolumab.
‡LFU for patient 2 was 4 d after the third injection of nivolumab.
§LFU for patient 3 was 1 wk after the second injection of nivolumab.

Magnetic resonance imaging was performed before each injection and a few days before death, but images showed no signs of immune reconstitution inflammatory syndrome. Conversely, images did show progression of PML features. As expected, the percentage of T cells expressing PD1, which was assessed for 2 patients, dramatically decreased after receipt of nivolumab (Appendix Figure 4), whereas other inhibitory receptors tested (2b4 and CD160) remained stable or increased. In addition, functional analysis showed a reduction of cytokine production by CD4+ and CD8+ T cells and an improvement of cytotoxic ability, a phenotype compatible with more terminally differentiated exhausted cells, which are less likely to respond to anti-PD1 immune checkpoint inhibitor (*2*).

Research has suggested that PML could occur at any time after transplantation (*3*), even several years after engraftment, which was the case for the 3 patients reported here. As opposed to the results reported by Cortese et al. (*1*), the outcomes for the 3 patients we report, who received nivolumab, was very bad and in line with the PML outcomes usually reported after solid-organ transplant patients (i.e., median survival time <6 months) (*3*). The difference between the patients reported by Cortese et al. and the patients that we report is probably use of immunosuppressive agents (calcineurin inhibitors or costimulation blockers) that can lead to persistent T-cell dysfunction, despite withdrawal of these treatments, resulting in refractory T-cell dysfunction after use of anti-PD1 blockers, as reported in ex vivo experiments (*4*). This hypothesis is supported by the absence of kidney rejection in 2 of the 3 patients. Of note, all 5 patients reported by Cortese et al. (*1*) for whom anti-PD1 blockers were efficient were not receiving immunosuppressive therapy at PML diagnosis.

Moreover, the 3 patients reported here had profound lymphopenia at diagnosis, which for 2 patients did not improve after receipt of nivolumab (Table). Although there is no established relationship between the severity of lymphopenia and the response to anti-PD1, the 3 patients with unfavorable outcomes reported by Cortese et al. (*1*) also had severe lymphopenia. This finding suggests that immunotherapies can be ineffective in patients with severe lymphopenia. The use of ex vivo expanded, BK virus–specific T cells (*5*) should be tested in this setting. For the kidney transplant patients with PML reported here, use of nivolumab, associated with immunosuppressive therapy withdrawal, did not restore efficient immune response and did not improve the outcomes.

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References

1. Cortese I, Muranski P, Enose-Akahata Y, Ha SK, Smith B, Monaco M, et al. Pembrolizumab treatment for progressive multifocal leukoencephalopathy. N Engl J Med. 2019;380:1597–605. https://doi.org/10.1056/NEJMoa1815039
2. Blackburn SD, Shin H, Freeman GJ, Wherry EJ. Selective expansion of a subset of exhausted CD8 T cells by alphaPD-L1 blockade. Proc Natl Acad Sci U S A. 2008;105:15016–21. https://doi.org/10.1073/pnas.0801497105
3. Mateen FJ, Muralidharan R, Carone M, van de Beek D, Harrison DM, Aksamit AJ, et al. Progressive multifocal leukoencephalopathy in transplant recipients. Ann Neurol. 2011; 70:305–22. https://doi.org/10.1002/ana.22408
4. Dekeyser M, de Goër de Herve MG, Hendel-Chavez H, Labeyrie C, Adams D, Nasser GA, et al. Refractory T-Cell anergy and rapidly fatal progressive multifocal leukoencephalopathy after

prolonged CTLA4 therapy. Open Forum Infect Dis. 2017;4:ofx100. https://doi.org/10.1093/ofid/ofx100
5. Muftuoglu M, Olson A, Marin D, Ahmed S, Mulanovich V, Tummala S, et al. Allogeneic BK virus-specific t cells for progressive multifocal leukoencephalopathy. N Engl J Med. 2018;379:1443–51. https://doi.org/10.1056/NEJMoa1801540

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Endemicity of Yaws and Seroprevalence of *Treponema pallidum* Antibodies in Nonhuman Primates, Kenya

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Human yaws has historically been endemic to Kenya, but current epidemiologic data are lacking. We report seroprevalence for *Treponema pallidum* antibodies in olive baboons (*Papio anubis*) and vervet monkeys (*Chlorocebus pygerythrus*) in Laikipia County, Kenya. Our results suggest endemicity of the yaws bacterium in monkeys, posing a possible zoonotic threat to humans.

Yaws is a disease caused by the bacterium *Treponema pallidum* subsp. *pertenue*, which is believed to be an exclusively human pathogen (*1*). However, this bacterium has recently been identified in African nonhuman primates (NHPs) (*2*), raising concerns about a possible zoonotic reservoir for human infection. Kenya is 1 of 76 countries that the World Health Organization categorizes as previously endemic for yaws, but no current data support its presence or absence (http://apps.who.int/gho/data/node.main.NTDYAWSEND). However, sustainable yaws eradication will rely on information about transmission dynamics and potential links between human and NHP *T. pallidum* strains (*3*).

In the early 1960s, Fribourg-Blanc and Mollaret tested 150 serum samples from wild-caught baboons (*Papio* sp.) from Guinea and Kenya (*4*). Although 72 (65%) of 111 serum samples from Guinea were positive for *T. pallidum* antibodies, none of the samples from Kenya were positive. In subsequent years, an additional 276 serum samples from baboons in Kenya supported the absence of *T. pallidum* infection. However, a more recent study of baboon samples collected during 1977–1994 in Kenya reported serologic evidence of *T. pallidum* infection in Nanyuki, Laikipia County (prevalence 57.5%) (*5*). For our study, we hypothesized that 39 years after the first samples were positive for antibodies against *T. pallidum* in Nanyuki (*5*), infection is still present in the NHP population.

All animal protocols were approved by the Kenya Wildlife Service (permit #4004), the Institute of Primate Research Scientific and Ethics Review Committee, and the Smithsonian Institution Animal Use and Care Committee. In October 2016, we sampled 65 olive baboons (*Papio anubis*) and 2 vervet monkeys (*Chlorocebus pygerythrus*) at sites surrounding the Mpala Research Centre in Laikipia County, Kenya. We performed a preliminary serologic screening by using the immunochromatographic Dual Path Platform (DPP) HIV-Syphilis Assay (Chembio Diagnostic Systems, Inc., http://chembio.com) according to the manufacturer guidelines. This syphilis (*T. pallidum*) assay is a useful screening tool because antibodies against *Treponema* subspecies are cross-reactive (*6*). We tested 67 samples with the DPP assay; 49 were positive and 18 negative.

However, because this test is not certified for use with NHPs, we subsequently confirmed results by using the *T. pallidum* Particle Agglutination Assay (TPPA) (SERODIA TPPA, https://www.fujirebio-us.com), which has been validated for use in baboons (*7*). Of the 52 samples tested with the TPPA assay, there were 33 positive, 6 negative, and 13 inconclusive results. Inconclusive TPPA results indicate nonspecific antibodies reacting with nonsensitized particles. Because of limited sample material, we were unable to perform repeated testing with a preabsorption step to remove all nonspecific binding antibodies (as described in the assay manual) and therefore excluded the inconclusive TPPA results from our analysis.

If we defined seropositive monkeys as those with positive results for the TPPA or DPP, 1 of 2 vervet monkeys and 53 (85.5%) of 62 baboons were seropositive. Male baboons (90.4%, 38/42) had a relative seropositivity risk ratio of 1.3 (95% CI 0.984–1.858) when compared with female baboons (72.2%, 13/18); however, this difference was not significant (p = 0.111 by Fisher exact test). If we included age, in addition to sex, in the analysis, adult male and female baboons both showed 100% seropositivity (21/21 and 10/10, respectively). Subadult males and females also showed seropositivity of 100% (6/6 and 1/1, respectively). Juveniles had a combined seropositivity of 61.1%: a total of 81.8% (9/11) of males and 28.6% (2/7) of females were seropositive. Infants had the lowest seroprevalence rate (50%, 2/4) (Table).

None of the tested NHPs had overt clinical signs of infection, such as skin lesions, which might have contained *T. pallidum* DNA. However, several other studies found that NHPs are frequently seropositive for *T. pallidum* antibodies without clinical lesions (*5*,*8*,*9*). Because wild NHPs are not treated and bacterial clearance is unlikely, the absence of lesions presumably corresponds to the latency stage of infection, which is also a key characteristic of human treponematoses (*10*). Future molecular investigations should include nontreponemal tests to further support the assumption that animals are in the latency stage and should target the DNA of the pathogen, which would enable comparison of *T. pallidum* strains of NHP origin from Kenya with those infecting NHPs in neighboring countries and possibly humans. In Tanzania, a country that has a similar history of previous yaws endemicity in humans and lacks current prevalence data, clinical lesions have been documented in olive baboons, vervet monkeys, yellow baboons, and blue monkeys, in addition to widespread seroprevalence in NHPs closely matching previous human infection geographic distribution (*9*).

Our results suggest that evidence of *Treponema* exposure in NHPs continues to be present in Laikipia County almost 4 decades after it was first detected. Our data provide further evidence that, in East Africa, *T. pallidum* infection is endemic to NHPs and that multiple NHP taxa contain antibodies indicating latent infection. Providing reliable information on the epidemiology of treponematoses in humans and NHPs has major programmatic implications for yaws eradication. Under a One Health approach, we call for additional yaws surveillance in communities in Kenya, especially in regions where NHPs and humans coexist.

Table. Demographic data and serologic results for nonhuman primates sampled for *Treponema pallidum* antibodies, Laikipia County, Kenya, October 2016*

Species, age group†	No. positive/no. tested (%) Male	Female
Olive baboon (*Papio anubis*)		
Adult	21/21 (100)	10/10 (100)
Subadult	6/6 (100)	1/1 (100)
Juvenile	9/11 (82)	2/7 (29)
Infant	2/4 (50)	ND
Subtotal	38/42 (90)	13/18 (72)
Vervet monkey (*Chlorocebus pygerythrus*)		
Adult	0/1 (0)	ND
Juvenile	1/1 (100)	ND
Subtotal	1/2 (50)	ND
Total	39/44 (89)	13/18 (72)

*Samples were tested by using the Dual Path Platform Assay or the *Treponema pallidum* Particle Agglutination Assay. ND, not done.
†Age ranges for *P. anubis* baboons, infant, <1.3 y; male juvenile, 1.3–6 y; female juvenile, 1.3–5 y; male subadult, 6–9 y; female subadult 5–6 y; male adult, >10 y; female adult, >6 y (Appendix reference *1*, https://wwwnc.cdc.gov/EID/article/25/11/19-0716-App1.pdf). Age ranges for *C. pygerythrus* monkeys: juvenile, 22–40 mo; adult, ≥40 months (Appendix reference *2*).

