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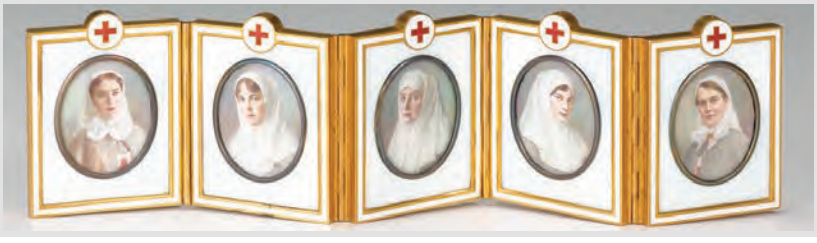

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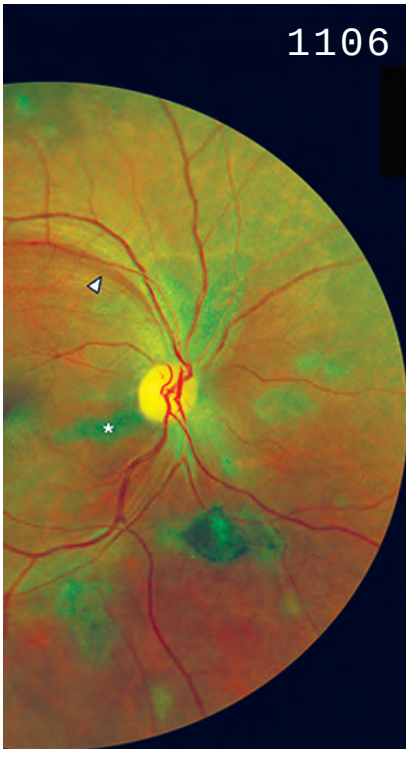

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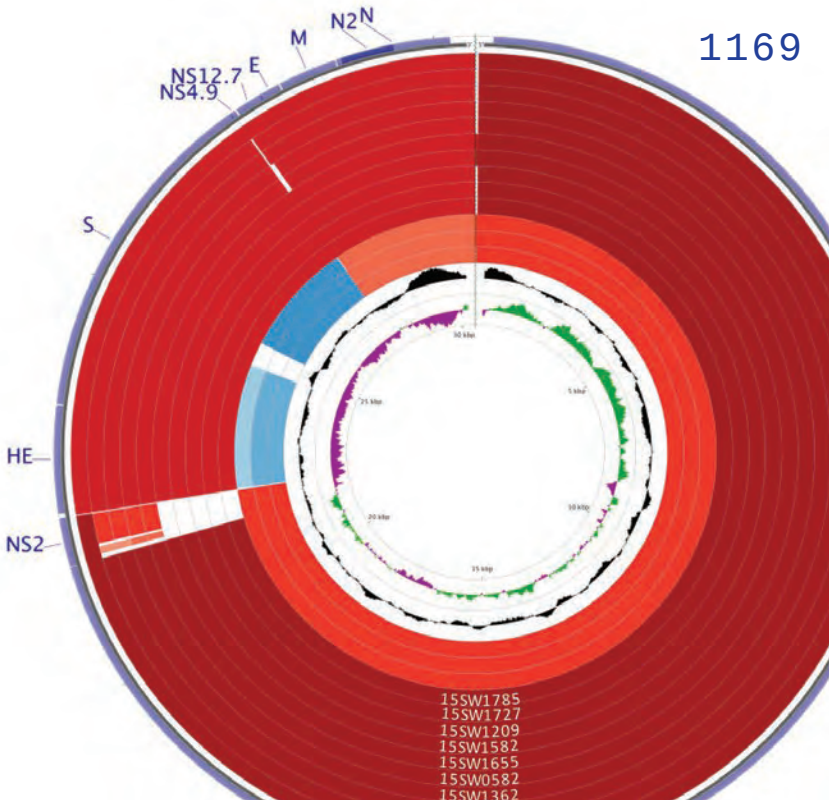

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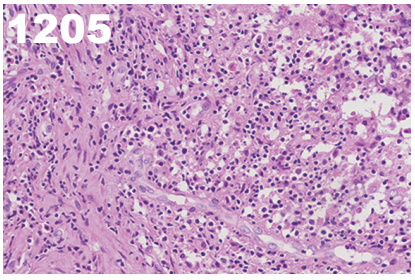

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Operational Research during the Ebola Emergency

Gabriel Fitzpatrick, Tom Decroo, Bertrand Draguez, Rosa Crestani, Axelle Ronsse, Rafael Van den Bergh, Michel Van Herp

Operational research aims to identify interventions, strategies, or tools that can enhance the quality, effectiveness, or coverage of programs where the research is taking place. Médecins Sans Frontières admitted ≈5,200 patients with confirmed Ebola virus disease during the Ebola outbreak in West Africa and from the beginning nested operational research within its emergency response. This research covered critical areas, such as understanding how the virus spreads, clinical trials, community perceptions, challenges within Ebola treatment centers, and negative effects on non-Ebola healthcare. Importantly, operational research questions were decided to a large extent by returning volunteers who had first-hand knowledge of the immediate issues facing teams in the field. Such a method is appropriate for an emergency medical organization. Many challenges were also identified while carrying out operational research across 3 different countries, including the basic need for collecting data in standardized format to enable comparison of findings among treatment centers.

Operational research is defined as the search for knowledge on interventions, strategies, or tools that can enhance the quality, effectiveness, or coverage of programs in which the research is being done (*1*). During the recent Ebola outbreak in West Africa, operational research was integrated into the response of Médecins Sans Frontières (MSF) to the emergency with the aim of controlling spread of the virus, improving patient outcomes, assessing the feasibility of new interventions, and advocating for policy change based on findings. Importantly, most operational research questions were decided by those in the field who had first-hand experience of the challenges encountered on a daily basis. This policy helped focus operational research to produce findings that were relevant to the emergency response.

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MSF, in close collaboration with other actors such as the World Health Organization (WHO) and various national ministries of health, has been detecting and controlling Ebola outbreaks for decades and uses 6 pillars for its approach:

- Isolation of Ebola patients and supportive medical and mental health care in dedicated Ebola treatment centers (ETCs);
- Contact tracing;
- Raising awareness in the community;
- A functioning surveillance and alert system;
- Infection control in communities and ETCs; and
- Maintaining healthcare for non-Ebola patients.

ETCs operated by MSF admitted ≈5,200 patients with laboratory-confirmed Ebola virus infection, ≈2,500 of whom survived. Consequently, the organization was in the unique position of being able to use its data and experience to answer operational research questions that had an impact on the 6 pillars of the Ebola response. This article aims to summarize the key findings of this published operational research and identify lessons learned and knowledge gaps.

Areas of Operational Research

Understanding Patients and How the Virus Spreads

Operational research has provided detailed information on the clinical signs and symptoms of infected patients arriving at ETCs (*2*–*6*), which has allowed health professionals to anticipate what they should look for when assessing patients. In addition, the level of virus in the blood (viral load) was shown to be the strongest predictor of patient survival (*7*,*8*), and this measure was used to target care and counsel family members regarding expectations for their loved ones (*9*); a higher viral load represented a greater risk for death of the patient.

A single case of sexual transmission of Ebola virus was identified in early 2015 (*10*). During the outbreak, sexual transmission of Ebola virus probably accounted for only a small proportion of overall cases. However, this mode of transmission might also be responsible for the flare up of cases that occurred after the declaration of the end of the outbreak in Sierra Leone and Liberia. MSF has advocated the importance of not stigmatizing survivors of

Ebola (based on risk for sexual transmission) because to do so would cause new Ebola patients to avoid seeking help (*11*).

Childbirth can also pose a risk for exposure in an Ebola setting. In one case of an Ebola virus–negative pregnant woman (who recently recovered), the fetus and surrounding amniotic fluid still harbored the virus (*12*). At delivery, there is considerable risk that those assisting in childbirth could be infected (*13*,*14*). Consequently, researchers have advocated that pregnant women who survived Ebola should be readmitted to the ETC when labor starts (*15*). The possible risk of Ebola passing from an infected mother to child through breast-feeding was also identified, resulting in a follow-up recommendation that breast-feeding be stopped and not restarted (*16*,*17*). The unexpected finding of a pregnant patient who initially was asymptomatic yet had a detectable Ebola viral load (*18*) showed how complex the study of Ebola virus transmission could be (*19*).

Psychologic counseling teams were employed throughout the outbreak to follow up and support the mental health of survivors. In Sierra Leone, an estimated one fifth of survivors were at high risk for developing posttraumatic stress disorder (*20*). These findings were used to advocate for more comprehensive care for survivors.

Clinical Trials

Clinical trials, although not considered actual operational research, formed a substantial component of the research undertaken in the field. MSF, in partnership with other organizations, highlighted that a trial drug called Favipiravir was of some benefit to patients who arrived to ETCs with a low Ebola viral load (*21*,*22*). In a previous outbreak of Ebola, decades ago, convalescent-phase plasma from survivors was transfused into infected patients with inconclusive results. MSF, as part of a team of national and international organizations, launched the largest-ever trial of convalescent-phase plasma in Guinea. The interim results showed there was no significant increase in survival among those who received the plasma (*23*). Of note, the study's findings are subject to some limitations and, consequently, further research is needed.

The Community

MSF anthropologists had the important role of finding out what the affected communities thought of the Ebola virus and government control measures, such as quarantine and cremation. This research identified areas of misunderstanding and rumor within the community. Health promotion messages and outbreak control measures were then targeted to address these knowledge gaps. In addition, anthropologists were crucial for identifying the beliefs and behaviors within communities that facilitated further spread of the Ebola virus (*24*).

Challenges within Ebola Treatment Centers

Laboratory Testing

The traditional way of diagnosing Ebola virus disease involved taking a blood sample from the patient by venipuncture and analyzing it by using PCR. At times, healthcare staff had difficulty performing venipuncture on very young children who were dehydrated (*25*). Staff performing venipuncture while wearing personal protective equipment (PPE) were also at risk for needle stick injuries that could result in nosocomial infections. Finger stick blood samples are much easier, faster, and safer to take than venipuncture samples. MSF questioned whether finger stick samples could be used instead of venipuncture for diagnosing Ebola virus disease. Staff in Guinea collected data on patients being screened for admission using both venipuncture and finger stick blood tests and found that finger stick samples were able to detect 87% of the Ebola cases confirmed using venipuncture samples (*25*). As a result of this research, it was recommended that finger stick blood sampling, although less accurate than venipuncture for diagnosing Ebola virus disease, could be used in situations where performing venipuncture was not possible.

The time between obtaining a blood sample and getting a PCR result can be considerable (*26*). MSF assessed the feasibility of using the Xpert Ebola Assay (Cepheid Inc., Sunnyvale, CA, USA) in the ETC setting and found that, when compared to traditional PCR testing, this assay reduced the waiting time between sampling and result notification by ≈50% (*26*). This difference was a major improvement in turnaround time for test results, especially for patients with suspected Ebola virus disease who were waiting to be admitted or discharged.

Different laboratories in the field used different types of PCR tests, which can give varying values for the viral load. As a result, comparing viral load results between laboratories for research purposes was occasionally difficult. MSF, in partnership with others, has advocated that standardized tests be employed so that viral load results can be compared among laboratory sites (*27*). Very rarely, the Ebola virus PCR test can give incorrect results; this fact was highlighted in an Ebola case from Monrovia with a false-negative PCR result (*28*). This complication underscores the need to always interpret test results in combination with the clinical and epidemiologic history of each patient.

Triage

Triage was used to determine which patients arriving to the ETC were likely to have Ebola virus infection or not. Patients who met the criteria of a suspect case using the WHO/MSF case definition were admitted to the suspect area of the ETC for blood testing. Patients whose illnesses did not meet the case definition were discharged from triage. If the triage step was not carried out appropriately with

an accurate case definition, then some potentially infected persons could be sent home and noninfected persons could be admitted to the area reserved for patients with suspected Ebola virus infection. Such a scenario posed a threat for further spread of the virus (*29*). An accurate point-of-care test by which staff could determine without delay whether a patient has Ebola virus infection would greatly improve the triage process (*30*).

Infection Prevention and Control

The minimum level of PPE that is required when treating Ebola patients is still not fully understood. In general, the higher the level of PPE, the more difficult it is for staff to attend to patients in tropical environments because of heat stress and loss of dexterity. MSF participated with several organizations to explore what the optimal PPE should be, and this research is ongoing (*31*).