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References

1. Marks M, Lebari D, Solomon AW, Higgins SP. Yaws. Int J STD AIDS. 2015;26:696–703. https://doi.org/10.1177/0956462414549036
2. Knauf S, Gogarten JF, Schuenemann VJ, De Nys HM, Düx A, Strouhal M, et al. Nonhuman primates across sub-Saharan Africa are infected with the yaws bacterium *Treponema pallidum* subsp. *pertenue.* Emerg Microbes Infect. 2018;7:157. https://doi.org/10.1038/s41426-018-0156-4
3. Bodimeade C, Marks M, Mabey D. Neglected tropical diseases: elimination and eradication. Clin Med (Lond). 2019;19:157–60. https://doi.org/10.7861/clinmedicine.19-2-157
4. Fribourg-Blanc A, Mollaret HH. Natural treponematosis of the African primate. Primates Med. 1969;3:113–21.
5. Harper KN, Fyumagwa RD, Hoare R, Wambura PN, Coppenhaver DH, Sapolsky RM, et al. *Treponema pallidum* infection in the wild baboons of East Africa: distribution and genetic characterization of the strains responsible. PLoS One. 2012;7:e50882. https://doi.org/10.1371/journal.pone.0050882
6. Chi KH, Danavall D, Taleo F, Pillay A, Ye T, Nachamkin E, et al. Molecular differentiation of *Treponema pallidum* subspecies in skin ulceration clinically suspected as yaws in Vanuatu using real-time multiplex PCR and serological methods. Am J Trop Med Hyg. 2015;92:134–8. https://doi.org/10.4269/ajtmh.14-0459

7. Knauf S, Dahlmann F, Batamuzi EK, Frischmann S, Liu H. Validation of serological tests for the detection of antibodies against *Treponema pallidum* in nonhuman primates. PLoS Negl Trop Dis. 2015;9:e0003637. https://doi.org/10.1371/journal.pntd.0003637
8. Knauf S, Batamuzi EK, Mlengeya T, Kilewo M, Lejora IA, Nordhoff M, et al. *Treponema* infection associated with genital ulceration in wild baboons. Vet Pathol. 2012;49:292–303. https://doi.org/10.1177/0300985811402839
9. Chuma IS, Batamuzi EK, Collins DA, Fyumagwa RD, Hallmaier-Wacker LK, Kazwala RR, et al. Widespread *Treponema pallidum* infection in nonhuman primates, Tanzania. Emerg Infect Dis. 2018;24:1002–9. https://doi.org/10.3201/eid2406.180037
10. Marks M, Mitjà O, Solomon AW, Asiedu KB, Mabey DC. Yaws. Br Med Bull. 2015;113:91–100. https://doi.org/10.1093/bmb/ldu037

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Middle East Respiratory Syndrome Coronavirus, Saudi Arabia, 2017–2018

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We characterized exposures and demographics of Middle East respiratory syndrome coronavirus cases reported to the Saudi Arabia Ministry of Health during July 1–October 31, 2017, and June 1–September 16, 2018. Molecular characterization of available specimens showed that circulating viruses during these periods continued to cluster within lineage 5.

Middle East respiratory syndrome (MERS) coronavirus (MERS-CoV) epidemiology in Saudi Arabia is characterized by healthcare-associated outbreaks (*1*,*2*), occasional household-contact transmission (*3*), and sporadic cases without apparent links to other known cases (*4*,*5*). Since 2015, healthcare-associated transmission has decreased as infection prevention and control practices have improved (*6*); however, sporadic cases continue to be reported, often associated with contact with dromedaries (*4*,*7*). Surveillance and routine investigation of recent MERS cases are critical to monitor the epidemiology of this emerging pathogen. We characterized exposures and demographics of MERS cases reported to the Saudi Arabia Ministry of Health during July 1–October 31, 2017, and June 1–September 16, 2018, and performed molecular characterization of available specimens to describe circulating viruses during these periods.

We summarized demographics and exposures using Ministry of Health investigation data. To further characterize exposures among sporadic cases (no known epidemiologic link to a hospital outbreak or known case) reported during July 1–October 31, 2017, we conducted telephone interviews using a standardized questionnaire addressing demographics and activities during the 14 days before symptom onset (exposure period). For deceased or unavailable patients, we interviewed proxies. We did not conduct interviews for cases reported during June 1–September 16, 2018; this period was selected because of specimen availability. Cases were confirmed by testing respiratory specimens with MERS-CoV real-time reverse transcription PCR assays (*8*). We shipped 20 specimens to the US Centers for Disease Control and Prevention for genome sequence analysis (*9*).

During July 1–October 31, 2017, a total of 61 MERS cases were reported from 12 of 13 administrative regions. Median patient age was 50 (range 10–89) years; 43 (70%) were male, and 23 (38%) died. Nine (15%) cases were associated with a hospital outbreak, 10 (16%) were household contacts of known cases, and 42 (69%) were classified as sporadic and further investigated. During November 2017, we interviewed 35 (83%) sporadic case-patients, 9 directly and 26 by proxy; 7 were unavailable. Among the 42 sporadic case-patients, median age was 57 (range 25–89) years; 35 (83%) were male, and 33 (79%) reported underlying conditions, most commonly diabetes (n = 24) and hypertension (n = 23). All were symptomatic and hospitalized; 22 (52%) died. During the exposure period, 21 (50%) sporadic case-patients reported camel contact: 12 had direct contact (touching), 5 indirect contact (visiting a setting with camels or exposure to others with direct camel contact), and 4 contact that could not be further classified. Among patients with camel contact, 6 also reported visiting a healthcare facility without a known

MERS-CoV outbreak for a reason unrelated to their subsequent MERS illness. Of the 21 sporadic case-patients without camel contact, 8 (38%) visited a healthcare facility without a known MERS-CoV outbreak, 5 (24%) denied high-risk (camel- or healthcare-related) exposures, 1 (2%) was reclassified after interview as a contact of a previously identified case-patient, and 7 (17%) had insufficient exposure data to further characterize.

We analyzed MERS-CoV whole-genome sequences from 4 sporadic cases from 2017 (Figure). All sequences demonstrated 99.9% nucleotide (nt) sequence identity and clustered together within lineage 5, the predominant circulating lineage since 2015 (*9*). Three were from the same region and had an identical 3-nt in-frame deletion in open reading frame (ORF) 4b (Figure). Among case-patients with the deletion, 1 had incomplete exposure data, but 2 reported visiting the same hospital during their nonoverlapping exposure periods. None reported camel contact. The fourth patient did not report high-risk exposures. All 4 patients died; interviews for 3 were conducted via proxy.

During June 1–September 16, 2018, a total of 32 MERS cases were reported from 10 administrative regions (*10*). Median patient age was 56 (range 29–84) years; 28 (88%) were male, and 23 (72%) reported underlying conditions,

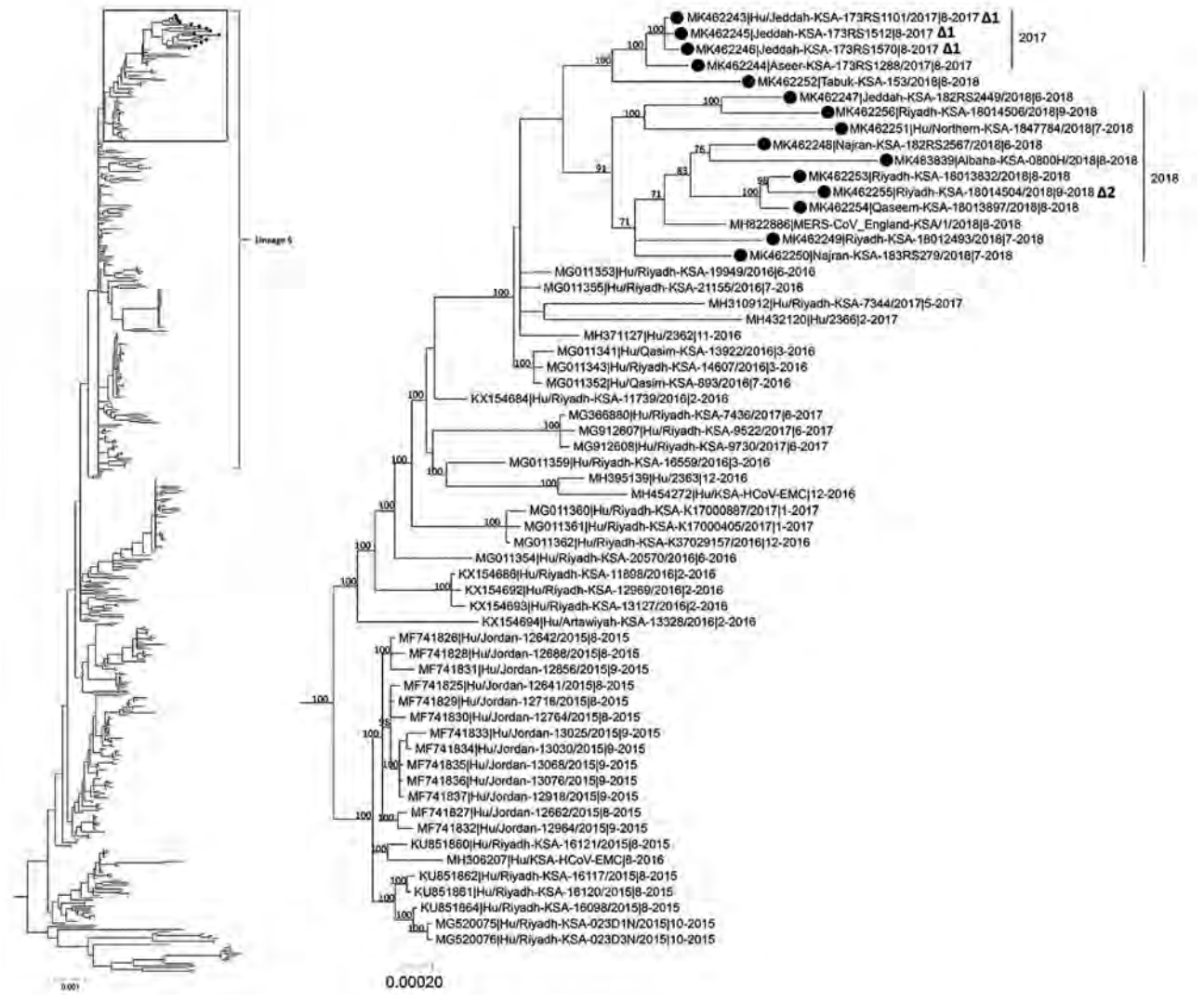

Figure. Phylogenetic tree of MERS-CoV whole-genome sequences obtained in Saudi Arabia (black dots) compared with 472 previously published human and camel genome sequences from GenBank. Tree inferred using MrBayes version 3.2.6 (https://nbisweden.github.io/MrBayes) under a general time-reversible model of nucleotide substitution with 4 categories of γ-distributed rate heterogeneity and a proportion of invariant sites. Box at the top of the tree on the left shows location of the tree on the right, showing lineage 5. Δ1 indicates 3-nt in-frame deletion in open reading frame 4a and Δ2 indicates 66-nt in-frame deletion in open reading frame 1a. Clade-credibility values ≥70% are indicated at selected nodes. Sequences from this study include GenBank accession numbers MK462243–MK462256 and MK483839. Scale bars indicate nucleotide substitutions per site. MERS-CoV, Middle East respiratory syndrome coronavirus; Hu, human; KSA, Kingdom of Saudi Arabia.

most commonly diabetes (n = 19) and hypertension (n = 15). Eleven (34%) patients died. Seven (22%) cases were in household contacts of known cases, 2 (6%) were healthcare-associated, and 23 (72%) were considered sporadic. Twelve (52%) sporadic case-patients reported camel contact. We obtained whole-genome sequences from 11 specimens and full or partial spike gene sequences from 5. One virus sequence obtained in 2018 from a patient from the Tabuk region was most similar to those in the 2017 clade; the other 10 virus sequences showed 99.5%–99.9% nt identity and formed a separate clade. This clade included a sequence from a 2018 case-patient from Saudi Arabia who traveled to the United Kingdom. Sequence variability among the 2018 viruses supports the disparate geographic origin of these cases. A case-patient in the Riyadh region who reported no high-risk exposures had a 66-nt in-frame deletion in ORF1a (Figure; GenBank accession no. MK462255).

In conclusion, dromedary contact was common for recent case-patients with sporadic MERS-CoV in Saudi Arabia, indicating continued zoonotic transmission to humans. MERS-CoV infection continues to be reported periodically among those without known high-risk exposures, warranting further investigation. Recently circulating viruses remain in lineage 5, the predominant circulating strain in Saudi Arabia since 2015. Sporadic unique ORF sequence deletions continue to be observed, highlighting the importance of ongoing molecular virology surveillance.

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References

1. Alanazi KH, Killerby ME, Biggs HM, Abedi GR, Jokhdar H, Alsharef AA, et al. Scope and extent of healthcare-associated Middle East respiratory syndrome coronavirus transmission during two contemporaneous outbreaks in Riyadh, Saudi Arabia, 2017. Infect Control Hosp Epidemiol. 2019;40:79–88. https://doi.org/10.1017/ice.2018.290
2. Oboho IK, Tomczyk SM, Al-Asmari AM, Banjar AA, Al-Mugti H, Aloraini MS, et al. 2014 MERS-CoV outbreak in Jeddah—a link to health care facilities. N Engl J Med. 2015;372:846–54. https://doi.org/10.1056/NEJMoa1408636
3. Drosten C, Meyer B, Müller MA, Corman VM, Al-Masri M, Hossain R, et al. Transmission of MERS-coronavirus in household contacts. N Engl J Med. 2014;371:828–35. https://doi.org/10.1056/NEJMoa1405858
4. Alhakeem RF, Midgley CM, Assiri AM, Alessa M, Al Hawaj H, Saeed AB, et al. Exposures among MERS case-patients, Saudi Arabia, January–February 2016. Emerg Infect Dis. 2016;22:2020–2. https://doi.org/10.3201/eid2211.161042
5. Assiri AM, Biggs HM, Abedi GR, Lu X, Bin Saeed A, Abdalla O, et al. Increase in Middle East respiratory syndrome–coronavirus cases in Saudi Arabia linked to hospital outbreak with continued circulation of recombinant virus, July 1–August 31, 2015. Open Forum Infect Dis. 2016;3:ofw165. https://doi.org/10.1093/ofid/ofw165
6. World Health Organization. MERS situation update. Cairo (Egypt): Eastern Mediterranean Regional Office (EMRO); 2019 [cited 2019 Feb 26]. http://www.emro.who.int/pandemic-epidemic-diseases/mers-cov/mers-situation-update-january-2019.html
7. Alraddadi BM, Watson JT, Almarashi A, Abedi GR, Turkistani A, Sadran M, et al. Risk factors for primary Middle East respiratory syndrome coronavirus illness in humans, Saudi Arabia, 2014. Emerg Infect Dis. 2016;22:49–55. https://doi.org/10.3201/eid2201.151340
8. World Health Organization. Laboratory testing for Middle East respiratory syndrome coronavirus: interim guidance. 2018 [cited 2019 Feb 26]. https://www.who.int/csr/disease/coronavirus_infections/mers-laboratory-testing
9. Assiri AM, Midgley CM, Abedi GR, Bin Saeed A, Almasri MM, Lu X, et al. Epidemiology of a novel recombinant Middle East respiratory syndrome coronavirus in humans in Saudi Arabia. J Infect Dis. 2016;214:712–21. https://doi.org/10.1093/infdis/jiw236
10. World Health Organization. Middle East respiratory syndrome coronavirus (MERS-CoV)—Saudi Arabia. 2018 [cited 2019 Apr 22]. https://www.who.int/csr/don/03-october-2018-mers-saudi-arabia

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Mycobacterium microti Infection in Free-Ranging Wild Boar, Spain, 2017–2019

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Mycobacterium microti is a member of the *Mycobacterium tuberculosis* complex that causes pathology in many mammals. *M. microti* infections have been found in some countries in Europe. We report an outbreak of tuberculosis caused by *M. microti* in wild boars in Spain.

Mycobacterium microti is a member of the Mycobacterium tuberculosis complex (MTBC), which also includes *M. tuberculosis* and *M. bovis*, the main causes of human and animal tuberculosis (TB), respectively. Even though voles and other wild small rodents were initially identified as its natural hosts (*1*), *M. microti* can cause pathology in a wide range of mammals, including pets, livestock, wildlife (*2*–*5*), and humans (*6*). *M. microti* infections have been previously reported in several countries in Europe, including Switzerland, Italy, and France (*3*,*7*–*9*). We report an outbreak of tuberculosis caused by *M. microti* in free-ranging wild boars in the Iberian Peninsula in Spain.

During June 2017–March 2019, a total of 9 free-ranging wild boars with lesions associated with TB were detected in the outbreak area, covering ≈3,000 hectares in the Catalan Pyrenees (Figure). TB was confirmed histologically, first by hematoxylin and eosin staining (9/9) and then by Ziehl-Neelsen staining of acid-fast bacilli (7/9). In all cases, submandibular lymph nodes showed granulomatous necrotizing lymphadenitis, sometimes with scant acid-fast bacilli, similar to that found in *M. microti* infections previously described in wild boar, which were generally confined to lymph nodes in the head (*7*,*8*).

To confirm the causative agent for these infections, we extracted DNA from tissue samples (DNAExtract-VK, Vacunek, http://vacunek.com) and performed real-time PCR (TBC-VK, Vacunek), which confirmed MTBC in 6 of 9 suspected cases. DVR spoligotyping at VISAVET Health Surveillance Centre, Universidad Complutense de Madrid, identified the pathogen in 4 of the 6 confirmed MTBC cases as *M. microti* (spoligopattern SB0423; *Mycobacterium bovis* spoligotype database, http://www.mbovis.org). In the other

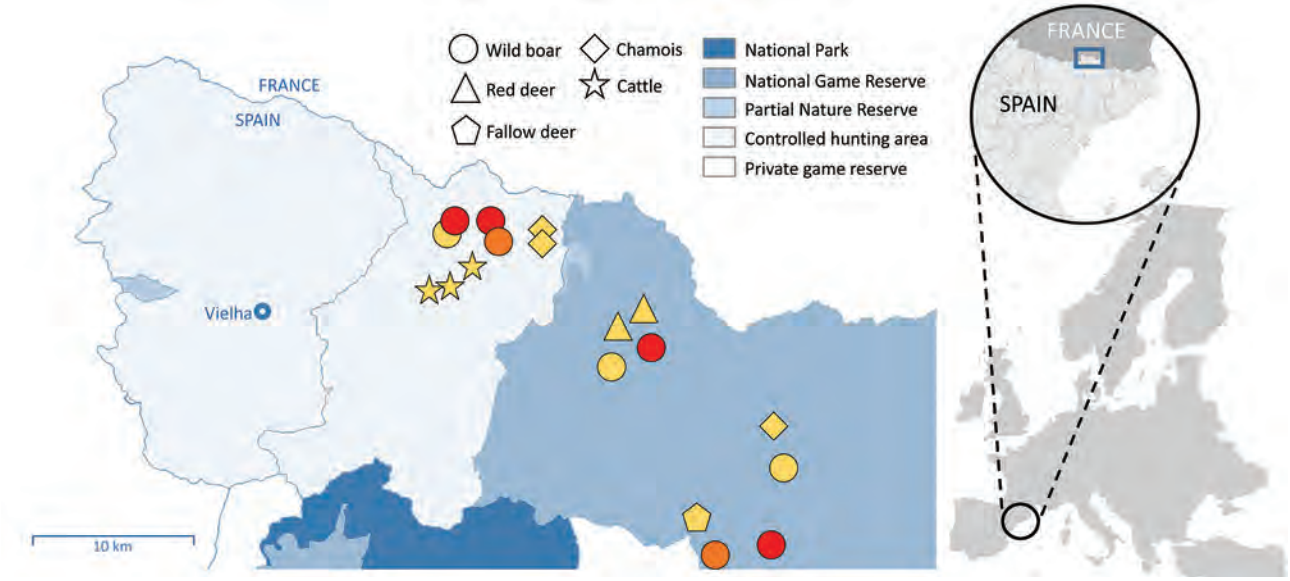

Figure. Outbreak area for wild boar tuberculosis (TB) cases, Spain, June 2017–March 2019. Circles show cases of wild boars with TB lesions. Stars indicate the location of cattle herds with positive skin tests (not confirmed at slaughterhouse); triangles, pentagons, and diamonds show locations of *Mycobacterium tuberculosis* complex–seropositive ungulates (no tissue samples were obtained from these animals). Colors indicate etiologic agent identification: red, *M. microti*; orange, any *M. tuberculosis* complex; yellow, unidentified. Different hunting areas are indicated. The border between Spain and France and the main village (Vielha) are labelled. Inset maps show location of the study area on the Iberian Peninsula.

2 cases, the laboratory was unable to determine the species because of low DNA load from the sample. This result likely was due to the slow in vitro growth rate of *M. microti* in infected animals, which makes it difficult to isolate in routine diagnostic laboratories.

Spoligopattern SB0423 is included in a phylogenetic cluster with spoligopattern SB0112, also associated with *M. microti*, on the basis of neighbor joining. Both spoligotypes are localized in the eastern French Pyrenees, close to the borders with Spain and Andorra (*3*). Most *M. microti* cases in France, found in cats, dogs, and llamas during 2005–2016 and, since 2015, in wild boars and badgers, have been found within 50 km of the outbreak area in Spain. The 2017 *M. microti* cases described in this report were found closer to the border with France; the remaining 2 cases, detected in 2019, were localized near the southern limit of the outbreak area (Figure).