Infection control was critical for patients admitted from triage into the area of the ETC reserved for patients with suspected Ebola virus infection. While in this area, patients had phlebotomy performed to check for the presence of Ebola virus; a positive test result resulted in the patient being admitted to the confirmed ward and a negative test resulted in discharge. Therefore, patients with and without Ebola virus disease were gathered together in the same space at the same time. If infection control measures were inadequate, noninfected persons awaiting PCR results could contract the virus from infected persons also awaiting results. However, investigation found no evidence of this kind of spread occurring in ETCs (*32*).

Effects of the Outbreak on Non-Ebola Healthcare

During the Ebola outbreak, researchers noted a major drop in clinic attendance by newly diagnosed HIV-positive patients and newly HIV-infected patients entering care in the affected countries (*33*). This trend was attributable to a combination of patients being afraid to attend clinics because of the known risk of contracting Ebola virus, clinics closing because of a lack of staff, and clinics reluctant to see new patients who might have symptoms compatible with Ebola virus disease.

Concerns were also raised that the control of tuberculosis (TB) in the region was jeopardized because of healthcare resources and personnel being focused solely on Ebola (*34*) and that operational research was required to document this problem and suggest strategies for better sustaining TB care during future epidemics. MSF's experience in the affected countries showed that basic non-Ebola healthcare, including maternal (*35*) and child healthcare, was adversely affected during the epidemic. National ministries of health in the affected countries, supported by various organizations, are currently running operational research courses to study the impact of the outbreak on health systems.

Evaluating Operational Research

The success of operational research is generally recognized to be measured within 4 domains (*36*). First, effective dissemination involves research findings being reported directly back to the field teams where the operational research was carried out. Second, the research should ideally be published in a peer-reviewed scientific journal. This step affords the work a level of acceptance within the wider community and can be a powerful aid when applied in the third domain, advocating for change to policy and practice. The fourth and most important measure of success is whether implementing the findings had a positive or negative impact on program performance and patient outcomes. Unfortunately, measuring impact is challenging and is often overlooked.

Strengths of MSF Operational Research

Important findings from MSF operational research were disseminated back to the field in a timely and appropriate manner to maximize patient welfare. The operational research produced by MSF was mostly decided by returning field staff. These persons noticed particular operational issues while working in the field and then committed to carrying out research to address them on their return. Such a method for selecting research topics can make the results particularly relevant for field teams caring for patients but can also bias research output by only selecting topics that returning persons bring forward. To balance this, the operational research unit also supported the development of research questions that needed to be answered during the course of the outbreak. The combination of allowing both field and office staff to develop and carry out research created a productive environment for scientific inquiry.

The operational research output was prioritized with pressing field questions that could be answered by using routine program data fast-tracked for investigation (*25*). The priority areas aimed to reflect the 6 pillars of Ebola outbreak control, but this was not always possible. Collection of data relevant to some of the pillars was limited; therefore, operational research within these domains was not feasible. MSF advocated that research be published in open-access format to maximize readership. In addition, all operational research articles were placed on the organization's field research website (http://fieldresearch.msf.org/msf).

Challenges for Operational Research

Operational research requires the collection of accurate, harmonized, routine data, and one of the issues with the large number of MSF ETCs spread across 3 countries was that information was not collected in a standardized way. This discrepancy led to difficulties when trying to amalgamate and analyze patient data. Additionally, clinical interventions, such as the use of intravenous fluids,

were not recorded systematically across ETCs. This lack of records proved to be a lost opportunity because retrospectively assessing what effect this intervention and others had on patient outcome was not possible. The use of personal digital assistants (i.e., small, mobile, handheld device that can store and retrieve information) has the potential to avert the problem of missed data collection in future outbreaks.

Research that was completed faced the challenge of finding journals that would review, accept, and publish the results in an appropriate timeframe. Some journals had the capacity to make quick decisions about publication, whereas others were slower and delayed the eventual dissemination of research to the wider scientific community. However, competing work priorities resulted in delays to some publications because authors were slow to finalize manuscripts.

The clinical trials were challenging to introduce in an emergency humanitarian setting and required impressive teamwork by all involved. Regrettably, even though the trials were fast-tracked compared with traditional timeframes, they were started very late in the outbreak, when case numbers were dwindling and efficacy was becoming more difficult to establish. In fact, many operational research questions across the 6 pillars of Ebola outbreak control still require comprehensive answers (Table).

Future Directions for Operational Research

The need to collect continuous accurate routine program data must be fundamental for any future outbreaks. This collection of data should not just focus on clinical outcomes but must include all disciplines, such as water and sanitation and health promotion. In view of limited resources, the choice of operational research priorities for further investigation should be decided by a scientific committee of medical, operational, and external experts. This step would potentially avoid the introduction of bias when selecting operational research questions.

Similarly, a support team involving an editor, operational researcher, and statistician could greatly facilitate field staff translating their operational research questions and findings into peer-reviewed scientific publications. Consideration should be given to the creation of a web-based, open-access scientific journal for MSF that acts as a repository for all relevant operational research. Such a journal would avoid the delays encountered with the peer-review process among certain publications while also maintaining scientific standards.

MSF advocacy continues to support the development of internationally recognized protocols and ethical guidelines for clinical trials during emergencies so when the next emergency occurs, trials can commence much sooner. Several international organizations have already developed emergency preparedness plans that allow for the rapid activation of research and development activities during large-scale epidemics (*37*,*38*). The objective of these organizations is to expedite the availability of effective tests, vaccines, and medications that can be used to save lives and contain serious outbreaks. The future challenge is that, although different organizations can be united by the common humanitarian objective of stopping an outbreak, different partners might have different priorities and interests that can threaten collaboration.

Conclusions

MSF has produced a comprehensive collection of published and unpublished operational research on the Ebola outbreak in West Africa. Importantly, the categories of research closely correspond to the 6 pillars of outbreak control described in this article. Operational research enables us to continually assess if new approaches are more effective than the ones currently in use and always aims to directly improve the care provided to those in need. The current model for deciding research topics within MSF is targeted toward addressing issues in the field. This approach is appropriate for an emergency medical organization. In this

Table. Operational research questions corresponding with the 6 pillars of Ebola outbreak control as observed by Médecins Sans Frontières*

Pillar of Ebola outbreak control	Key questions for operational research
Isolation of cases and supportive medical and mental health care in dedicated ETCs	Is it possible to provide isolation for case-patients outside the ETC setting, such as in the community? Survivors of Ebola can suffer from physical and psychological side effects. How can their follow-up care be most effectively carried out?
Contact tracing	Which methods of contact tracing can provide the most comprehensive, relevant, and timely data in the field setting?
Raising awareness in the community	What novel methods of communications should be used for raising awareness and promoting health?
A functioning surveillance and alert system	How can communities be convinced to participate in the alert system in an effective manner?
Infection control in communities and ETCs	For how long does Ebola virus remain infectious in the environment of ETCs and the houses of case-patients?
Maintaining healthcare for non-Ebola patients	What is the efficacy, efficiency, safety, and feasibility of triage systems in non-Ebola health structures?

*ETC, Ebola treatment center.

ever more connected world, MSF has advocated through its operational research for the creation of a functioning, international, rapid response capability for infectious disease outbreaks (*39*,*40*).

G.F. wrote the first draft of this manuscript. All other authors submitted amendments to the draft. G.F. incorporated all comments into the final manuscript that was then agreed upon by all authors.

Dr. Fitzpatrick has worked as a medical doctor and epidemiologist in the field with MSF. He currently holds a governance position within the organization. His area of interest concerns infectious diseases outbreak control.

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Measles Outbreak with Unique Virus Genotyping, Ontario, Canada, 2015

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The province of Ontario continues to experience measles virus transmissions despite the elimination of measles in Canada. We describe an unusual outbreak of measles in Ontario, Canada, in early 2015 that involved cases with a unique strain of virus and no known association among primary case-patients. A total of 18 cases of measles were reported from 4 public health units during the outbreak period (January 25–March 23, 2015); none of these cases occurred in persons who had recently traveled. Despite enhancements to case-patient interview methods and epidemiologic analyses, a source patient was not identified. However, the molecular epidemiologic analysis, which included extended sequencing, strongly suggested that all cases derived from a single importation of measles virus genotype D4. The use of timely genotype sequencing, rigorous epidemiologic investigation, and a better understanding of the gaps in surveillance are needed to maintain Ontario's measles elimination status.

In Canada, the last endemic measles case was reported in 1997, and elimination status was achieved the following year (*1*,*2*). This status is maintained as long as measles virus does not establish a chain of transmission spanning ≥12 months within a region (*3*). Against the background of elimination, a detailed travel history from measles case-patients is crucial to determine the probable source of infection. Laboratory investigation, including virus identification and genotyping, is also critical. Molecular epidemiologic analysis can provide information about transmission patterns of circulating virus strains and help identify potential sources of infection (*3*).

The high risk for measles importation because of diverse and globally connected communities and the high infectivity of the measles virus make maintenance of immunity within the Ontario population critical. Two doses of measles vaccine is the most effective method of preventing disease, and elimination can only be achieved and maintained with high vaccination coverage. Two-dose measles-containing vaccination coverage in Ontario was estimated at 88.3% among 7-year-olds and 95.4% among 17-year-olds during the 2012–13 school year (*4*). Two-dose vaccination coverage of ≥95% is recommended to achieve measles herd immunity (*5*,*6*).

We describe an unusual measles outbreak that occurred in Ontario, Canada, in 2015. Our analysis focuses on the outbreak response and laboratory findings.

Methods

Epidemiologic Investigation

Measles is a reportable disease in Ontario, requiring physicians and laboratories to notify local Ontario public health units immediately of all suspected and confirmed cases. Data regarding cases are captured in the provincial reportable disease database, the integrated Public Health Information System (*7*). We analyzed data on all measles outbreak case-patients with rash onset dates during January 25–March 23, 2015. Confirmed cases were defined according to the provincial measles case definition (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/16-1145-Techapp1.pdf) (*8*), and then according to an outbreak case definition, to exclude imported index cases and facilitate monitoring of potential outbreak cases. We also describe case-patient and contact investigation by public health unit, focusing on the 2 public health units handling the most cases, given that policies varied by health unit. Immunization information was acquired through interviews with case-patients or their legal guardians; information was validated through 1 of 2 provincial immunization

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repositories or providers. We obtained ethics approval from Public Health Ontario's research ethics board.

Laboratory Testing

Urine, throat swab, or nasopharyngeal swab specimens were collected from persons with a compatible clinical illness and submitted for molecular testing to Public Health Ontario Laboratories (PHOL), which performs frontline measles diagnostic testing. Total nucleic acid extraction was performed by using the NucliSens easyMAG extraction system (bioMérieux Canada Inc., Québec, Canada). One-step real-time reverse transcription PCR (rRT-PCR) was performed by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) and the TaqMan RNA-to-Ct 1-Step Kit (Life Technologies Corporation, Carlsbad, CA, USA). A previously published rRT-PCR protocol was used (*9*). Detection of ≥1 of the gene targets is considered sufficient for laboratory detection of measles virus. Serum or plasma specimens submitted for diagnostic serology were tested for measles virus IgM and IgG by using an ELISA test kit according to the manufacturer's instructions (Euroimmun, Luebeck, Germany).

Urine, throat swab, or nasopharyngeal swab specimens collected from laboratory-confirmed case-patients were referred to the National Microbiology Laboratory (NML) for genotyping. Total nucleic acid was extracted by using the QIAamp Viral RNA kit (QIAGEN, Valencia, CA, USA) or the MPLC Total Nucleic Acid Isolation Kit–High Performance on the MagNA Pure LC 2.0 (Roche Applied Science, Indianapolis, IN, USA). The World Health Organization (WHO) standardized genotyping regions (450 nt of the nucleoprotein gene [N-450] and 1,854 nt of the hemagglutinin [H] gene) were amplified (*10*) with primer pairs MVN1109/MVN1698R (online Technical Appendix Table), H1/H6, and H5/H2 (modified from Kessler et al. [*11*] by using the QIAGEN OneStep RT-PCR kit. Purified amplicons were sequenced by using amplification primers and H gene internal primers H3, H4, H7, and H8 (also modified from Kessler et al. [*11*]). The hypervariable noncoding region between the matrix and fusion genes (MF-NCR) (i.e., 1,024 nt, from the stop codon of the matrix gene [nucleotide 4,443 of MVi/New York.USA/26.09/3; GenBank accession no. JN635402.1] to the start codon of the fusion gene [nucleotide 5,466 of MVi/New York. USA/26.09/3; GenBank accession no. JN635402.1] inclusive) was amplified in 1 fragment (primers 4200f/5609r) or in 2 overlapping fragments (primers 4200f/4869r and 4801f/5609r) (where additional sequencing primers also are noted; online Technical Appendix). Raw sequence data were assembled and trimmed by using SeqMan Pro software (DNASTAR, Madison, WI, USA) and maximum-parsimony phylogenetic trees generated by using MEGA6 software (*12*). Genotypes were assigned by highest homology of N-450 sequences to WHO genotype reference sequences (*11*). Rapid genotyping for vaccine strains (genotype A) was performed for 30 clinical specimens from 25 patients by using a genotype A–specific rRT-PCR developed in-house (A. Severini, pers. comm.) and confirmed by standard genotyping.