In the outbreak area, up to 18 animals in 3 cattle herds showed positive results for a single intradermal tuberculin skin test (Figure). However, none of these animals showed gross lesions in target tissues (i.e., lungs, pulmonary, and retropharyngeal lymph nodes) at a slaughterhouse or positive results to mycobacterial culture and PCR. Similarly, in a recent case in France, a cow reacting to a TB skin test did not show TB-like lesions in respiratory tissues and returned negative results from cultures, but *M. microti* DNA was finally detected in retropharyngeal lymph nodes only by using advanced molecular techniques (*5*). These results indicate that cattle exposed to *M. microti* may induce positive results to diagnostic tests performed in bovine TB eradication campaigns. *M. microti* infection can sometimes cause visible lesions in cattle (*2*), but the fact that *M. microti* are natural knockouts for the virulence-related RD1^{mic} genomic region (*10*) may indicate a lower pathogenicity compared with other MTBC species and account for these negative test results.

We tested additional wild ungulates in the outbreak area and found that 6 (2 red deer, 1 fallow deer, and 3 chamois) were seropositive for MTBC using a MPB83-specific IgG indirect ELISA test (Figure). Unfortunately, no tissue samples were submitted to examine for lesions or to detect and identify mycobacteria. However, overall positive results for *M. microti* and the absence of other MTBC strains during the 2017–2019 period in the outbreak area suggest a multihost circulation of *M. microti*. Because voles are known maintenance hosts of *M. microti* (*1*), further investigation of wild small rodent populations in the outbreak area could determine the epidemiology of this outbreak in greater detail.

These findings, together with previously reported cases nearer the border between France and Spain, indicate a transboundary circulation of *M. microti* across the Pyrenean border that should be taken into account for wildlife TB surveillance. Coordinated action between animal health authorities and laboratories in Spain and France is required, as well as the improvement of livestock management and biosecurity practices.

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References

1. Cavanagh R, Begon M, Bennett M, Ergon T, Graham IM, De Haas PE, et al. *Mycobacterium microti* infection (vole tuberculosis) in wild rodent populations. J Clin Microbiol. 2002;40:3281–5. https://doi.org/10.1128/JCM.40.9.3281-3285.2002
2. Smith NH, Crawshaw T, Parry J, Birtles RJ. *Mycobacterium microti*: more diverse than previously thought. J Clin Microbiol. 2009;47:2551–9. https://doi.org/10.1128/JCM.00638-09
3. Michelet L, de Cruz K, Karoui C, Hénault S, Boschiroli M. *Mycobacterium microti*, an unrecognized tubercular agent [in French]. Epidémiol Santé Anim. 2017;71:129–38.
4. Michelet L, de Cruz K, Phalente Y, Karoui C, Hénault S, Beral M, et al. Mycobacterium microti infection in dairy goats, France. Emerg Infect Dis. 2016;22:569–70. https://10.3201/eid2203.151870
5. Michelet L, De Cruz K. Mycobacterium microti infection in a cow in France. Vet Rec. 2017;180:429. PubMed http://dx.doi.org/10.1136/vr.j2041
6. Panteix G, Gutierrez MC, Boschiroli ML, Rouviere M, Plaidy A, Pressac D, et al. Pulmonary tuberculosis due to Mycobacterium microti: a study of six recent cases in France. J Med Microbiol. 2010;59:984–9. https://doi.org/10.1099/jmm.0.019372-0
7. Schöning JM, Cerny N, Prohaska S, Wittenbrink MM, Smith NH, Bloemberg G, et al. Surveillance of bovine tuberculosis and risk estimation of a future reservoir formation in wildlife in Switzerland and Liechtenstein. PLoS One. 2013;8:e54253. https://doi.org/10.1371/journal.pone.0054253
8. Boniotti MB, Gaffuri A, Gelmetti D, Tagliabue S, Chiari M, Mangeli A, et al. Detection and molecular characterization of *Mycobacterium microti* isolates in wild boar from northern Italy. J Clin Microbiol. 2014;52:2834–43. https://doi.org/10.1128/JCM.00440-14
9. Chiari M, Ferrari N, Giardiello D, Avisani D, Pacciarini ML, Alborali L, et al. Spatiotemporal and ecological patterns of

Mycobacterium microti infection in wild boar (*Sus scrofa*). Transbound Emerg Dis. 2016;63:e381–8. https://doi.org/10.1111/tbed.12313

10. Brodin P, Eiglmeier K, Marmiesse M, Billault A, Garnier T, Niemann S, et al. Bacterial artificial chromosome-based comparative genomic analysis identifies *Mycobacterium microti* as a natural ESAT-6 deletion mutant. Infect Immun. 2002;70:5568–78. https://doi.org/10.1128/IAI.70.10.5568-5578.2002

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Availability of Injectable Antimicrobial Drugs for Gonorrhea and Syphilis, United States, 2016

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We estimated the availability of the injectable antimicrobial drugs recommended for point-of-care treatment of gonorrhea and syphilis among US physicians who evaluated patients with sexually transmitted infections in 2016. Most physicians did not have these drugs available on-site. Further research is needed to determine the reasons for the unavailability of these drugs.

Rates of sexually transmitted infections (STIs) are on the rise in the United States. Compared with the incidence in 2013, the numbers of reported gonorrhea (+75%) and syphilis (primary and secondary, +153%) cases in 2017 were dramatically increased (*1*). Timely (optimally same-day) treatment of bacterial STIs with a highly effective regimen is critical for national STI control efforts and can help mitigate the development of drug resistance, a particularly pertinent issue for *Neisseria gonorrhoeae* (*2*). In addition, the high number of congenital syphilis cases in the United States in 2017 highlights the need for efficient and effective treatment of *Treponema pallidum* infection (*3*).

The recommended first-line treatment for uncomplicated gonorrhea is intramuscular ceftriaxone (250 mg) and for primary and secondary syphilis is intramuscular penicillin G benzathine (2.5 million units) (*4*). On-site access to these injectable medications in clinics facilitates point-of-care treatment and helps mitigate drug resistance to *N. gonorrhoeae* and *T. pallidum* when these drugs are used instead of the oral antimicrobial drug alternatives. Therefore, we set out to determine a nationally representative estimate of the availability of these medications and examine if differences existed by geographic location and between offices designated and not designated as patient-centered medical homes (PCMHs).

We used the 2016 Physician Induction File of the National Ambulatory Medical Care Survey (*5*) to assess the number of physicians who treat patients with STIs and had injectable antimicrobial drugs available on-site. The Physician Induction File is a nationally representative survey of office-based, nonfederal physicians in the United States and includes information regarding practice characteristics and habits. Use of the data is restricted, and access is facilitated through the Research Data Center, National Center for Health Statistics, Centers for Disease Control and Prevention, in Hyattsville, Maryland, USA. A total of 1,030 physicians (46.2% unweighted response rate), representing an estimated 330,581 (95% CI 326,994–334,168) physicians in the United States, completed the Physician Induction File in 2016. In this survey, physicians who reported evaluating or treating patients for STIs were asked which antimicrobial drugs they had available on-site for same-day management of gonorrhea and syphilis, including intramuscular ceftriaxone and penicillin G benzathine at the recommended doses. We determined national estimates of reported on-site, same-day availability for these antimicrobial drugs and stratified results by PCMH designation and US region. We used multiple logistic regression models to determine if PCMH designation and region were predictive of on-site availability of these 2 medications.

An estimated 45.2% (149,483, 95% CI 138,850–160,116) of office-based physicians indicated that they evaluate patients for STIs in their offices. Of these, 77.9% (116,479, 95% CI 105,360–127,598) reported not having penicillin G benzathine available on-site and 56.1% (83,827, 95% CI 73,709–93,945) reported not having ceftriaxone. Access to both of these drugs was generally higher in the South (Table). Physicians in offices not designated PCMHs were more likely than those in offices designated PCMHs to report lacking on-site availability of ceftriaxone

Table. On-site availability of drugs to manage gonorrhea and syphilis and likelihood of drug nonavailability by type and location of clinic, United States, 2016*

Category	Weighted population, no. (95% CI)	On-site drug availability, % (95% CI)		Likelihood of nonavailability†	
		Yes	No	β	OR (95% CI)
Ceftriaxone, 250 mg, intramuscular					
PCMH designation‡					
Yes	44,028 (35,296–52,760)	54.4 (42.1–66.3)	45.6 (33.7–57.9)	Referent	Referent
No	89,381 (79,356–99,406)	37.3 (30.0–45.0)	62.7 (55.0–70.0)	0.71	2.03 (1.15–3.57)
Region of office§					
Northeast	33,384 (28,666–38,102)	34.3 (22.7–47.5)	65.7 (52.5–77.3)	Referent	Referent
South	45,574 (39,694–51,454)	49.0 (38.4–59.6)	51.0 (40.4–61.6)	−0.63	0.53 (0.27–1.06)
Midwest	33,296 (28,374–38,218)	41.4 (28.9–54.8)	58.6 (45.2–71.1)	−0.28	0.76 (0.35–1.64)
West	37,228 (31,038–43,418)	48.6 (35.8–61.6)	51.4 (38.4–64.2)	−0.62	0.54 (0.26–1.14)
Penicillin G benzathine, 2.5 million units, intramuscular					
PCMH designation‡					
Yes	44,028 (35,296–52,760	34.2 (23.2–46.6)	65.8 (53.4–76.8)	Referent	Referent
No	89,381 (79,356–99,406)	14.6 (9.5–21.1)	85.4 (78.9–90.5)	1.16	3.20 (1.63–6.29)
Region of office§					
Northeast	33,384 (28,666–38,102)	17.8 (8.9–30.3)	82.2 (69.7–91.1)	Referent	Referent
South	45,574 (39,694–51,454)	32.1 (22.8–42.5)	67.9 (57.5–77.2)	−0.83	0.43 (0.19–1.00)
Midwest	33,296 (28,374–38,218)	15.5 (7.3–27.3)	84.5 (72.7–92.7)	0.22	1.25 (0.46–3.39)
West	37,228 (31,038–43,418)	19.6 (9.4–33.8)	80.4 (66.2–90.6)	−0.13	0.88 (0.32–2.40)

*The data source was the National Center for Health Statistics, National Ambulatory Medical Care Survey (*5*). All analyses were conducted by using SUDAAN (https://www.rti.org/impact/sudaan-statistical-software-analyzing-correlated-data) to account for the complex sampling design of the survey. OR, odds ratio; PCMH, patient-centered medical home.
†Likelihood determined by using a logistic regression model that included the factors PCMH designation and region of country. β is the coefficient of the variable, indicating direction and strength of the association.
‡PCMH designation of first-listed National Ambulatory Medical Care Survey–sampled office. An estimated weighted total of 149,483 office-based physicians indicated that they evaluate patients for or treat patients with sexually transmitted infections at their first-listed sampled office. PCMH data were missing (i.e., the physician refused to answer, did not know, or left blank or an instrument error occurred) for 10.8% of weighted office-based physicians treating gonorrhea and syphilis.
§Estimates might not sum to the estimated weighted total because of rounding of weighted numbers.