Statistical Analyses

We calculated incidence rates by using 2015 demographic data from Statistics Canada obtained through IntelliHealth Ontario. Descriptive analyses were performed by using Microsoft Excel 2010 (Microsoft, Redmond, WA, USA). Where appropriate, we excluded cases with missing data from analyses.

Results

Descriptive Epidemiology

Overall

Nineteen measles cases were reported during the period under surveillance; however, 1 case was excluded because it did not meet the outbreak case definition (an imported case in a patient with a travel history to Pakistan and a rash onset occurring after the outbreak was underway). Therefore, 18 confirmed cases of measles with rash onset occurring January 25–February 17, 2015, were reported by 4 public health units and met the outbreak case definition (Figure), representing an overall incidence of 1.3 cases/1 million population. Case demographic and immunization data were summarized (Table 1); 61% of case-patients were adults. Immunization status was known for 14 of the 18 case-patients, and of these, most (64.3%) were unimmunized (3 adults and 6 children). The 2 fully immunized case-patients

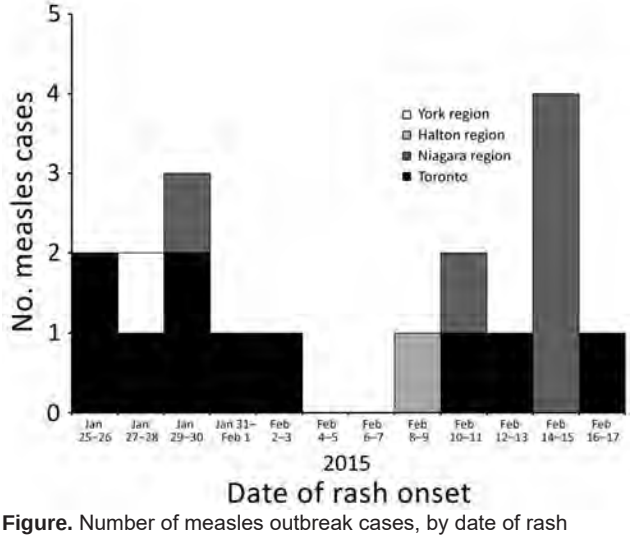

Figure. Number of measles outbreak cases, by date of rash onset, Ontario, Canada, January 25–March 23, 2015.

Table 1. Demographic characteristics of 18 case-patients identified during a measles outbreak, by health unit and overall, Ontario, Canada, January 25–March 23, 2015*

Characteristic	No. (%) patients			
	NRPH	TPH	Other PHU	Overall
Age group, y				
<18	4 (66.7)	3 (30.0)	0	7 (38.9)
≥18	2 (33.3)	7 (70.0)	2 (100)	11 (61.1)
Sex				
M	2 (33.3)	4 (40.0)	2 (100)	8 (44.4)
F	4 (66.7)	6 (60.0)	0	10 (55.6)
Hospitalized				
Yes	1 (16.7)	1 (10.0)	0	2 (11.1)
No	5 (83.3)	9 (90.0)	2 (100)	16 (88.9)
Immunization status				
Unknown	0	3 (30.0)	1 (50)	4 (22.2)
Known	6 (100.0)	7 (70.0)	1 (50)	14 (77.8)
Unimmunized	6	3	0	9
1 dose	0	3	0	3
2 doses	0	1	1	2

*NRPH, Niagara Region Public Health; PHU, public health unit; TPH, Toronto Public Health.

were adults (Table 2). Two adult case-patients were hospitalized, and a third sought emergency department treatment. All case-patients recovered without complications.

Toronto Public Health

On January 28, 2015, the first measles case of this outbreak was reported to Toronto Public Health (TPH). Three additional cases were reported during the following 3 days, triggering extensive public health investigations, a local outbreak declaration, and activation of the Incident Management System within TPH to help manage the response. Six additional cases were confirmed during the following 3 weeks, yielding a total of 10 cases (Figure).

Each case-patient was asked about daily activities during the 21 days before symptom onset (*13*) to identify locations where the virus might have been acquired or transmitted. Initial case-patient interviews focused on travel, contact with others who traveled or anyone known to be ill with measles, and visits to healthcare providers or hospitals. A list of acquisition and transmission exposures was updated daily and used to inform questions during interviews of subsequent case-patients and to search for epidemiologic links between cases.

None of the confirmed case-patients reported travel to a measles-endemic area. Some community exposures were identified for subsets of cases, but evidence was insufficient to confirm links in both place and time. Because epidemiologic links were not identified through traditional methods, 3 case-patients were reinterviewed with an exposure-focused questionnaire. The questionnaire was supplemented by reference to publicly available social media information (e.g., Instagram posts) to facilitate recall and by requests to review online banking records to identify additional acquisition and exposure locations. Concurrently, a daily updated list of potential acquisition and transmission exposures from the previous day's interviews was imported into Pajek social networking software (*14*) to automate the identification of overlapping exposures. This step increased efficiency and ensured a more systematic search for potential linkages. Nonetheless, no epidemiologic links could be confirmed for TPH's cases. Given the rash onset dates (January 25–February 17, 2015), there might have been 2 or 3 generations of cases (*15*).

TPH investigated an additional 140 suspected measles cases during the outbreak period. Clinical presentations of patients with suspected infection were compared with the provincial case definition, and clinical samples were collected from these patients to confirm or rule out measles. Patients were provided with health education and asked to temporarily self-isolate from group settings. In a break from usual policy, persons living in Toronto who were recently vaccinated before onset of a measles-like rash and suspected of having an adverse event after immunization were investigated by genotyping to rule out the possibility of an actual measles infection, given that measles might be circulating in parts of the community.

TPH identified 1,548 contacts who were potentially exposed to the 10 confirmed measles case-patients, including 223 persons who resided outside of Toronto. Almost all (96.7%) were associated with exposure at healthcare institutions, with the number of contacts identified ranging from 3 at a physician's office to 414 at a major acute care hospital. In addition, 51 contacts (3.3%) were identified in association with noninstitutional exposures. Contacts were categorized as high- or low-risk, according to Ontario Ministry of Health and Long-Term Care protocol (*7*) and TPH Measles Policy. High-risk contacts included household contacts, susceptible pregnant women, infants <12 months of age, immunocompromised persons, healthcare workers, or children 1–6 years of age who might not have received their second dose of measles, mumps, and rubella (MMR) vaccine. All other contacts were considered

Table 2. Selected characteristics of 18 case-patients identified during a measles outbreak, Ontario, Canada, January 25–March 23, 2015*

Case-patient no.	Date of rash onset	Age, y	Health unit	Immunization status, no. doses	IgM	Sequencing of N-450, H gene, and MF-NCR†
1	Jan 25	22	Toronto	0	+	Type sequence to which sequences from all other cases are compared
2	Jan 26	39	Toronto	1	+	1 SNP in the H gene (C961T)
3	Jan 27	1	Toronto	0	IND	No changes
4	Jan 27	24	York	2	+	No changes
5	Jan 29	20	Niagara	0	+	No changes
6	Jan 29	55	Toronto	Unknown	+	H gene sequence not determined; otherwise no changes
7	Jan 30	1	Toronto	0	NT	1 SNP in the H gene (C1497T)
8	Jan 31	39	Toronto	2	+	1 SNP in the MF-NCR (G932A)
9	Feb 2	46	Toronto	Unknown	+	No changes
10	Feb 9	35	Halton	Unknown	+	No changes
11	Feb 10	14	Niagara	1 (PEP)	–	No changes
12	Feb 11	34	Toronto	1	+	No changes
13	Feb 13	2	Toronto	Unknown	+	No changes
14	Feb 14	10	Niagara	1 (PEP)	NT	No changes
15	Feb 14	17	Niagara	1 (PEP)	NT	No changes
16	Feb 14	10	Niagara	1 (PEP)	NT	No changes
17	Feb 14	23	Niagara	0	NT	Not determined (specimens unavailable)
18	Feb 17	41	Toronto	1	+	No changes

*H, hemagglutinin; IND, indeterminate; MF-NCR, noncoding region between the matrix and fusion genes; N-450, 450 nt of the nucleoprotein gene; NT, not tested; PEP, postexposure prophylaxis, SNP, single-nucleotide polymorphism.
†All sequence results are in reference to the earliest detected sequence: MVs/Ontario.CAN/3.15 (GenBank accession nos. KU218405, KU218406, and KX396596 for the N-450, H gene, and MF-NCR, respectively), which is designated the "type sequence" for the purposes of this outbreak. The N-450 sequences, which were identical for all 17 cases with sequences, did not match any named lineage in MeaNS, the World Health Organization's measles sequence database.

low-risk. High-risk and household contacts were interviewed by telephone or a home visit. Low-risk contacts were notified of their exposure and provided with information and directions in letters couriered to their home addresses. A measles hotline was established to take calls from healthcare providers, contacts who had received letter notification, or the general public. The line received 280 calls during its period of operation.

In addition to making 578 phone calls and sending 808 exposure notification letters, TPH issued 153 exclusions to unimmunized and underimmunized persons associated with high-risk settings (e.g., daycare attendees and healthcare workers) and provided either MMR vaccine or immunoglobulin as postexposure prophylaxis for 132 persons, depending on the timing of the exposure and risk condition in the contact, according to provincial guidelines (*7*). No known secondary cases were found among identified contacts.

Niagara Region Public Health

During the outbreak period, Niagara Region Public Health (NRPH) identified 6 confirmed cases of measles (Figure) and investigated links among the cases. Five cases had laboratory confirmation and the remaining case had an epidemiologic link to a laboratory-confirmed case. The index case-patient had traveled to Toronto during her incubation period. Although no direct link could be found to any TPH case-patient, the NRPH patient traveled on public transport and went to a large entertainment venue while in Toronto. The other 5 case-patients, all of whom were unimmunized, were family members of the index case-patient and were deemed secondary cases. Four of the 5 secondary case-patients received 1 dose of MMR vaccine after exposure and before becoming case-patients; however, these doses were not administered within 72 hours. An additional 25 suspected cases were reported and investigated by NRPH during this period; none met the case definition.

NRPH staff identified and followed up on a total of 1,837 contacts who were potentially exposed to the 6 case-patients. Two exposure sites outside of the region were identified, and additional notification was given to the other health units involved. Most (88%) of the Niagara contacts were associated with exposure at schools and school-related activities. Contact management was conducted by telephone, record review, or in-person interview. Panorama, the provincial immunization repository, was used to identify that 79 NRPH students were either not fully immunized or did not have complete immunization records reported. Health education and recommendations were provided to all students. Most students (55 [70%]) submitted appropriate documentation, whereas 24 (30%) received exclusion letters and were not able to return to school until NRPH received documentation that the student was fully immunized. A total of 279 contacts (15%) who were associated with other exposure settings (e.g., private residences, community centers, retail stores, and healthcare institutions) were identified. Contact management involved a review of

the contact's measles susceptibility, immunization status, and health education and recommendations for further action (e.g., vaccine), if needed.

In February 2015, a total of 367 doses of MMR or measles, mumps, rubella, and varicella (MMRV) vaccine were administered at NRPH general immunization clinics. This number is 350 doses more than the annual historical average during the previous 2 years. The increased demand resulted in increases in the capacity and service hours of the general immunization clinics and the addition of 3 supplementary MMR/MMRV vaccine–only clinics and 2 school-based clinics. Additionally, 4,826 doses of measles-containing vaccine were distributed to community health providers. This number is 3,885 more than the annual historical average during the previous 2 years. At the same time, staff received ≈12,000 incoming calls regarding measles, compared with the typical average of ≈3,700 calls per month. NRPH also made ≈8,000 outgoing calls for measles follow-up actions compared with the average of ≈3,200 calls per month. A measles hotline was activated to assist in managing call volumes.

Additional Cases

Two additional outbreak cases were reported among residents of Halton Region and York Region public health units (Figure). Both case-patients were male adults, and neither had recently traveled. One case-patient had received 2 doses of MMR vaccine, and the other had an unknown immunization status. Staff from these public health units instituted disease control measures to manage susceptible contacts and exposure settings and to rule out epidemiologic links to cases from TPH or NRPH.

Laboratory Testing

Diagnostic Testing

During the outbreak period, PHOL received 966 specimens from 610 patients for measles rRT-PCR testing, including the 17 outbreak-related case-patients. Measles RNA was detected in 58 specimens from 36 persons; NML detected measles virus by PCR in specimens from 33 of those persons. Of the specimens in which measles RNA was detected by PCR at PHOL but not reproduced at NML, 1 was considered a false positive at PHOL, and 2 had received measles-containing vaccine before onset of symptoms and, like most patients with vaccine-associated cases, probably had viral loads near the threshold for detection and therefore were missed upon retesting at NML. NML identified wild-type virus in 17 of 33 persons and genotype A vaccine strain by conventional N-450 sequencing in the remaining 16. In addition, NML rapidly detected vaccine strain in 15 of these 16 persons by using a laboratory-developed rRT-PCR specific for measles vaccine strain, providing genotypic information up to several days before traditional N-450 sequencing.

A total of 1,484 serologic specimens were submitted for diagnostic testing (IgM and IgG) during the outbreak period, compared with 262 specimens during the corresponding period in 2014. In addition, 34,708 specimens were submitted to check immune status (IgG serologic tests only), representing a 155% increase in submissions to PHOL compared with the same period in 2014, when 13,606 specimens were received for immunity screening. In total, 47 (3%) of the 1,484 specimens submitted for IgM testing were reactive. IgM serologic specimens were submitted for 14 of the 17 PCR-confirmed measles cases; 11 of these 14 cases were IgM reactive, and 1 was IgM indeterminate. Among the 8 PCR-positive patients with measles vaccine strain, 6 were IgM positive, 1 was IgM indeterminate, and 1 was IgM negative. Excluding the 7 IgM-reactive specimens in persons documented to have been shedding vaccine strain, most (28/40 [70%]) of IgM-reactive specimens reported did not represent measles infection.

Genotyping

Seventeen outbreak-related measles cases were genotyped using the WHO-recommended targets to gather evidence of relationships between cases and the possible outbreak origin. All cases were genotype D4 infections, with identical N-450 sequences (GenBank accession no. KU218405). Full-length H gene sequences (1,854 nt) were successfully obtained from 16 case-patients. Sequences from 14 case-patients were identical to each other (GenBank accession no. KU218406), whereas 2 were each 1 nucleotide different from the majority sequence (Table 2). A search of the N-450 sequences deposited into MeaNS, the WHO measles sequence database (*16*), revealed that the identified D4 viral strain was not associated with any reported measles activity globally, although it had been identified in 2 contemporary imported cases in New York state (GenBank accession nos. KP797976 and KP797977). In the absence of clear travel history or an epidemiologic link between most case-patients, extended genotyping of the MF-NCR was performed to seek additional evidence about possible chains of transmission. With the exception of 1 sequence that differed by a single nucleotide (Table 2), all cases had identical MF-NCR sequences (GenBank accession no. KX396596) and shared a characteristic previously unreported pattern of insertions and deletions.

Discussion

This outbreak was unusual as no travel history or common case-patient exposures were noted, despite intensive investigation, and laboratory evidence strongly suggested that all

cases formed the same chain of transmission. Despite rapid increases in reported cases early in the outbreak, the total number of cases was relatively small and transmission was limited. The relatively high immunization coverage in Ontario (*4*) probably played a role in this respect; most cases occurred in either unimmunized people or those who were not fully immunized. This outbreak occurred in an area of the province where there is a large multicultural population and a high volume of international travelers (*17*). Limited transmission might also reflect that the outbreak occurred in areas of Ontario with a high proportion of immigrants from measles-endemic countries, who are more likely to have natural immunity from previous infection (*17*).

We never identified a source case in this outbreak, and an interval of >5 months elapsed between report of the first case in the outbreak and report of the previous measles case in the province. Typically, we are able to identify linkages with travel or potential exposure sites for recent measles cases in Ontario. Although some overlapping exposures were identified, no epidemiologic links could be confirmed among the index case in NRPH or any of the cases in Toronto, York Region, or Halton Region. This finding might reflect an exposure that involved a casual interaction not deemed worth mentioning by the case-patient but one that was actually critical given the highly communicable nature of the measles virus. Alternatively, a source case-patient might have been exposed other case-patients in several locations while moving through the city during the infectious period (i.e., a point-source case but not a point-source exposure). The absence of a source case indicates that not all cases were reported to public health (*15*,*18*).

Many clinicians might not consider a diagnosis of measles in a patient with measles-like symptoms, either because of lack of familiarity or because its rarity results in omission from the differential diagnosis. Clinicians might also be unfamiliar with appropriate diagnostic testing. During this outbreak, we produced guidance for physicians about best practices for assessing suspected measles cases (*19*). These guidelines indicated that most patients should receive measles-containing vaccine when doubt exists about their immune/immunization status, rather than serologic testing, given that serologic testing can delay protecting a nonimmune person and can result in unnecessary healthcare utilization (*19*). Despite this guidance, a large volume of samples were sent to PHOL for IgG serologic testing. IgM serologic testing is also problematic because there is a high likelihood of false-positive tests in a low-prevalence setting (*20*). Our findings substantiated this; >70% of the positive measles IgM tests were from persons who were not ultimately reported as having measles. Serologic testing cannot differentiate between the immune response after wild-type infection and recent immunization; therefore, it is not recommended for determining immunity in well persons during an outbreak or among persons who have been recently vaccinated.

All 17 cases that were genotyped using the WHO-recommended N-450 and H gene targets showed identical or minimally variable sequence. Because measles viral strains associated with large outbreaks globally can result in repeat importations of the same sequence, our findings cannot be taken as absolute proof of a single importation. A single nucleotide difference can be enough to suggest separate importation events (*21*). Consequently, the possibility of multiple importations cannot be excluded. Nonetheless, the hypothesis of a single importation was supported by sequencing of MF-NCR, which has been identified as a hypervariable region within the measles genome (A. Severini, pers. comm.). With the exception of 1 case that differed by a single nucleotide, this region was identical in this outbreak and showed a characteristic pattern of insertion/deletion. This pattern clearly distinguished this outbreak from those associated with the most closely related reported isolates MVi/New York.USA/26.09/3 and MVi/Florida.USA/19.09, strongly suggesting that all cases derived from a single importation event. The genetic diversity of measles virus decreases as progress is made toward global elimination. As a result, extended genotyping beyond the WHO standard targets, as was required in this outbreak, will probably be needed more often to define the molecular epidemiology of measles outbreaks (*22*).

In conclusion, until measles is eradicated worldwide, Ontario's public health system continues to respond to measles activity. The level of response is challenging from a public health perspective. The use of timely genotype sequencing, rigorous epidemiologic investigation, and a better understanding of the gaps in surveillance are needed to maintain Ontario's measles elimination status. Molecular epidemiologic analysis beyond WHO-recommended targets will probably play an increasing role in the future.

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At the time of submission, Ms. Thomas was an epidemiologist at Public Health Ontario in Toronto, Canada. Her primary areas of research interest are applied immunization and vaccine-preventable diseases, with the objective of informing public health policy.

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Case–Control Study of Risk Factors for Meningococcal Disease in Chile

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An outbreak of meningococcal disease with a case-fatality rate of 30% and caused by predominantly serogroup W of *Neisseria meningitidis* began in Chile in 2012. This outbreak required a case–control study to assess determinants and risk factors for infection. We identified confirmed cases during January 2012–March 2013 and selected controls by random sampling of the population, matched for age and sex, resulting in 135 case-patients and 618 controls. Sociodemographic variables, habits, and previous illnesses were studied. Analyses yielded adjusted odds ratios as estimators of the probability of disease development. Results indicated that conditions of social vulnerability, such as low income and overcrowding, as well as familial history of this disease and clinical histories, especially chronic diseases and hospitalization for respiratory conditions, increased the probability of illness. Findings should contribute to direction of intersectoral public policies toward a highly vulnerable social group to enable them to improve their living conditions and health.

Meningococcal disease has a case-fatality rate of 50% for patients not given treatment and 10%–20% for those given treatment (*1*,*2*). The causative agent is *Neisseria meningitidis*, for which 14 serogroups have been identified; 6 (A, B, C, W, X, and Y) can cause human disease (*3*). Geographic distribution and epidemic potential differ for each serogroup (*3*). Serogroups A, B, and C are responsible for 80%–90% of cases worldwide, and serogroups Y and W account for the remaining 10%–20%. The extended meningitis belt of sub-Saharan Africa has the highest frequencies of this disease. Before 2010 and mass preventive vaccination campaigns during the MenAfriVac project (http://www.meningvax.org/), group A meningococcus accounted for ≈80%–85% of all cases in the meningitis belt; epidemics occur at intervals of 7–14 years. Since that time, the frequency of serogroup A has decreased, including carriage (*4*), but other meningococcal serogroups, such as W, X, and C, still cause epidemics, albeit at a lower frequency and smaller size (*2*).

The principal clinical forms of meningococcal disease are meningeal syndrome, meningococcal sepsis, and pneumonia. Mortality rates increase for meningococcal sepsis (8%–13%), and rates for sepsis with shock can reach 34%–73% (*5*). Sequelae are present in 10%–20% of patients; the most common ones are limb necrosis, neurologic impairment, and deafness (*1*). Risk factors for disease development and death include individual characteristics, environmental and living conditions, and access to healthcare. Socially disadvantaged persons are at greater risk for disease development and have less access to healthcare resources; thus, illness and death rates for these persons are higher than for those in more privileged social positions (*6*,*7*).

In the 1990s in Chile, the incidence rate of meningococcal disease was stable (≈3.5 cases/100,000 persons). This rate began to decrease gradually in 2001 and eventually reached 0.5 cases/100,000 persons by 2010 (a low level of endemicity) (*5*). The predominant serogroup until 2012 was serogroup B. During this time, there were also small outbreaks of serogroup C disease that were controlled with vaccines and isolated cases of serogroup W disease, which made up only 2% of the total. However, the incidence of meningococcal disease began to increase in 2010 because of an increase in serogroup W (*5*), which by 2012 represented 58% of all cases (Figure). This increase in serogroup W occurred simultaneously with an increased mortality rate (from 10% to 30%) and nonspecific clinical symptoms and sequelae such as amputations, hearing loss, and neurologic damage in 10% of case-patients (*8*). As a result of this increase in meningococcal disease, a vaccination campaign was implemented in Chile at the end of 2012 with a quadrivalent meningococcal conjugate vaccine for serotypes A, C, W, and Y for children <5 years of age.

In this context, the Ministry of Health of Chile considered it necessary to conduct a study to identify factors associated with development of meningococcal disease. This

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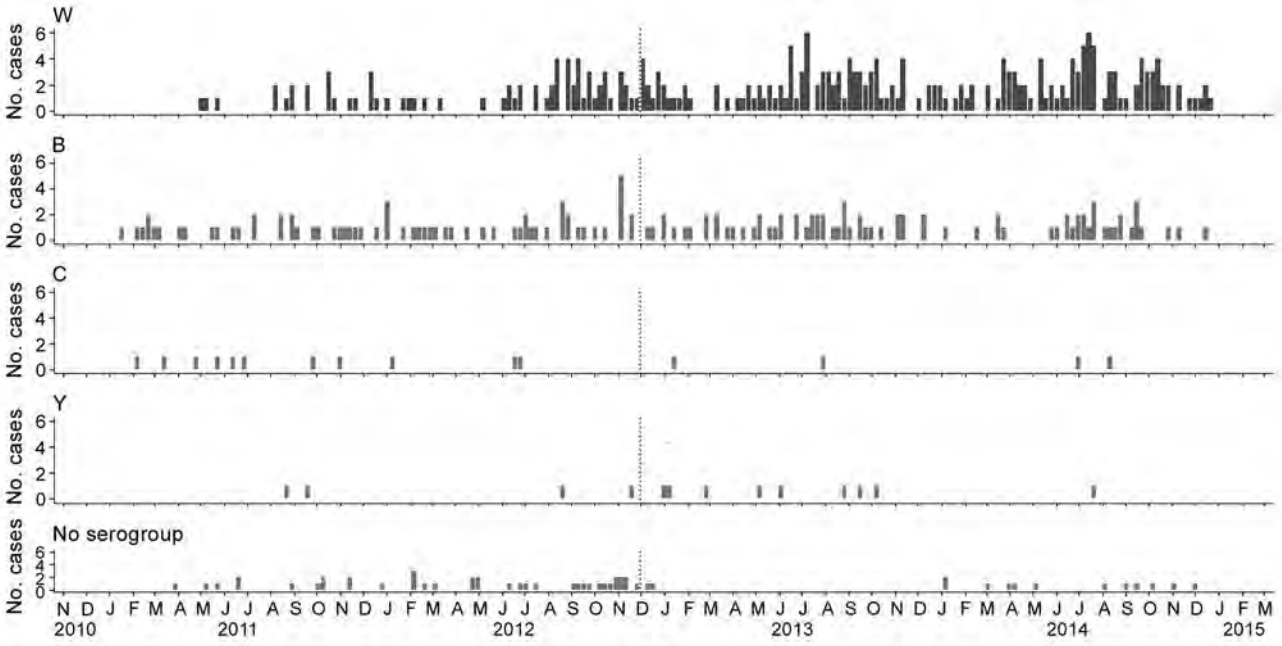

Figure. Monthly cases of meningococcal disease by serogroup, Chile 2011–2014. Dotted vertical line indicates beginning of the vaccination campaign against *Neisseria meningitidis* serogroups A, C, W, and Y. Data were obtained from the Department of Epidemiology, Ministry of Health of Chile.

study was based on the hypothesis that occurrence of this disease is related to living conditions, lifestyles, and medical histories of infected persons.