(odds ratio 2.03, 95% CI 1.15–3.57) and penicillin G benzathine (odds ratio 3.20, 95% CI 1.63–6.29) (Table).

Our nationally representative analysis demonstrates that most office-based physicians who provide STI services reported not having on-site access to the recommended injectable medications for gonorrhea and syphilis management. The PCMH designation has been noted as an indicator for quality of care by the American Medical Association (*6*) and thus might be an indication for greater access to more complex treatments and supplies, such as injectable antimicrobial drugs. In addition, previous research has found that PCMHs have larger provider staffs and are affiliated with larger medical groups or health systems that may have access to greater resources, characteristics that might result in these practices having greater access to injectable medications to treat gonorrhea and syphilis patients (*7*). Differences in drug availability by region were not statistically significant; however, we note that the point estimates of on-site availability were highest among physicians in areas with the highest levels of STIs (the southern United States) and where congenital syphilis has been most commonly observed (the western United States) (*1*).

We provide a national estimate of the percentage of US physicians treating patients with STIs that report not having the recommended antimicrobial drugs on-site for same-day treatment of gonorrhea and primary and secondary syphilis. In light of our findings, we believe that future research should focus on determining the factors preventing physicians from providing these medications on-site. The costs of obtaining and carrying these medications, as well as issues of storage and shelf life, should be explored to determine if these factors are barriers. In addition, the implications of prescribing alternative treatments or delaying care in situations when medications are not readily available on-site should be further explored. Mitigating the lack of medication availability to treat these infections will help public health officials stop the rise in STI disease.

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References

1. Centers for Disease Control and Prevention. Sexually transmitted disease surveillance 2017. Atlanta: US Department of Health and Human Services; 2018.
2. Kirkcaldy RD, Bolan GA, Wasserheit JN. Cephalosporin-resistant gonorrhea in North America. JAMA. 2013;309:185–7. https://doi.org/10.1001/jama.2012.205107
3. Nurse-Findlay S, Taylor MM, Savage M, Mello MB, Saliyou S, Lavayen M, et al. Shortages of benzathine penicillin for prevention

of mother-to-child transmission of syphilis: an evaluation from multi-country surveys and stakeholder interviews. PLoS Med. 2017;14:e1002473. https://doi.org/10.1371/journal.pmed.1002473
4. Workowski KA, Bolan GA; Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines, 2015. MMWR Recomm Rep. 2015;64(No. RR-3):1–137.
5. Centers for Disease Control and Prevention National Center for Health Statistics. Ambulatory health care data. 2019 Jul 3 [cited 2019 May 29]. https://www.cdc.gov/nchs/ahcd/index.htm
6. American Medical Association. Principles of the patient-centered medical home H-160.919. 2018 [cited 2019 May 29]. https://policysearch.ama-assn.org/policyfinder/detail/Principles%20of%20the%20Patient-Centered%20Medical%20Home%20H-160.919?uri=%2FAMADoc%2FHOD.xml-0-734.xml
7. McHugh M, Shi Y, Ramsay PP, Harvey JB, Casalino LP, Shortell SM, et al. Patient-centered medical home adoption: results from aligning forces for quality. Health Aff (Millwood). 2016;35:141–9. https://doi.org/10.1377/hlthaff.2015.0495

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Host Switching of Zoonotic Broad Fish Tapeworm (*Dibothriocephalus latus*) to Salmonids, Patagonia

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Diphyllobothriosis is a reemerging zoonotic disease because of global trade and increased popularity of eating raw fish. We present molecular evidence of host switching of a human-infecting broad fish tapeworm, *Dibothriocephalus latus*, and use of salmonids as intermediate or paratenic hosts and thus a source of human infection in South America.

Diphyllobothriosis is an emerging zoonotic disease caused by broad fish tapeworms. Except for the Pacific broad tapeworm (*Adenocephalus pacificus*), whose life cycle is completed in the sea, all species of the genus *Dibothriocephalus* (formerly in *Diphyllobothrium*) were limited to the freshwaters in the Northern Hemisphere (*1*). However, some of these tapeworms also were reported in the Southern Hemisphere, including South America, especially Patagonia, in the 20th century. Although the introduction routes of these human parasites remain unknown, their larvae (plerocercoids) have appeared in South America in nonnative but economically important salmonids, such as rainbow, brown, and brook trout (*2*,*3*).

Several cases of diphyllobothriosis have been reported from South America, and plerocercoids of tapeworms identified as *Dibothriocephalus latus* and *D. dendriticus* have been reported in fish (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/11/19-0792-App1.pdf). However, species identification was based almost exclusively on morphologic characteristics. Considering general uniformity, intraspecific variability, and shortage of species-specific morphologic traits (especially in plerocercoids), all previous reports of *D. latus* and *D. dendriticus* tapeworms from South America need verification (*4*). Reports concerning the most commercially important species of salmonids being infected with *D. latus* tapeworms are especially dubious because this species most likely uses only freshwater percid, esocid, and gadid fish as its second intermediate hosts in the Northern Hemisphere (*1*,*4*).

Reliable identification of plerocercoids, which are the source of diphyllobothriosis, is crucial from the epidemiologic point of view because salmonids are of great economic value in South America as a food source for local populations, sport fishing, and exportation (*5*). We provide molecular evidence of second intermediate or paratenic host switching of human-infecting *D. latus* tapeworms in Patagonia, South America.

We found a total of 44 plerocercoids in 3 salmonid species: from Lake Gutiérrez, Rio Negro, Argentina (October 2017), rainbow trout (*Oncorhynchus mykiss*), of which 2/7 fish examined were infected; brown trout (*Salmo trutta*), of which 3/4 were infected; and brook trout (*Salvelinus fontinalis*), of which 5/10 were infected; and from Lake Alicura, Neuquén, Argentina (April 2018), brown trout, of which 3/4 were infected. Most plerocercoids were encysted in the body cavity, mainly among the pyloric ceca, and only a few were free in the muscle. We selected, photographed, and sequenced the partial *cox*1 gene of 22 larvae in accordance with the procedure described by Wicht et al. (*6*). We also photodocumented morphologic vouchers (hologenophores) of sequenced specimens (Figure).

Our morphologic and molecular evaluation revealed the presence of *D. dendriticus* plerocercoids in 12 fish (8

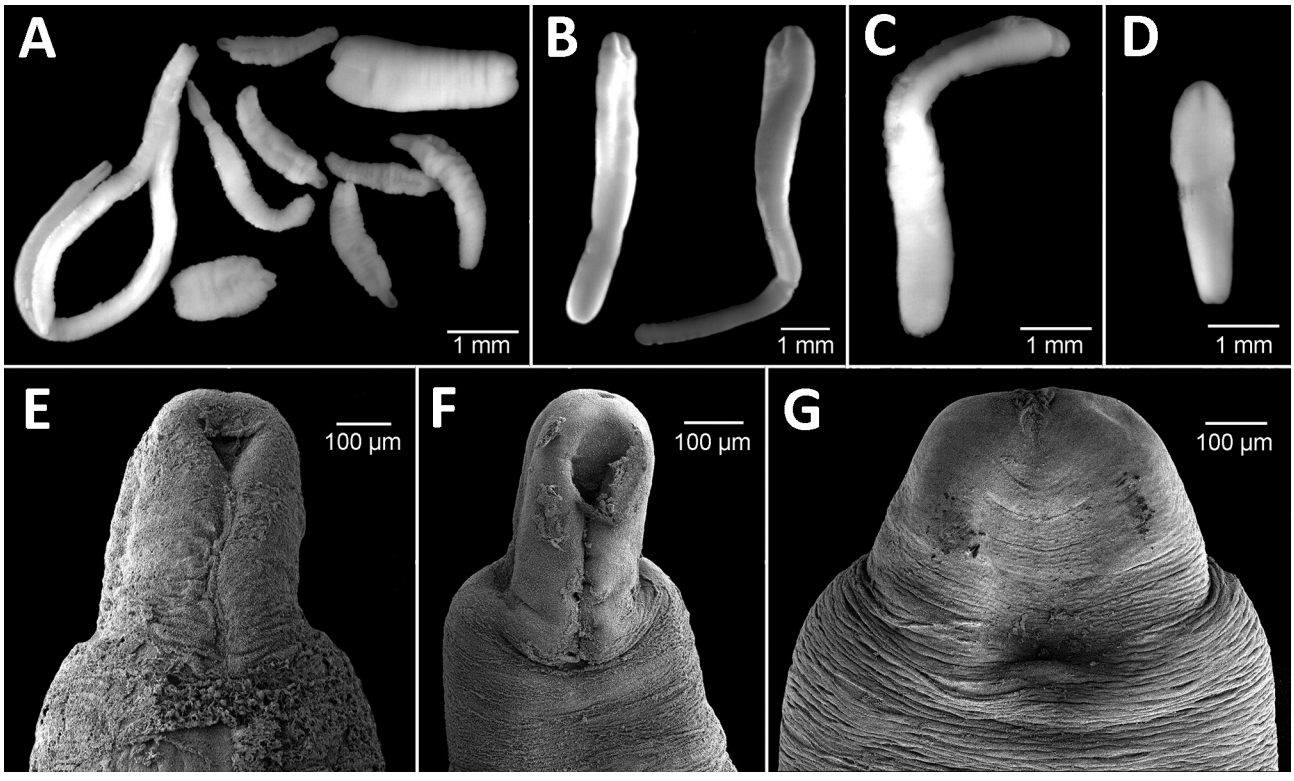

Figure. Microphotographs of *Dibothriocephalus* spp. plerocercoids from 3 salmonid species in Lago Gutiérrez, Patagonia, Argentina. A) *D. dendriticus* and *D. latus* plerocercoids from *Oncorhynchus mykiss* rainbow trout. B) *D. latus* plerocercoids from *Salvelinus fontinalis* brook trout. C) *D. dendriticus* plerocercoids from *Salmo trutta* brown trout. D) *D. latus* plerocercoids from *S. trutta* brown trout. E, F) *D. dendriticus* plerocercoids from *O. mykiss* rainbow trout. G) *D. latus* plerocercoids from *S. fontinalis* brook trout.

in *O. mykiss* rainbow trout, 2 in *S. trutta* brown trout, and 2 in *S. fontinalis* brook trout); their sequences were identical with those of *D. dendriticus* tapeworms from Chile (GenBank accession nos. AB623150 and AB623149). We also detected the presence of *D. latus* plerocercoids in 10 fish (1 in *O. mykiss* rainbow trout, 3 in *S. trutta* brown trout, and 6 in *S. fontinalis* brook trout); these sequences were identical with those of *D. latus* tapeworms from Italy (GenBank accession no GU997614) (Appendix Table 2).

D. dendriticus plerocercoids have been reported in >50 species of freshwater fish of 12 families, and salmonids represent the principal, most common fish hosts (*7*). In contrast, *D. latus* plerocercoids have never been confirmed reliably in salmonids in the Northern Hemisphere, where they occur in relatively few freshwater fish species, such as perch (*Perca* spp.), pike (*Esox* spp.), ruffe (*Gymnocephalus cernua*), burbot (*Lota lota*), and walleye (*Sander* spp.) (*4*), which are not present in South America. Therefore, *D. latus* tapeworms had to adapt to new second or paratenic intermediate hosts (i.e., salmonids) after their introduction to the Southern Hemisphere, even though salmonids are not suitable hosts in the Northern Hemisphere, where these tapeworms occurred originally (*1*,*4*).

The origin of freshwater, human-infecting broad fish tapeworms in South America remains unknown. Salmonids were introduced to Chile (from Germany) and Argentina (from the United States) at the beginning of the 20th century as eggs or juveniles from a hatchery (*8*,*9*). However, no evidence indicates that naturally infected salmonids were imported to South America. Introduction of adult tapeworms of *Dibothriocephalus* spp. through infected humans or dogs cannot be ruled out, nor can the introduction of *D. dendriticus* tapeworms by migratory birds (*1*,*4*).

Our findings provide evidence of host switching of *D. latus* plerocercoids in Patagonia. Adaptation to new fish hosts might have serious epidemiologic consequences because of the economic importance of salmonids and their consumption by humans locally and abroad. Moreover, these introduced salmonids currently represent a substantial proportion of the total fish population in most of the lakes in the Andes region (*5*,*10*). Therefore, parasitologic examination of fish before their exportation on ice is necessary to avoid emergence of new foci of human diphyllobothriosis.

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References

1. Kuchta R, Scholz T. Diphyllobothriidea. In: Caira JN, Jensen K, editors. Planetary biodiversity inventory (2008–2017): tapeworms from vertebrate bowels of the earth. Special publication no. 25. Lawrence (KS, USA): University of Kansas, Natural History Museum; 2017. p. 167–89.
2. Torres P, Franjola R, Pérez J, Auad S, Uherek F, Miranda JC, et al. Epidemiología de la difilobotriasis en la cuenca del Río Valdivia, Chile. Rev Saúde Pública. 1989;23:45–57. https://doi.org/10.1590/S0034-89101989000100007
3. Revenga J, Semenas L. Difilobotriasis en salmónidos introducidos en el Parque y Reserva Nacional Nahuel Huapi Argentina: morfológia de plerocercoides. Arch Med Vet. 1991;23:157–63.
4. Scholz T, Kuchta R. Fish-borne, zoonotic cestodes (*Diphyllobothrium* and relatives) in cold climates: a never-ending story of neglected and (re)-emergent parasites. Food and Waterborne Parasitology. 2016;4:23–38. https://doi.org/10.1016/j.fawpar.2016.07.002
5. Soto D, Arismendi I, Gonzáles J, Sanzana J, Jara F, Jara C, et al. Southern Chile, trout and salmon country: invasion patterns and threats for native species. Rev Chil Hist Nat. 2006;79:97–117. https://doi.org/10.4067/S0716-078X2006000100009
6. Wicht B, Yanagida T, Scholz T, Ito A, Jiménez JA, Brabec J. Multiplex PCR for differential identification of broad tapeworms (Cestoda: *Diphyllobothrium*) infecting humans. J Clin Microbiol. 2010;48:3111–6. https://doi.org/10.1128/JCM.00445-10
7. Kuchta R, Brabec J, Kubáčková P, Scholz T. Tapeworm *Diphyllobothrium dendriticum* (Cestoda)—neglected or emerging human parasite? PLoS Negl Trop Dis. 2013;7:e2535. https://doi.org/10.1371/journal.pntd.0002535
8. Golusda P. Aclimatación y cultivo de especies salmonídeas en Chile. Bol Soc Biol Concepc. 1927;1:80–100.
9. Tulián E. Acclimatization of American fishes in Argentina. Bull Bureau Fish. 1910;28:3–20.
10. Fernández MV, Lallement M, Rechencq M, Vigliano PH, Sosnovsky A, Macchi PJ. Top predator fish assemblages in Northern Patagonia, Argentina. What factors regulate their patterns of distribution and abundance? Austral Ecol. 2018;43:651–62. https://doi.org/10.1111/aec.12610

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LETTERS

Achromobacter xylosoxidans Infections after Prostate Biopsies, France, 2014

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To the Editor: We read with interest the article by Haviari et al. concerning a health care–associated outbreak of *Achromobacter xylosoxidans* infections after prostate biopsies (*1*). Although noteworthy, the description lacks some data.

First, the isolates of *Achromobacter* cannot be referred to as *A. xylosoxidans* from just the method used in this study, API 20 NE mass spectrometry (bioMérieux, https://www.biomerieux.com). Since 2012, a total of 18 species have been defined in the genus *Achromobacter* (*2*). Only multilocus sequence typing or sequencing 765 bp of the housekeeping gene *nrdA* enables the identification of the isolates to the species level (*3*). To date, in the few studies available, a great variety of species have been detected in clinical samples, with *A. xylosoxidans* the most predominant (*4*,*5*). Correct identification of the isolates involved in all types of infection is necessary to help understand the epidemiology, pathogenicity, and resistance pattern of the various species.

Second, the antimicrobial drug resistance profiles are not given (except for ceftriaxone, which is an intrinsic resistance, and ofloxacin) but again are valuable epidemiologic data. This information might help in detecting the emergence of new cases in the unit or in other hospitals, as well as in discussing the therapeutic options.

Finally, all the bacteria recovered in the container belonged to environmental waterborne genera frequently encountered in wet sites in hospitals. As discussed by the authors, these microorganisms have been involved in contamination of antiseptic solutions containing quaternary ammonium compounds or chlorhexidine. Unfortunately, the authors did not mention which disinfectants were used in the biopsy room (for hands, sinks, surfaces, or containers)

and did not investigate for these potential sources of contamination. In the absence of identification of any reservoir and despite the new measures adopted, new cases might still occur.

In conclusion, these missing data are needed for other hospitals to identify epidemiogenic *Achromobacter* isolates. Complete information would help in implementing control measures to contain and prevent outbreaks.

References

1. Haviari S, Cassier P, Dananché C, Hulin M, Dauwalder O, Rouvière O, et al. Outbreak of *Achromobacter xylosoxidans* and *Ochrobactrum anthropi* infections after prostate biopsies, France, 2014. Emerg Infect Dis. 2016;22:1412–9. https://doi.org/10.3201/eid2208.151423
2. Vandamme PA, Peeters C, Inganas E, Cnockaert M, Houf K, Spilker T, et al. Taxonomic dissection of *Achromobacter denitrificans* Coenye et al. 2003 and proposal of *Achromobacter agilis* sp. nov., nom. rev., *Achromobacter pestifer* sp. nov., nom. rev., *Achromobacter kerstersii* sp. nov. and *Achromobacter deleyi* sp. nov. Int J Syst Evol Microbiol. 2016;66:3708–17. https://doi.org/10.1099/ijsem.0.001254
3. Spilker T, Vandamme P, Lipuma JJ. Identification and distribution of *Achromobacter* species in cystic fibrosis. J Cyst Fibros. 2013;12:298–301. https://doi.org/10.1016/j.jcf.2012.10.002
4. Amoureux L, Bador J, Verrier T, Mjahed H, DE Curraize C, Neuwirth C. *Achromobacter xylosoxidans* is the predominant *Achromobacter* species isolated from diverse non-respiratory samples. Epidemiol Infect. 2016;144:3527–30. https://doi.org/10.1017/S0950268816001564
5. Coward A, Kenna DT, Perry C, Martin K, Doumith M, Turton JF. Use of *nrdA* gene sequence clustering to estimate the prevalence of different *Achromobacter* species among cystic fibrosis patients in the UK. J Cyst Fibros. 2016;15:479–85. https://doi.org/10.1016/j.jcf.2015.09.005

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Macrolide-Resistant *Mycoplasma genitalium* in Southeastern Region of the Netherlands, 2014–2017

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To the Editor: We read with interest the article by Martens et al., which analyzed the frequency of macrolide resistance–mediating mutations in *Mycoplasma genitalium* infections in the southeastern region of the Netherlands during 2014–2017 (*1*). The authors reported high rates of macrolide resistance in *M. genitalium* infections (281/827; 34.0%) and observed a decrease in the rate in 2017 (115/290; 39.7%) compared with 2016 (92/207; 44.4%), after an increase in the number of tests of cure performed.

Increasing rates of macrolide resistance in *M. genitalium* are a problem not only in the Netherlands but also worldwide: 77.4% in New Zealand (*2*) and 41% in the United Kingdom (*3*). Macrolide resistance in *M. genitalium* as a consequence of single-dose azithromycin treatment has been previously reported (*4*). European Academy of Dermatology and Venereology guidelines recommend azithromycin in an extended regimen (500 mg day 1,250 mg days 2–5, orally) as a first-line treatment, followed by a test of cure 4–6 weeks after treatment (*5*).

In northern Spain, local protocols for the treatment of *M. genitalium* infections are based on the European guideline. We performed a prospective/retrospective study during August 2015–October 2018. We confirmed 173 cases of *M. genitalium* infection; mean patient age was 29.4 years, and 57.2% (99/173) were male. We found macrolide-resistant *M. genitalium* strains in 21.8% (27/124) patients, which is a lower rate than was found in the Netherlands. Most of the patients attending posttreatment follow-up showed wild-type *M. genitalium*, and only 10.9% (5/46) became resistant to azithromycin treatment, in contrast with 89.6% (60/67) reported by Martens et al. We suggest that the decrease in macrolide resistance resulted from the increased number of posttreatment follow-ups. Our data confirm this. We believe that giving local advice on the basis of extended azithromycin treatment and posttreatment follow-up can limit the spread of macrolide resistance.