Methods

We conducted a population-based, case-control study to identify risk factors for meningococcal disease. The study included regions in Chile where ≥3 cases of disease occurred during January 2012–March 2013. All confirmed cases of meningococcal disease during this period were included; there were no age or nationality restrictions, including deceased patients. Cases were identified by using the monitoring system of the Ministry of Health of Chile because regulations in Chile require that all cases in this country must be reported immediately (*9*). Cases were confirmed by isolating *N. meningitidis* from cerebrospinal fluid cultures or cultures of other normally sterile fluids or tissues, or by PCR for cerebrospinal fluid or plasma (*1*). All strains were confirmed and serotyped at Public Health Institute of Chile by the slide agglutination test (*10*).

We assigned 4 controls per case-patient, which enabled 98% power to detect 50% more overcrowding in case-patients than in controls, a variable that is a risk factor for meningococcal disease (*11–14*) and is reliable and available for Chile. Controls lived in the same municipalities as case-patients, resided in Chile during the study period, and had not been given a diagnosis of meningococcal disease or meningeal syndrome of unknown etiology. Group matching for sex and age was performed by using a proportional distribution of controls corresponding to the number of inhabitants in each municipality and the proportions of sex and age (<2, 2–6, 7–18, 19–45, 46–65, and ≥66 years) observed for case-patients. Controls were selected by using a 3-stage random sampling procedure (blocks, homes, and persons) based on the most recent census data available. Controls who refused to participate were replaced in the same area by means of a systematic skipping of dwellings.

We studied case-patients and controls by using a structured questionnaire that contained 118 questions, including variables related to social and environmental determinants (education, occupation, income, healthcare system, housing, overcrowding); individual factors (sex, age, ethnicity, habits such as alcohol and tobacco use, and medical history); and vaccination history for meningococcal disease (vaccine against serogroups A, B, and C and quadrivalent meningococcal conjugate vaccine against serogroups A, C, W, and Y). Vaccination status and date were verified by vaccination card for children <5 years of age and through self-report for persons ≥5 years of age. We explicitly asked case-patients about the period before their disease. We asked controls about their living conditions and lifestyles at the time of the survey and ≤2 years before to coincide with exposure periods of case-patients. For persons <14 years of age or those who died, the survey was conducted with a relative. Collection of information was conducted by physicians, public health professionals, and epidemiologists during August 16–November 22, 2013.

We captured information by using the SurveyToGo program (http://www.dooblo.net/stgi/downloads.aspx) and exported the database into SPSS version 21.0 (IBM, Armonk, NY, USA), which was validated data on a weekly basis. Descriptive and bivariate analyses were conducted. Measures of central tendency and dispersion were calculated for quantitative variables, and frequencies and percentages were used for categorical variables. Statistical tests were used to identify significant differences ($p<0.05$ in a 1-sided test) between the case-patients and controls. A Fisher exact or χ^2 test was used for categorical variables, and a Mann-Whitney test was used for continuous variables, after checking that these variables did not distribute normally by the Shapiro–Wilk test. Further analyses of significant results involved calculating raw odds ratios (ORs) and 95% CIs.

The effect of exposure variables was evaluated by using multivariate logistic regression models to estimate the probability of development of meningococcal disease, which produced adjusted ORs and 95% CIs. Multivariate models included variables that were significant in bivariate analysis, which was conducted by using the forward stepwise method. In each model, the goodness of fit (Nagelkerke R^2 value) and significance were evaluated. Matching enabled controlling by sex, age, and region of residence. Three models were used: 1 for children <5 years of age, 1 for persons ≥5 years of age, and 1 that considered only serogroup W disease for all ages in Santiago (Metropolitan Region). Serogroup W was not analyzed by age groups because of low numbers of cases.

The protocol and consent and assent forms were approved by the Ethics Committee of Faculty of Medicine, Clínica Alemana Universidad del Desarrollo (Santiago, Chile). All participants were informed of the objectives of the study and voluntarily agreed to participate anonymously and with their confidentiality protected by signing a consent form. Contact information for the cases was obtained from records of the Ministry of Health of Chile in compliance with current regulations (*11–13*). A psychological support strategy was also developed for relatives of deceased case-patients and those with serious sequelae, which included the presence of a psychologist during the interview and a referral to the corresponding health network when requested.

Results

Of 149 case-patients reported in regions with ≥3 cases, where ≈82% of the population resided, 135 (90.6%) were enrolled in the study; 8 case-patients could not be located, and 6 case-patients refused to participate. A total of 618 controls were enrolled in the study.

More than half (55%) of case-patients and controls were men. There were no age differences between case-patients and controls, except for infants <1 year of age, who had a higher proportion of cases (cases 29%, controls 13%). Of all case-patients and controls, 60% lived in the metropolitan region, 98% had Chilean nationality, and <9% belonged to the native population. A total of 46.6% of case-patients had disease caused by serogroup W, 31.8% by serogroup B, 2.5% by serogroup C, and 0.7% by serogroup Y; 18.2% of cases were not serogrouped. For outcomes, 27% of case-patients died <43 days after the first consultation (median 1 day), all during hospitalization for meningococcal disease; 14.2% survived and had sequelae (neurologic, 8 case-patients; amputations, 4; sensory, 3); and 58.8% survived without any short-term sequelae.

Because meningococcal disease differs in frequency by patient age and serogroups, we analyzed persons <5 years of age and those ≥5 years of age. We also analyzed case-patients who had serogroup W in the Metropolitan Region, which contained most cases with this serogroup.

Analysis for Persons <5 Years of Age

We analyzed 59 case-patients and 281 controls <5 years of age; of these persons <5 years of age, 66% of case-patients and 28% of controls were <1 year of age. Bivariate analysis showed a strong association between meningococcal disease and social and environmental characteristics, such as overcrowding (≥2.5 persons/bedroom), fewer years of mother's education, belonging to the public health insurance system, and monthly household income <US $517, which corresponded to the threshold of the lowest quintile income in the country in 2011 (*14*). Among habits, greeting ≥2 persons with a kiss on the mouth was associated with development of disease. For medical history of children, previous hospitalizations for asthma or acute lower respiratory tract infection (RTI), pertussis (whooping cough), and diarrhea were risk factors. A family history of meningococcal disease also increased risk for illness (Table 1).

Because the vaccination campaign began at the end of 2012, most case-patients had no access to the vaccine before the disease developed. A total of 70.6% of controls <5 years of age had received the vaccine, in contrast to only 1 (1.7%) child among case-patients. However, serogroup B was not included in the vaccine. There is strong evidence that vaccination with quadrivalent meningococcal conjugate vaccine is protective (*4,15–18*). Because case-patients did not have the same probability of being vaccinated as controls, vaccination status was not considered in the analysis.

The multivariate model included variables significant in the bivariate model. The multivariate model showed that being <1 year of age, having a monthly household income <US $517, sharing daily activities with other children, sharing the same bed with ≥2 persons, greeting ≥2 persons with

Table 1. Meningococcal disease determinants for persons <5 years age and controls for all *Neisseria meningitidis* serogroups, Chile, January 2012–March 2013*

Characteristic	Case-patients, n = 59	Controls, n = 281	p value	OR (95% CI)
Demographic				
Age, y†	0 (0–1)	1 (0–2)	<0.001‡	NA
Age <1 y†	39/59	80/281	<0.001§	4.9 (2.7–8.9)
Male sex	42/59	187/281	0.298§	1.2 (0.7–2.3)
Chilean nationality	58/59	278/281	0.535§	0.6 (0.1–6.1)
Native person	7/59	22/281	0.220§	1.6 (0.6–3.9)
Residence in Santiago (metropolitan region)	37/59	179/281	0.499§	1.0 (0.5–1.7)
Social and environmental				
No. bedrooms in home†	3 (2–3)	3 (2–4)	0.045‡	NA
No. persons/bedroom†	2 (1.7–3.0)	1.8 (1.3–2.1)	<0.001‡	NA
Square feet of housing	646 (484–1,023)	753 (517–1,076)¶	0.323‡	NA
Square feet/person	129 (97–180)	143 (93–230)¶	0.212‡	NA
Education for mother, y†	13 (111–13)	13 (12–15)	0.015‡	NA
Children not attending school in mother's care†	39/47	144/214	0.022§	1.4 (1.1–5.3)
Lived in crowded places†	3/59	0/281	0.005§	NA
Income <US $517†	34/59	101/262	0.006§	2.2 (1.2–3.8)
Public health insurance†	64/59	209/281	0.003§	2.2 (1.0–4.8)
Shared bedroom with ≥2 persons†	51/59	180/281	<0.001§	3.6 (1.6–7.8)
Shared bed with ≥2 persons†	24/59	59/281	0.002§	2.6 (1.4–4.7)
Shared space with other children†	54/59	212/281	0.003§	3.5 (1.4–9.1)
Overcrowding†	22/59	55/281	0.004§	2.4 (1.3–4.5)
Individual behavior and medical history				
No. hospitalizations†	0 (0–1)	0 (0–1)	0.004‡	NA
No. nonrespiratory chronic diseases	0 (0–0)	0 (0–0)	0.093‡	NA
Acute respiratory illness	33/59	186/281	0.090§	1.5 (0.9–2.7)
Bronchopneumonia†	8/59	7/281	0.001§	6.1 (2.1–17.7)
Greeted ≥2 persons with kiss on mouth†	24/59	70/281	0.012§	2.1 (1.2–3.7)
History of meningococcal disease in family†	13/59	24/281	0.004§	3.0 (1.4–6.4)
History of hospitalizations†	28/59	84/281	0.008§	2.1 (1.2–3.8)
Hospitalization for asthma or acute lower RTI†	19/59	44/281	0.004§	2.6 (1.4–4.8)
Hospitalization for whooping cough†	4/59	1/281	0.004§	20.4 (2.2–185.7)
Hospitalization for diarrhea†	4/59	4/281	0.033§	5.0 (1.2–20.7)
Nonrespiratory chronic disease	12/59	35/281	0.086§	1.8 (0.9–3.7)
Stressful event	14/59	42/281	0.076§	1.8 (0.9–3.5)

*Values are median (IQR) or no. positive/no. tested except as indicated. IQR, interquartile range; NA, not applicable; OR, odds ratio; RTI, respiratory tract infection.
†Significant difference.
‡By Mann-Whitney test.
§By Fisher exact test.
¶n = 276.

a kiss on the mouth, hospitalization for whooping cough and asthma or acute lower RTI, and a familial history of meningococcal disease were primary determinants for this disease in children <5 years of age (Table 2).

Analysis for Persons ≥5 Years of Age

We analyzed 76 case-patients and 337 controls ≥5 years of age. Bivariate analysis showed a strong association between the probability of development of meningococcal disease and overcrowding (fewer bedrooms, fewer overall square feet, and fewer square feet per person). Meningococcal disease was also strongly associated with having lived in crowded places, such as regiments, hospitals, or campgrounds; having had a stressful event (death of close person, moving, divorce, or losing a job); and excessive use of alcohol in persons >14 years of age (≥4 or more glasses

Table 2. Multivariate model of risk factors for development of meningococcal disease in persons <5 years of age for all *Neisseria meningitidis* serogroups, Chile, January 2012–March 2013*

Risk factor	β	SE	p value	OR (95% CI)
Hospitalization for whooping cough	2.94	1.30	0.024	18.86 (1.47–241.87)
Age <1 y	2.04	0.38	<0.001	7.68 (3.68–16.05)
Shared space with other children	1.78	0.63	0.005	5.91 (1.73–20.14)
Hospitalization for asthma or acute lower RTI	1.44	0.41	<0.001	4.24 (1.91–9.44)
History of meningococcal disease in family	1.39	0.46	0.002	4.02 (1.63–9.92)
Shared bed with >2 persons	1.22	0.38	0.001	3.37 (1.62–7.03)
Income <US $517	0.95	0.35	0.007	2.59 (1.30–5.17)
Greeted ≥2 persons with kiss on mouth	0.85	0.37	0.023	2.35 (1.13–4.89)
Constant	–6.87	1.56	<0.001	0.00

*OR, odds ratio; RTI, respiratory tract infection; R^2 = 39%.

of alcohol at each drinking opportunity or until drunk). For medical history, having had an episode of acute respiratory illness, having been hospitalized for asthma or acute lower RTI, and having ≥1 nonrespiratory chronic illness increased the probability of development of meningococcal disease. For use of medications, an association was found between disease and regular use of corticoids and antidepressants (Table 3).