References

1. Martens L, Kuster S, de Vos W, Kersten M, Berkhout H, Hagen F. Macrolide-resistant *Mycoplasma genitalium* in southeastern region of the Netherlands, 2014–2017. Emerg Infect Dis. 2019;25: 1297–303. https://doi.org/10.3201/eid2507.181556
2. Anderson T, Coughlan E, Werno A. *Mycoplasma genitalium* macrolide and fluoroquinolone resistance detection and clinical implications in a selected cohort in New Zealand. J Clin Microbiol. 2017;55:3242–8. https://doi.org/10.1128/JCM.01087-17
3. Pond MJ, Nori AV, Witney AA, Lopeman RC, Butcher PD, Sadiq ST. High prevalence of antibiotic-resistant *Mycoplasma genitalium* in nongonococcal urethritis: the need for routine testing and the inadequacy of current treatment options. Clin Infect Dis. 2014;58:631–7. https://doi.org/10.1093/cid/cit752
4. Jensen JS, Bradshaw CS, Tabrizi SN, Fairley CK, Hamasuna R. Azithromycin treatment failure in *Mycoplasma genitalium*-positive patients with nongonococcal urethritis is associated with induced macrolide resistance. Clin Infect Dis. 2008;47:1546–53. https://doi.org/10.1086/593188
5. Jensen JS, Cusini M, Gomberg M, Moi H. 2016 European guideline on *Mycoplasma genitalium* infections. J Eur Acad Dermatol Venereol. 2016;30:1650–6. https://doi.org/10.1111/jdv.13849

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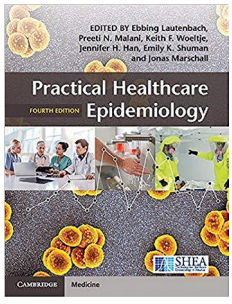
Practical Healthcare Epidemiology, 4th Edition

Ebbing Lautenbach, Preeti N. Malani, Keith F. Woeltje, Jennifer H. Han, Emily K. Shuman, Jonas Marschall; Cambridge University Press, Cambridge, UK; 2018; ISBN-10: 1107153166; ISBN-13: 978-1107153165; Pages: 452; Price: $185.00



On September 28, 2014, a 42-year-old Liberian man was admitted to a Texas hospital and quickly placed in isolation with a presumptive diagnosis of Ebola virus disease (EVD), a diagnosis that was subsequently confirmed. When caring for him, staff were advised to use personal protective equipment as recommended by the Centers for Disease Control and Prevention. Unfortunately, the patient died on October 8, and 2 nurses who had cared for him contracted EVD (*1*). This failure of infection control contributed to the public spread of panic, served as a wake-up call to the public health community, and helped convince the Centers for Disease Control and Prevention that EVD should be treated only in specialized hospitals with highly trained personnel (*2*).

The following spring, a different deadly virus spread rapidly through a modern healthcare system. In South Korea, over the course of 2 months, an outbreak of Middle East respiratory syndrome led to 186 confirmed cases and 38 deaths; most of the patients contracted the virus within healthcare facilities (*3*).

These examples may be dramatic, but healthcare-associated infections are acquired daily and affect an estimated 4% of all hospitalized patients (*4*). Patients are infected by microbes that are introduced by our actions (e.g., catheter-associated urinary tract infections), are made more deadly for patients with a frail medical status (e.g., influenza), or are emerging and resistant (e.g., *Candida auris,* carbapenem-resistant *Enterobacteriaceae*).

Although we may never win the war against disease-causing microorganisms, we can celebrate victories. Since the practice of infection prevention and control became a specialty in the 1960s, healthcare-associated infections have decreased substantially (*5*). New leaders in the field of infection prevention and control will always be needed. It is for these emerging experts that Lautenbach and colleagues have updated their book, Practical Healthcare Epidemiology, to a fourth edition. They set forth to provide "a pragmatic, easy-to-use reference" that provides "real-world advice" as if they were speaking to a person who would be running an infection prevention program and who was just starting in this field. They have succeeded.

Practical Healthcare Epidemiology covers a broad range of topics, organized into appropriately themed sections. The authors speak with authority, and there are no weak chapters. The chapter "Ethical Aspects of Infection Prevention," a subject that is often given short shrift, is itself worth the price of admission.

The 56 listed contributors are a major source of strength, but they also contribute to the book's minor weaknesses. Organizational consistency is lacking across chapters and even within chapters (e.g., the terms "antibiotic" and "antimicrobial" are used interchangeably). I found it slightly annoying that despite being aimed at "fledgling healthcare epidemiologists," the book contains no job description for this position and uses "healthcare epidemiologist" and "hospital epidemiologist" synonymously, without explanation.

But enough nit-picking. Practical Healthcare Epidemiology is valuable not just for its intended audience of new practitioners but for anyone involved in infection prevention and control. I especially recommend it for those looking for a practical, easy-to-understand—but not simple—resource.

Edward Lifshitz

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References

1. Cortese D, Abbott P, Chassin M, Lyon GM III, Riley WJ. The expert panel report to Texas health resources leadership on the 2014 Ebola events [cited 2019 Mar 28]. https://www.texashealth.org/assets/documents/system/public_relations/expert_panel_report_to_thr_on_evd_response.pdf
2. Centers for Disease Control and Prevention. Interim guidance for preparing Ebola Treatment Centers. 2014 Dec 15 [cited 2019 Mar28]. https://www.cdc.gov/vhf/ebola/healthcare-us/preparing/treatment-centers.html
3. Kim KH, Tandi TE, Choi JW, Moon JM, Kim MS. Middle East respiratory syndrome coronavirus (MERS-CoV) outbreak in South Korea, 2015: epidemiology, characteristics and public health implications. J Hosp Infect. 2017;95:207–13. https://doi.org/10.1016/j.jhin.2016.10.008
4. Magill SS, Edwards JR, Bamberg W, Beldavs ZG, Dumyati G, Kainer MA, et al.; Emerging Infections Program Healthcare-Associated Infections and Antimicrobial Use Prevalence Survey Team. Multistate point-prevalence survey of health care-associated infections. N Engl J Med. 2014;370:1198–208. https://doi.org/10.1056/NEJMoa1306801
5. Centers for Disease Control and Prevention. Data summary of HAIs in the US: assessing progress 2006–2016. 2017 Dec 5 [cited 2019 Mar 28]. https://www.cdc.gov/hai/data/archive/data-summary-assessing-progress.html

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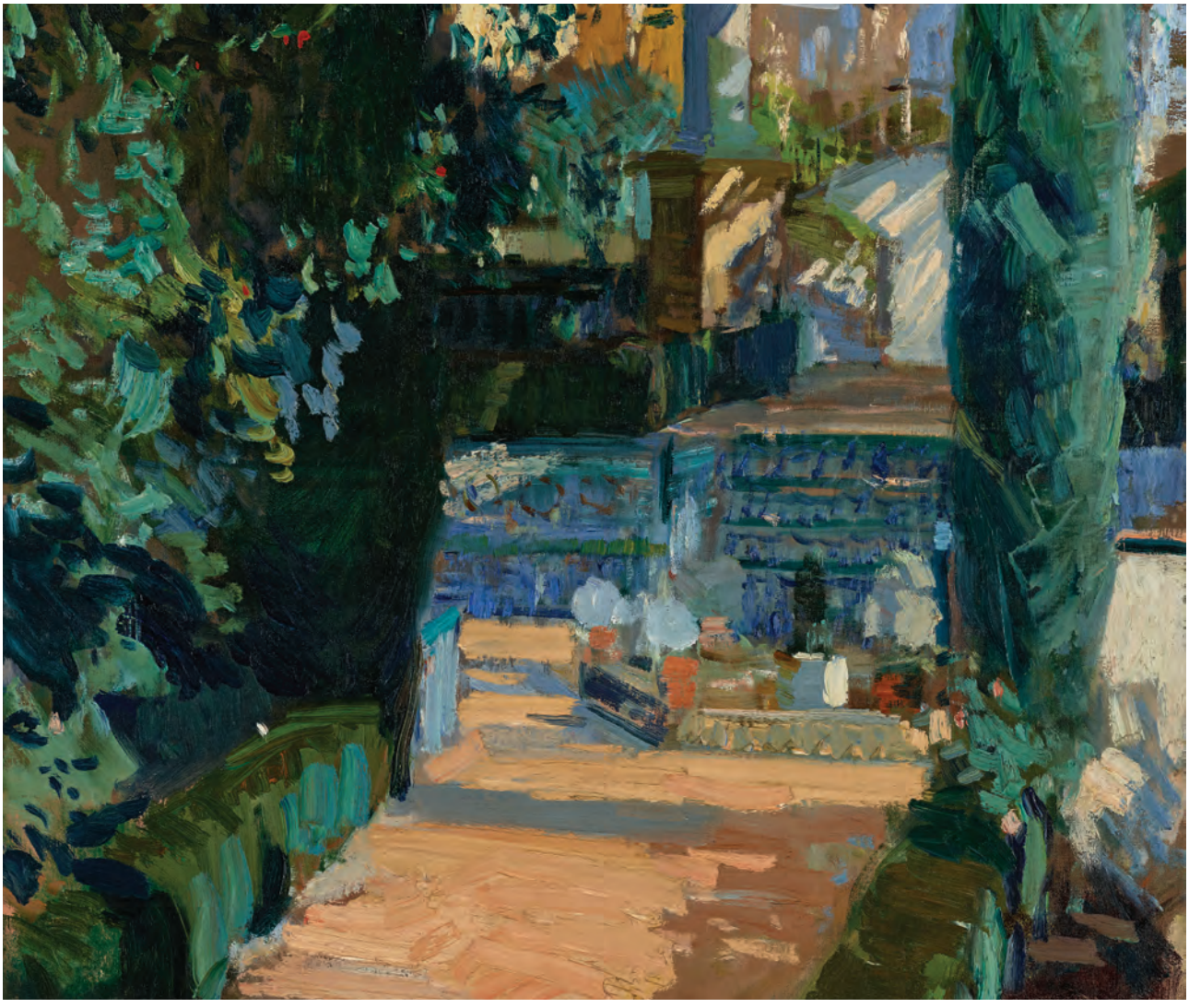

Joaquin Sorolla y Bastida (1863–1923). *Court of the Dances, Alcázar, Sevilla*, 1910 (detail)**.** Oil on canvas, 37.5 × 25 in./95.2 × 63.5 cm. Digital image courtesy of the Getty's Open Content Program, The J. Paul Getty Museum, Los Angeles, CA, USA.

"Many Things Grow in the Garden That Were Never Sown There"

Byron Breedlove

Among Europe's oldest gardens are those at the Real Alcázar Palace in the heart of Seville, Spain. This palace and its gardens were designated as an UNESCO World Heritage Site in 1987. During 1910, King Alfonso XIII summoned artist Joaquin Sorolla y Bastida to Seville to paint a portrait of the king for the Hispanic Society of America. While waiting 3 weeks for the king's arrival, Sorolla completed 18 paintings, including *Court of the Dances, Alcázar, Sevilla*, this issue's cover art, one of a pair of paintings that shows different views of the same resplendent garden known for its fruit trees, produce, and fragrant flowers.

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Sorolla scholars Blanca Pons-Sorolla and Monica Rodriguez Subirana describe Sorolla—frequently referred to as the "master of light" for his luminous, shimmering canvases—as "one of the most estimable, prolific, and fascinating personalities in the history of modern Spanish painting." Although Sorolla is best known for "depicting the color, movement, and play of light on the sea like no one else," Pons-Sorolla and Subirana point out that he also was one of the finest portraitists of his time and a noted landscape artist who "excelled at depicting gardens, one of the motifs he reproduced with the greatest splendor and sensibility."