We also performed multivariate analysis for this group. Multivariate analysis showed that living lived in overcrowded household or in collective places, excessive use of alcohol for persons >14 years of age, regular use of corticoids, having ≥1 nonrespiratory chronic illness, and having been hospitalized for asthma or acute lower RTI were related to development of disease (Table 4).

Analysis for Serogroup W in Metropolitan Region of Chile

For case-patients with meningococcal disease caused by serogroup W, 82% occurred in the metropolitan region of Santiago. This region had a greater risk for disease caused by serogroup W than other regions, where serogroup B predominated. Thus, we analyzed cases caused by serogroup W in the metropolitan region (50 case-patients and 366 controls).

The most affected group was children <1 year of age (28% for cases; 15% for controls). Meningococcal disease was strongly associated with overcrowding and conditions that increased proximity between persons, such as sharing the same bedroom and sharing daily activities with other children <5 years of age. For medical history, risk for meningococcal disease was increased in persons who

Table 3. Meningococcal disease determinants for persons ≥5 years of age and controls for all *Neisseria meningitidis* serogroups, Chile, January 2012–March 2013*

Characteristic	Case-patients, n = 76	Controls, n = 337	p value	OR (95% CI)
Demographic				
Age, y	35 (6–57)	36 (18–55)	0.849†	NA
Age <25 y	26/76	115/337	0.544‡	1.0 (0.6–1.7)
Male sex	33/76	153/337	0.427‡	0.9 (0.6–1.5)
Chilean nationality	75/76	329/337	0.484‡	1.8 (0.2–14.8)
Native person	5/76	30/337	0.347‡	0.7 (0.3–1.9)
Residence in Santiago (metropolitan region)	44/76	190/337	0.457‡	1.1 (0.6–1.8)
Social and environmental				
No. bedrooms in home§	3 (2–4)	4 (2-4)	0.042†	NA
No. persons/bedroom	1.3 (1–2)	1.3 (1–1.7)	0.146†	NA
Square feet of housing§	753 (505–1,103)¶	861 (581–1,173)#	0.038†	NA
Square feet/person§	179 (108–288)¶	215 (143–375)#	0.031†	NA
Education for mother of persons <18 y of age, y	13 (9–14)**	13 (13–16)††	0.063†	NA
Lived in crowded places§	5/76	7/337	0.050‡	3.3 (1.0–10.8)
Income <US $517	33/74	126/317	0.263‡	1.2 (0.7–2.0)
Public health insurance§	64/76	246/337	0.026‡	2.0 (1.0–3.8)
Shared bedroom with ≥2 persons	20/76	71/337	0.198‡	1.3 (0.8–2.4)
Shared bed with ≥2 persons	3/76	18/337	0.439‡	0.7 (0.2–2.5)
Overcrowding§	12/76	24/337	0.018‡	2.4 (1.2–5.1)
Individual behavior and medical history				
No. hospitalizations	1 (0–2)	1 (0–2)	0.707†	NA
No. nonrespiratory chronic diseases§	1 (0–2)	1 (0–1)	0.005†	NA
Acute respiratory illness§	42/76	141/337	0.023‡	1.7 (1.0–2.8)
Bronchopneumonia	3/76	6/337	0.219‡	2.3 (0.6–9.3)
Person >14 y of age consuming ≥4 alcoholic drinks§	14/59	35/269	0.034‡	2.1 (1.0–4.2)
Depression§	16/76	43/337	0.050‡	1.8 (1.0–3.4)
History of meningococcal disease in family	11/76	42/337	0.378‡	1.2 (0.6–2.4)
History of hospitalizations	46/76	200/337	0.478‡	1.1 (0.6–1.7)
Hospitalization for asthma or acute lower RTI§	12/76	26/337	0.029‡	2.2 (1.1–4.7)
Hospitalization for diarrhea	3/76	10/337	0.439‡	1.3 (0.4–5.0)
Hypertension§	14/76	34/337	0.037‡	2.0 (1.0–4.0)
Nonrespiratory chronic disease§	49/76	158/337	0.004‡	2.1 (1.2–3.4)
Stressful event§	49/76	179/337	0.047‡	1.6 (1.0–2.7)
Used corticoids§	8/76	11/337	0.012‡	3.5 (1.4–9.0)
Used antidepressants§	10/76	22/337	0.049‡	2.2 (1.0–4.8)

*Values are median (IQR) or no. positive/no. tested except as indicated. IQR, interquartile range; NA, not applicable; OR, odds ratio; RTI, respiratory tract infection.
†By Mann-Whitney test.
‡By Fisher exact test.
§Significant difference.
¶n = 74.
#n = 333.
**n = 22.
††n = 82.

Table 4. Multivariate model of risk factors for development of meningococcal disease in persons ≥5 years of age for all *Neisseria meningitidis* serogroups, Chile, January 2012–March 2013*

Risk factor	β	SE	p value	OR (95% CI)
Overcrowding	1.61	0.50	0.001	5.01 (1.87–13.45)
Lived in crowded places	1.51	0.66	0.022	4.54 (1.24–16.67)
Used corticoids	1.46	0.57	0.011	4.29 (1.39–13.21)
Person 14 y of age consuming ≥4 alcoholic drinks	1.30	0.41	0.001	3.68 (1.66–8.19)
Nonrespiratory chronic disease	1.20	0.36	0.001	3.32 (1.64–6.73)
Hospitalization for asthma or acute lower RTI	1.08	0.49	0.028	2.94 (1.12–7.71)
Constant	−6.73	1.69	<0.001	0.00

*OR, odds ratio; RTI, respiratory tract infection; R^2 = 19%.

had previous hospitalizations, especially for respiratory diseases. A history of chronic diseases (diabetes, obesity, depression, and hypertension) and use of medications also increased the risk for meningococcal disease (Table 5).

Multivariate analysis showed that the demographic and social factors that remained associated with development of disease were age <1 year (≈4 times higher) and overcrowding. For clinical history, the probability of development of meningococcal disease was 5.8 times higher for those who had had ≥1 nonrespiratory chronic illness and ≈3 times higher for those previously hospitalized for asthma or acute lower RTI (Table 6).

Discussion

We sought to understand the principal determinants and risk factors for development of meningococcal disease in Chile and included 91% of the cases of this disease in Chile in 2012 and the first 3 months of 2013. Our study identified some risk factors previously reported and some that were not previously reported. These factors were a combination of social conditions, habits, and host health status, which are fundamental for the infectious process.

Evidence indicates that living conditions are one of the key factors in the likelihood of development of meningococcal disease (*19*–*22*). This evidence was especially true when linked with a lower socioeconomic level, as was the finding in our study for beneficiaries of the public health system, those who had monthly incomes <US $517, and those children whose mothers had less education.

For housing, 1 study showed that the number of persons per bedroom (>1.5) or those sharing the room or the bed were risk factors for meningococcal disease (*23*). In our study, we found that the average number of bedrooms was smaller and that overcrowding was greater for case-patients than for controls. Controls had an average of 41 more square feet per person in their houses than case-patients. We also found a higher probability of development of meningococcal disease in persons <5 years of age who shared a bedroom or bed with ≥2 persons.

For habits of persons, excessive use of alcohol in case-patients >14 years of age was twice as common as in controls, which is consistent with results of other studies (*19*,*24*,*25*). For exposure to tobacco, in contrast with findings of other studies (*19*,*21*,*26*–*30*), we found no association in our study.

Another major risk factor was proximity to other persons, in which a higher amount of contacts is associated with a greater likelihood of development of meningococcal disease (*19*,*22*,*25*–*27*,*31*–*36*). Our study showed that having contact with other children during the day and greeting ≥2 persons with a kiss on the mouth were risks for development of disease in preschoolers. Persons ≥5 years of age who had lived in crowded places also had a greater likelihood for development of meningococcal disease than persons who had not lived in such places.

For previous clinical history, other studies reported that patients with serious medical problems have a greater risk for development of meningococcal disease, especially patients with nephrotic syndrome, systemic lupus erythematosus and liver disease (*19*,*26*), HIV or other immunosuppressive diseases (*25*,*26*), a history of corticoid use (*26*), decreased endothelial thrombomodulin expression (*19*,*26*,*35*–*37*), and anatomic or functional asplenia (*19*,*25*,*35*). Stress and depression (*34*) have also been shown to be risk factors, and low weight or obesity (*26*) might predispose a person to development of meningococcal disease.

We found a greater probability of development of meningococcal disease in persons with a history of previous hospitalization for asthma or acute lower RTI in both age groups studied. In persons ≥5 years of age, we showed that those who have a chronic disease and use more medications, especially corticoids, on a regular basis had a higher probability of development of meningococcal disease. For patients ≥5 years of age who had chronic diseases, hypertension was present in 18.4% of case-patients but only 10.1% of controls. This finding might be the result of persons who had meningococcal disease and more previous diseases or hospitalizations; thus their history of hypertension was better known.

Family history has been reported as a risk factor for meningococcal disease (*21*,*35*–*38*), which indicates that genetic polymorphisms might be associated with disease in case–control studies (*38*) and affect susceptibility to and severity of meningococcal disease. In our study, we found that persons <5 years of age who had a familial history of this disease had a 4-fold greater probability of developing it.

An age <1 year and being infected with serogroup W were strongly associated with the likelihood of meningococcal disease. Other risk factors were similar to those found by analysis of all other serogroups.

Strengths of this study were including 91% of all cases that occurred in the study period, selecting 4 population controls/case-patient, and sampling that was independent of exposure conditions. These features ensured comparability and that the controls could show development of the disease because they resided in municipalities in which cases of meningococcal disease were still occurring.

One limitation of this study was that, because of the case–control design, information about exposure was collected retrospectively. As a result, there might have been memory bias, the probability of which increases when information about several factors is collected simultaneously, as was the case in this study. A considerable amount of time passed between occurrence of disease and when the study was conducted. Another limitation was that controls were questioned about a period 2 years before they were interviewed, However, the study was conducted 6 months after the last case included in the study had occurred. The period during which case-patients

Table 5. Meningococcal disease determinants for case-patients and controls for *Neisseria meningitidis* serogroup W, Santiago (Metropolitan Region) of Chile, January 2012–March 2013*

Characteristic	Case-patients, n = 50	Controls, n = 366	p value	OR (95% CI)
Demographic				
Age, y	3.5 (0–51.5)	5 (1–38)	0.765†	NA
Age <1 y‡	14/50	56/366	0.005§	2.6 (1.3–5.0)
Age <5 y	26/50	179/366	0.398§	1.1 (0.6–2.4)
Male sex	28/50	203/366	0.534§	1.0 (0.6–1.9)
Chilean nationality	50/50	357/366	0.312§	NA
Native person	9/50	37/366	0.082§	2.0 (0.9–4.3)
Social and environmental				
No. bedrooms in home	3 (2–4)	3 (2–4)	0.273†	NA
No. persons/bedroom	1.7 (1.2–2.5)	1.5 (1.2–2.0)	0.135†	NA
Square feet of housing	614 (503–1,076)	823 (546–1,206)	0.053†	NA
Square feet/person	157 (93–273)	189 (120–301)	0.146†	NA
Education for mother of persons <18 y of age, y	13 (11–15)¶	13 (13–15)#	0.274†	NA
Children <5 y of age not attending school in mother's care	13/19	89/137	0.492§	1.2 (0.4–3.3)
Lived in crowded places	0/50	3/366	0.680§	NA
Income <US $517	22/50	111/366	0.073§	1.6 (0.9–3.0)
Public health insurance‡	42/50	259/366	0.032§	2.2 (1.0–4.8)
Shared bedroom with ≥2 persons‡	26/50	137/366	0.035§	1.8 (1.0–3.3)
Shared bed with ≥2 persons	8/50	45/366	0.294§	1.4 (0.6–3.1)
Children <5 y of age sharing space with other children‡	25/26	134/179	0.008§	8.4 (1.1–63.7)
Overcrowding‡	14/50	48/366	0.008§	2.6 (1.3–5.1)
Individual behavior and medical history				
No. hospitalizations	1 (0–2)	0 (0–1)	<0.001†	NA
No. nonrespiratory chronic diseases	1 (0–2)	0 (0–1)	0.002†	NA
Acute respiratory illness	32/50	204/366	0.170§	1.4 (0.8–2.6)
Bronchopneumonia	4/50	11/366	0.093§	2.8 (0.9–9.2)
Person >14 y of age consuming ≥4 alcoholic drinks	5/22	22/148	0.246§	1.7 (0.6–5.0)
Children <5 y of age greeting ≥2 persons with kiss on mouth	11/26	45/179	0.058§	2.2 (0.9–5.1)
Depression‡	9/50	26/366	0.015§	2.9 (1.3–6.5)
Diabetes mellitus ‡	6/50	14/366	0.023§	3.4 (1.3–9.4)
History of meningococcal disease in family	8/50	43/366	0.256§	1.4 (0.6–3.2)
History of hospitalizations‡	33/50	162/366	0.003§	2.4 (1.3–4.5)
Hospitalization for asthma or acute lower RTI‡	15/50	42/366	0.001§	3.3 (1.7–6.6)
Hospitalization for whooping cough‡	3/50	0/366	0.002§	NA
Hospitalization for diarrhea	2/50	6/366	0.248§	2.5 (0.5–12.7)
Hypertension‡	7/50	19/366	0.026§	3.0 (1.2–7.5)
Nonrespiratory chronic disease‡	30/50	112/366	<0.001§	3.4 (1.9–6.2)
Obesity‡	7/50	21/366	0.038§	2.7 (1.1–6.6)
Stressful event	23/50	126/366	0.076§	1.6 (0.9–2.9)
Used medicines‡	22/50	101/366	0.015§	2.1 (1.1–3.8)
Used corticoids	4/50	18/366	0.265§	1.7 (0.5–5.2)
Used antidepressant drugs‡	5/50	8/366	0.013§	5.0 (1.6–15.9)
Used antihypertension drugs‡	10/50	25/366	0.005§	3.4 (1.5–7.6)
Used hypoglycemic agents‡	6/50	17/366	0.045§	2.8 (1.0–7.5)

*Values are median (IQR) no. or no. positive/no. tested. IQR, interquartile range; NA, not applicable; OR, odds ratio; RTI, respiratory tract infection.
†By Mann-Whitney test.
‡Significant results.
§By Fisher exact test.
¶n = 29.
#n = 224.

Table 6. Multivariate model of risk factors for development of meningococcal disease caused by *Neisseria meningitidis* serogroup W, Santiago (metropolitan region) of Chile, January 2012–March 2013*

Risk factor	β	SE	p value	OR (95% CI)
Nonrespiratory chronic disease	1.76	0.37	<0.001	5.78 (2.78–12.04)
Age <1 y	1.44	0.42	0.001	4.23 (1.86–9.60)
Hospitalization for asthma or acute lower RTI	1.04	0.38	0.006	2.83 (1.35–5.95)
Overcrowding	0.84	0.40	0.034	2.32 (1.07–5.06)
Constant	–4.38	1.04	<0.001	0.01

*OR, odds ratio; RTI, respiratory tract infection; R^2 = 20%.

and controls were investigated was different, although it might have included a common time frame.

The question of vaccination status was also a problem because a vaccination campaign occurred once the outbreak was recognized. Therefore, controls had an opportunity to be vaccinated that case-patients did not. Because the likelihood of vaccination was different for case-patients and controls, we did not analyze this variable.

If one considers that prospective designs are ideal, it would be useful to conduct a new study of incident cases during an outbreak to further explore the causality of associations identified in this study. In addition, the protective role of vaccination against meningococcus, which has been demonstrated worldwide, should stimulate discussion about which groups should be targeted for vaccination. Although children 12 months of age have been vaccinated in Chile since 2014, a booster vaccination during adolescence might be advisable, as recommended by the World Health Organization (*15*). Therefore, analysis of age groups most affected by serogroup W should be performed to determine whether vaccination is affecting the target group or a shift to other groups has occurred at the present time. Vaccination can compensate for risk factors and protect against this disease. These findings indicate the need for health systems to move toward health equity by targeting actions to the most vulnerable groups.

Results of this study have contributed to understanding the epidemiology of the disease and identified determinants and risk factors that increase the probability of development of meningococcal disease in the population in Chile. Unfavorable living conditions, such as poverty, small spaces, overcrowding, and a previous clinical history, especially respiratory sequelae, are major risk factors. Positive familial history of meningococcal disease also seems to be a major factor associated with development of the disease, especially in children, a finding that should be addressed in future research. Most of these indicators point to a highly vulnerable social group that should be the target of intersectoral public policies, enabling them to improve their living conditions and health.

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MERS-CoV Antibody Responses 1 Year after Symptom Onset, South Korea, 2015

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We investigated the kinetics of the Middle East respiratory syndrome coronavirus (MERS-CoV) neutralizing and spike protein antibody titers over the course of 1 year in 11 patients who were confirmed by reverse transcription PCR to have been infected during the outbreak in South Korea in 2015. Robust antibody responses were detected in all survivors who had severe disease; responses remained detectable, albeit with some waning, for ≤1 year. The duration of viral RNA detection (but not viral load) in sputum significantly correlated with the antibody response magnitude. The MERS S1 ELISA antibody titers correlated well with the neutralizing antibody response. Antibody titers in 4 of 6 patients who had mild illness were undetectable even though most had evidence of pneumonia. This finding implies that MERS-CoV seroepidemiologic studies markedly underestimate the extent of mild and asymptomatic infection. Obtaining convalescent-phase plasma with high antibody titers to treat MERS will be challenging.

Middle East respiratory syndrome (MERS) remains a disease of global public health concern for which no proven specific countermeasures are available. As of December 5, 2016, ≈1,800 laboratory-confirmed cases have been reported (*1*). MERS coronavirus (MERS-CoV) is an enzootic pathogen present in dromedary camels in many parts of the world, including the Middle East, Iran, Pakistan, and Africa (*2*,*3*). Zoonotic infections have been repeatedly reported on the Arabian Peninsula and have led to large nosocomial outbreaks. One notable example occurred in South Korea in 2015, initiated by a traveler returning home from the Arabian Peninsula (*4*). The infection in this traveler led to an outbreak of 186 cases and 36 deaths that had a substantial impact on the local economy. A cohort of 17 patients from this outbreak was intensively followed up to obtain detailed clinical, immunologic, and virologic characterization of their disease course (*5*,*6*). The kinetics of the serologic responses during the acute phase have already been reported, and they showed that robust but delayed antibody responses could be detected in patients who were more severely ill (*7*). Another study reported a significant linear correlation between the $\log_{10}$ viral loads and the serologic response in the acute phase of illness (*8*). The kinetics of the long-term serologic responses to MERS-CoV infections is poorly understood and remains of clinical interest. We report the results of a 1-year follow-up on the antibody responses in 11 of these patients.

Material and Methods

Patients

The acute-phase serologic responses of a cohort of 17 patients with reverse transcription PCR (RT-PCR)–confirmed MERS-CoV disease admitted to Seoul National University (SNU) Hospital in Seoul, South Korea; SNU Boramae Medical Center in Seoul; and SNU Bundang Hospital in Seongnam, South Korea, were previously reported (*7*). Nine of these patients had severe disease (defined as requiring supplemental oxygen or mechanical ventilation). The clinical, viral load, and cytokine profiles were previously reported (*5*,*6*).

We followed up 11 of these patients, 5 with severe disease (patients C, D, F, G, and I) and 6 with mild disease (patients K, L, M, N, O, and P), for 1 year. Their serum samples were collected at ≈6 months and ≈12 months after disease onset and used to investigate the long-term kinetics and duration of antibody responses that form the basis of this report. The clinical characteristics and early immunologic responses of the original and present cohorts of patients are summarized (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/7/17-0310-Techapp1.pdf). The reasons for the lack of follow-up for

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the other 6 patients were transfer of care to another clinical unit (patient A), refusal of follow-up (patients J and Q), and death (patients B, E, and H). Patients B and E died during the acute phase of the illness, and patient H was discharged to receive rehabilitation care but was then given a diagnosis of aspiration pneumonia and died 2 months after disease onset. This study was approved by the Institutional Ethics Review Board of Seoul National University Hospital (approval no. 1506-093-681).

Viruses

The human CoV-EMC/2012 strain was used for 50% tissue culture infectious dose assays, microneutralization assays, and plaque-reduction neutralization tests (PRNTs). A subset of serum samples was also tested with a strain from the outbreak in South Korea, MERS-CoV Hu/KOR/SNU1_035/2015.

Serologic Tests

We heat inactivated serum samples for 30 min at 56°C before carrying out serologic tests. We performed the MERS-CoV PRNT (using a ≥90% plaque-reduction cutoff [$PRNT_{90}$]), microneutralization test, and pseudoparticle neutralization test (ppNT) as described (*7*,*9*) (online Technical Appendix).

We used the MERS-CoV S1 ELISA kit (EI 2604-9601G; EUROIMMUN, Luebeck, Germany) for the detection of human IgG against MERS-CoV spike protein. We assayed serum samples in duplicate and performed the assay according to the manufacturer's instructions. The assay included a calibrator, which defines the upper limit of the reference range for noninfected humans, and this upper limit served as the cutoff value. The assay was made semiquantitative by calculating the ratio of the extinction of the patient sample over the extinction of the calibrator. Ratios <0.8 were considered negative, ratios ≥1.1 were considered positive, and ratios ≥0.8 to <1.1 were considered borderline. We included known positive and negative control serum samples in all assays and denoted antibody titers in the reciprocal.

Statistical Analysis

We calculated peak viral loads in sputum; $PRNT_{90}$, ppNT, and microneutralization antibody titers; and MERS S1 ELISA optical density (OD) ratios of the 11 MERS patients by using descriptive statistics. Spearman correlation coefficients were calculated to correlate the peak viral loads in sputum during the acute phase of illness with serologic responses ($PRNT_{90}$ antibody titers and MERS S1 ELISA OD ratios) at different times after disease onset. We also tested the correlation between duration of viral shedding and serologic responses at a 5% significance level. We excluded missing data from the main analyses. A sensitivity analysis was performed by imputing values from the most recent tests.

Results

All 5 patients with severe disease, but only 2 (33%) of 6 with mild disease ($p = 0.06$ by Fisher exact test), had $PRNT_{90}$ antibody titers ≥40 at the 1-year follow-up (Figure, panel A). $PRNT_{90}$ antibody titers of patients C and F, who had acute-phase antibody titers of ≥320, declined ≥4-fold 1 year later. Patients D, G, K, and N who had acute phase peak antibody titers in the range of 80–160 only had ≤2-fold declines in titer. Patients C, D, F, G, I, K, and N (the 5 patients with severe disease and 2 of the 6 with mild disease who had $PRNT_{90}$ antibody titers of ≥40 in their acute-phase serum samples [collected 21–50 days after disease onset]) continued to have detectable antibodies by $PRNT_{90}$ (titer ≥1:40), ppNT (titer ≥1:40), microneutralization assay (titer ≥1:20), and S1 ELISA (ratio ≥1.1) 1 year after illness onset (Table; Figure, panel B). MERS antibody titers waned during the first 6 months after disease onset, especially in patients who had had high antibody titers. The waning of antibody titers between 6 months and 1 year after disease onset was less steep.

At 1 year after infection, the 4 patients who had mild disease (or who did not require supplemental oxygen or mechanical ventilation) all had negative results by microneutralization assay and S1 ELISA, but 1 was positive by ppNT (titer of 10) and 2 by $PRNT_{90}$ (titer 1:10) (Table). Although designated as having mild disease, all of these patients, with 1 exception (patient P), had chest infiltrates on x-ray, indicating lung parenchymal pathology.

The kinetics of antibody production seen with the $PRNT_{90}$, ppNT, microneutralization test, and S1 ELISA were comparable (Table), suggesting that any of these tests could be used for detection of MERS-CoV antibodies in patients with past infection. One year after infection, all patients who had antibody titers of ≥20 by $PRNT_{90}$ also had antibodies detectable by ppNT, microneutralization assay, and S1 ELISA. One patient (L) with a marginal $PRNT_{90}$ titer of 1:10 was not positive by ppNT, microneutralization assay, or ELISA. At 1 year after infection, the correlation coefficients were 0.89 between $PRNT_{90}$ and ppNT titers, 0.94 between $PRNT_{90}$ and microneutralization assay titers, and 0.96 between $PRNT_{90}$ and S1 ELISA titers.