In *Court of the Dances, Alcázar, Sevilla*, Sorolla's characteristically thick, dynamic brushstrokes capture the fecundity and textures of this sun-speckled garden. The intense blue sky glimpsed through the tangled vegetation contrasts with the deep green foliage. Spanish sunlight filters through tangles of flora, making leaves and fronds glow and shadows spill across the walkway, columns, and walls of this well-tended garden. Pons-Sorolla and Subirana note Sorolla "was interested in showing the sight lines around which the gardens were arranged, and how one entered these spaces through the leafy trees, placed in perspective with the background so as to convey a feeling of an infinite path."

According to the J. Paul Getty Museum, which houses this work, "The courtyard of Seville's Alcázar Palace, the city's most splendid example of Moorish architecture, sparkles in the dappled summer sunlight. As always, Joaquín Sorolla y Bastida was concerned with color and light, brilliance and atmosphere. The colored reflections of the light animate the scene and help to define the forms, creating a sense that nature is ever-changing."

"Many things grow in the garden that were never sown there" is among the enduring adages that English physician and writer Thomas Fuller included in his book *Gnomologia* (1732). That maxim, in its most literal sense, applies to various weeds, invasive plants, and other unwelcome flora that can surreptitiously infiltrate cultivated gardens such as those Sorolla depicted at the Alcázar Palace.

More broadly, this saying also provides a metaphor for the complex interactions of factors driving the surge in emerging infections across the globe, including climate change, human population growth and migration, antimicrobial resistance, and modern agricultural practices. Public health officials and researchers must be vigilant and focused in preparing for and responding to pathogens emerging in geographic locations and in populations where they were rare, nonexistent, or unknown.

Bibliography

1. Carlson CJ, Mendenhall E. Preparing for emerging infections means expecting new syndemics. Lancet. 2019;394:297. https://doi.org/10.1016/S0140-6736(19)31237-1
2. Fuller T. Gnomologia: adages and proverbs; wise sentences and witty saying, ancient and modern, foreign and British. London: Barker and Bettesworth Hitch; 1732. p. 143.
3. Huneker J. Eight essays on Joaquín Sorolla y Bastida, Volume 1. The art of Joaquin Sorolla by Leonard Williams. New York: Hispanic Society of America; 1909. p. 88–93, 209.
4. J. Paul Getty Museum [cited 2019 Sept 5]. http://www.getty.edu/art/collection/objects/694/joaquin-sorolla-y-bastida-corner-of-the-garden-alcazar-sevilla-spanish-1910/
5. Joaquin Sorolla y Bastida: the master of light [cited 2019 Sep 25] http://www.sorollapaintings.com/index.html
6. Morens DM, Folkers GK, Fauci AS. The challenge of emerging and re-emerging infectious diseases. Nature. 2004;430:242–9. https://doi.org/10.1038/nature02759
7. Pons-Sorolla B, Subirana MR. Sorolla: painted gardens. New York: Rizzoli Electa: 2019. p 7–16, 46–53.
8. Richman-Abdou K. Rediscovering Joaquín Sorolla: the Spanish impressionist known as a "master of light." My Modern Met [cited 2019 Sep 23]. https://mymodernmet.com/joaquin-sorolla-spanish-impressionism/
9. Sovek C. Light and color, Sorolla style. The Artist's Magazine, December 1990 [cited 2019 Sep 25]. http://www.sovek.com/publications/articles/sorolla/

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- Epidemiologic, Entomologic, and Virologic Factors of 2014–2015 Ross River Virus Outbreak, Southeast Queensland, Australia
- High Prevalence of Macrolide-Resistant *Bordetella Pertussis* and *ptxP1*
- Cost-effectiveness of Prophylactic Zika Virus Vaccine in the Americas
- Multicountry Analysis of Spectrum of Clinical Manifestations of Children <5 Years of Age Hospitalized with Diarrhea
- Zika Virus–Specific IgM 25 Months after Illness Onset, Miami–Dade County, Florida, USA
- Bagaza Virus Detected in Himalayan Monal Pheasants, South Africa, 2016–2017
- Divergent Barmah Forest Virus from Papua New Guinea
- Half-Life of African Swine Fever Virus in Shipped Feed
- Animal Exposure and Human Plague, United States, 1970–2017
- Sheep as Host Species for Zoonotic *Babesia venatorum*, United Kingdom
- Predicting Dengue Outbreaks in Cambodia
- Highly Diverse Rotaviruses in Common Shrews, Germany, 2004–2014
- Rhombencephalitis and Myeloradiculitis Caused by a European Subtype of Tickborne Encephalitis Virus
- Molecular Confirmation of *Rickettsia parkeri* in *Amblyomma ovale* Ticks from Veracruz, Mexico
- *Aspergillus felis* in Patient with Chronic Granulomatous Disease
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- Hemorrhagic Fever with Renal Syndrome, Russia
- Human Parasitism by *Amblyomma parkeri* Ticks Infected with *Candidatus Rickettsia* Paranaensis, Brazil

Complete list of articles in the December issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

November 20–24, 2019
American Society of Tropical Medicine and Hygiene
68th Annual Meeting
National Harbor, MD, USA
https://www.astmh.org/

January 28–30, 2020
American Society for Microbiology
2020 ASM Biothreats
Arlington, VA, USA
https://www.asm.org/Events/ASM-Biothreats/Home

February 20–23, 2020
International Society for Infectious Diseases
Kuala Lumpur, Malaysia
https://www.isid.org/

March 8–11, 2020
Conference on Retroviruses and Opportunistic Infections
Boston, MA, USA
https://www.croiconference.org/

March 9–13, 2020
African Society for Laboratory Medicine
7th African Network for Influenza Surveillance Epidemiology
Livingstone, Zambia
https://www.anise2020.org

April 18–21, 2020
The European Congress of Clinical Microbiology and Infectious Diseases
Paris, France
https://www.eccmid.org/eccmid_2020/

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Article Title

Lack of Efficacy of High-Titered Immunoglobulin in Patients with West Nile Virus Central Nervous System Disease

CME Questions

1. Your patient is a 57-year-old man with West Nile virus (WNV) neuroinvasive disease. According to the phase 2, randomized, double-blind, multicenter study by Gnann and colleagues, which of the following statements about safety and tolerability of Omr-IgG-am compared with that of intravenous immunoglobulin (IVIG) and normal saline (NS) in patients with WNV neuroinvasive disease is correct?

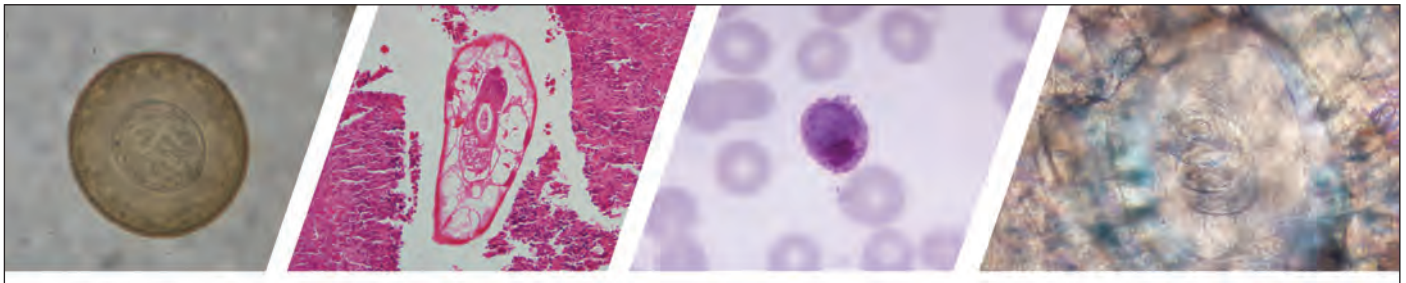
A. IVIG was significantly safer than Omr-IgG-am, according to recorded serious adverse events (SAEs), deaths, and laboratory testing
B. There was a persistent, nonsignificant trend toward better morbidity and mortality outcomes in the NS group than in the immunoglobulin (Ig) groups
C. Mortality in the study population was 5.9%
D. The most commonly reported treatment-related adverse event (TRAE) was seizure

2. According to the phase 2, randomized, double-blind, multicenter study by Gnann and colleagues, which of the following statements about efficacy of Omr-IgG-am compared with that of IVIG and NS in patients with WNV neuroinvasive disease is correct?

A. The battery of 4 neuropsychological tests showed significantly better efficacy with Omr-IgG-am than with NS
B. "Favorable" vs. "unfavorable" outcomes at day 90 occurred in significantly more patients receiving IVIG vs. Omr-IgG-am
C. Median duration of hospital stay was 10, 12, and 8.5 days for Omr-IgG-am, IVIG, and NS groups, respectively
D. On day 90, three-quarters of patients had normal/unimpaired neuropsychological function

3. According to the phase 2, randomized, double-blind, multicenter study by Gnann and colleagues, which of the following statements about clinical implications of safety and efficacy of Omr-IgG-am compared with that of IVIG and NS in patients with WNV neuroinvasive disease is correct?

A. The study authors recommended treatment with Ig products for patients with neuroinvasive WNV disease
B. Rash was the most common presenting symptom
C. Cerebrospinal fluid showed a predominance of neutrophils
D. Delays in enrollment and study drug administration could have reduced the potential efficacy of Omr-IgG-am



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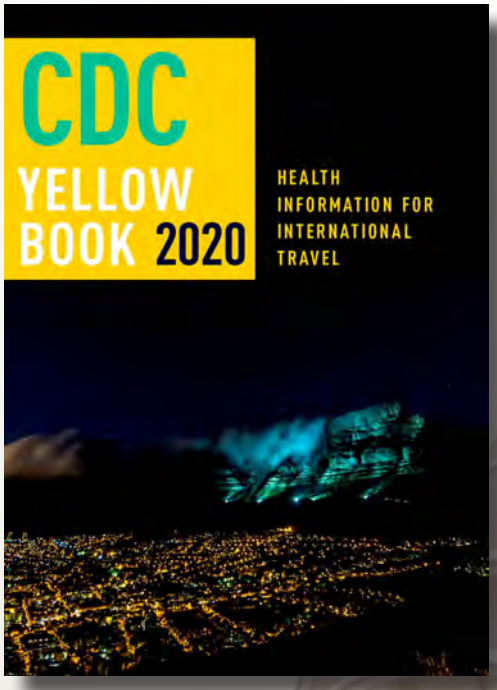

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Types of Articles

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.

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.

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.

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). 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. 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. Email to eideditor@cdc.gov.

December 2019 *Emerging Infectious Diseases* marks the final issue that will be published in print. We encourage you to make the transition with us. Read the journal and sign up for table of contents alerts: https://wwwnc.cdc.gov/eid

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