The virus we used for serologic testing was the prototype MERS-CoV EMC clade A virus, and the virus that patients were exposed to (and that caused the outbreak in South Korea) was a clade B virus. To confirm that the neutralizing antibody titers against the clade A and B viruses were not significantly different, we tested 10 paired serum samples (from 5 MERS-CoV patients who had various levels of microneutralization antibody responses to EMC)

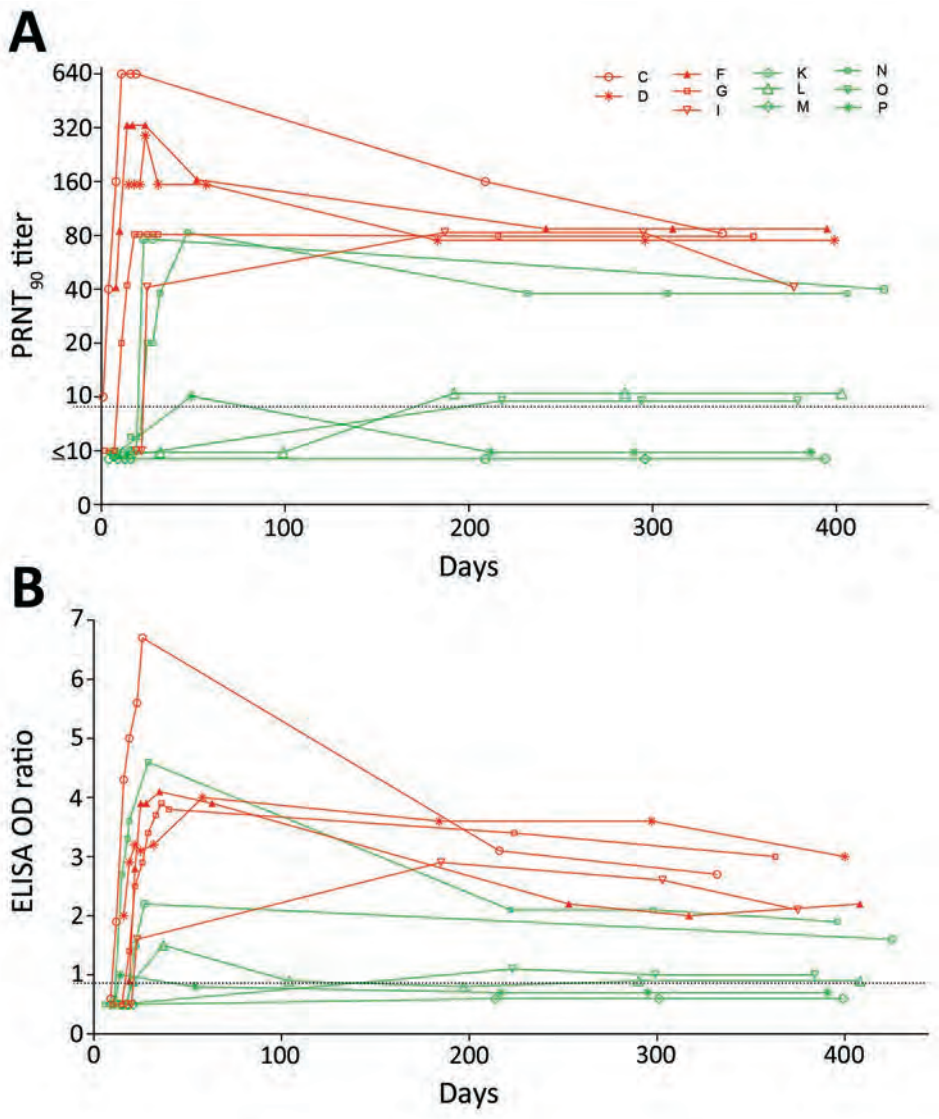

Figure. Middle East respiratory syndrome (MERS) coronavirus antibody titers in serially collected serum samples from 11 patients with reverse transcription PCR–confirmed symptomatic MERS, South Korea, 2015. $PRNT_{90}$ titers (A) and MERS spike protein (S1) ELISA OD ratios (B) were determined at multiple time points 0 to >400 days after disease onset. The limit of detection was 10 for the PRNT, and the cutoff between negative and borderline samples for the S1 ELISA was an OD ratio of 0.8. Letters in key indicate patients; red indicates those with severe disease, and green indicates those with nonsevere disease. OD, optical density; $PRNT_{90}$, ≥90% plaque-reduction neutralization test.

by microneutralization assay using the clade A virus and a clade B virus from the outbreak in South Korea. The titers were similar (within a 2-fold dilution), confirming that the neutralizing epitopes of MERS-CoV are antigenically conserved (online Technical Appendix Table 2).

The peak viral loads in sputum did not correlate with $PRNT_{90}$ antibody titers or S1 ELISA OD ratios at the acute phase of illness, at ≈6 months after illness, or at ≈12 months after illness (online Technical Appendix Table 3). However, we found strong positive correlations between duration of virus detection and antibody titers (as measured by the $PRNT_{90}$ and S1 ELISA) at these time points (online Technical Appendix Table 4). We defined the duration of virus detection as the day from symptom onset to negative PCR conversion. The median duration of virus shedding was 19 days (interquartile range 16.5–27.5 days). Only 2 of the patients (D and F) received corticosteroid therapy, and both had robust antibody responses (Table). However, because of the small number of patients given this treatment, evaluating its quality with a statistical analysis was not possible.

Discussion

With this cohort of patients, we had previously reported that the severity of illness was associated with higher neutralizing antibody responses and ELISA ODs in the acute phase of illness (*7*). The analysis of antibody titers at ≈1 year after illness shows that these higher antibody titers continue to persist for at least 1 year. In the patients with the highest peak antibody titers, antibody titers waned during the first 6 months after infection but then stabilized over the next 6 months. This pattern of antibody production contrasts with the pattern reported for 1 patient with a microneutralization titer of 400, whose titers declined

to an undetectable level within months by microneutralization test, immunofluorescent antibody (IFA) assay, and ELISA (*10*).

Patients K, L, M, N, O, and P were designated as having nonsevere disease because they were not given supplemental oxygen therapy, even though all of them, with the exception of patient P, had evidence of lung parenchymal disease. Only 2 of these patients (K and N) manifested robust antibody responses during the acute phase of the illness and early convalescence, and these titers were still present 1 year later. For the other patients, robust serologic antibody titers did not develop during the acute phase of the illness, and the patients remained seronegative or with marginal antibody titers at 1 year after infection. Because of the poor antibody response that resulted from symptomatic disease, persons with asymptomatic or mild infection without severe lung parenchymal disease are not expected to develop detectable MERS-CoV antibodies, a conclusion with implications for seroepidemiologic studies.

Three other studies have investigated the kinetics of long-term antibody persistence in patients with MERS-CoV illness. Survivors of a MERS-CoV outbreak in Jordan in April 2012 have been followed up with serologic testing at 13 months and 34 months after infection (*11*). These patients did not have RT-PCR–confirmed MERS-CoV infection, but they were considered to probably have MERS-CoV infections because they had positive serologic results together with an epidemiologic link to a confirmed MERS patient. Seven patients had symptomatic acute upper respiratory disease during the outbreak; of these, 5 had radiologic evidence of lower lung pathology, and 2 did not have chest radiologic examination data. All 7 patients had detectable antibodies by ELISA and IFA assay, and 6 of them had detectable antibodies by microneutralization test at 13 months and 34 months after infection with titers ranging from 20 to 80. However, only patients with robust serologic responses were included in this study (selection bias), and those without were excluded by definition.

In a study in Saudi Arabia, 9 patients with RT-PCR–confirmed MERS-CoV infection were followed up at 3 months, 10 months, and 18 months (for only 2 patients) after infection and tested by MERS-CoV S1 ELISA and IFA assay (*12*). These 9 patients (2 with severe pneumonia, 3 with nonsevere pneumonia, 1 with upper respiratory symptoms, and 3 with asymptomatic infections) were identified through contact tracing. All 5 patients with pneumonia were antibody positive by ELISA and IFA assay at 3 months after infection, 4 were positive by ELISA at 10

Table. Characteristics and serologic responses of patients from MERS-CoV outbreak by time after disease onset, South Korea, 2015*

				Titers and ODs at various time points after disease onset											
				$PRNT_{90}$ antibody titer			MERS-CoV spike ppNT			Microneutralization assay titer			MERS-CoV S1 ELISA OD‡		
Pt.	Severe disease	Peak viral load†	Steroid use	21–50 d	180–248 d§	285–403 d§	21–50 d	180–248 d	285–403 d	21–50 d	180–248 d	285–403 d	21–50 d	180–248 d	285–403 d
C	Yes	6.1	No	640	160 (216 d)	80 (332 d)	640	160	80	320	160	40	6.2	2.9	2.5
D	Yes	8.2	Yes (11–23)¶	160	80 (181 d)	80 (294 d), 80 (397 d)	160	160	80	80	80	80	2.6	3.0	2.5
F	Yes	4.8	Yes (14–16)¶	320	80 (248 d)	80 (312 d), 80 (403 d)	320	320	40	160	40	40	3.6	1.7	1.5
G	Yes	7.2	No	80	80 (219 d)	80 (358 d)	80	80	160	80	80	80	3.4	2.9	2.5
I	Yes	5.2	No	NA#	80 (180 d)	80 (298 d), 40 (370 d)	40	40	40	40	40	40	1.1	2.4	1.6
K	No	6.7	No	80	NA	40 (420 d)	160	NA	40	40	NA	20	1.7	NA	1.1
L	No	4.6	No	<10	10 (192 d)	10 (285 d), 10 (403 d)	10	<10	<10	<10	<10	<10	1.0	0.3	0.4
M	No	5.4	No	NA**	<10 (209 d)	<10 (296 d), <10 (394 d)	<10	<10	<10	<10	<10	<10	0	0.1	0.1
N	No	6.6	No	80	40 (220 d)	40 (296 d), 40 (394 d)	40	80	40	80	40	20	4.1	1.6	1.4
O	No	8.2	No	NA	10 (218 d)	10 (294 d), 10 (379 d)	NA	<10	10	NA	<10	<10	NA	0.6	0.5
P	No	5.5	No	10	<10 (212 d)	<10 (290 d), <10 (386 d)	<10	<10	<10	<10	<10	<10	0.3	0.1	0.1

*MERS-CoV, Middle East respiratory syndrome coronavirus; NA, not assayed; OD, optical density; ppNT, pseudoparticle neutralization test; $PRNT_{90}$, ≥90% plaque-reduction neutralization test; Pt., patient; S1, spike protein; steroid, corticosteroid; upE, region upstream of the E gene.
†Viral loads (log_{10} upE RNA copies/mL) were quantified from sputum samples.
‡MERS-CoV S1 ELISA OD ratios <0.8 were considered negative, ≥0.8 to <1.1 were considered borderline, and ≥1.1 were considered positive.
§Exact day(s) after disease onset that serum was collected are indicated in parentheses.
¶Days after disease onset during which steroids were used.
#$PRNT_{90}$ antibody titer was 40 on day 18.
**$PRNT_{90}$ antibody titer was 10 on day 16.

months after infection, and 3 were positive by IFA assay at 10 months after infection. In contrast, 0 of 4 patients with mild upper respiratory tract infection or asymptomatic infection were positive by ELISA or IFA assay at 3 or 10 months after infection. These data are comparable with our own, suggesting that milder infections are less likely to elicit serologic responses. However, that study did not provide data on virus neutralizing antibodies (*12*). A second study in Saudi Arabia reported that only 4 of 11 healthcare workers with real-time RT-PCR–confirmed MERS-CoV infection had detectable ELISA antibody titers ≈1 year after infection; of these 4 healthcare workers, 3 had detectable microneutralization antibody titers and only 1 had a high antibody titer (800) (*10*).

Waning antibody titers have also been demonstrated in patients with severe acute respiratory syndrome–CoV infection (*13*). Antibody titers peaked at 4 months after disease onset and declined to undetectable levels in 19% and 11% of serum samples by IgG test and microneutralization test, respectively, 30 months after infection. Geometric mean microneutralization antibody titers dropped from 1,232 at month 4 to 32 at month 30 after infection and remained at that level until month 36 (*13*). In volunteers experimentally infected with human CoV 229E, neutralizing antibody titers peaked at 3 weeks after infection and fell considerably by 12 weeks after infection, declining close to baseline levels by 1 year (*14*). However, human CoV 229E infects mainly the upper respiratory tract, unlike the pathogens of severe acute respiratory syndrome and MERS, which are more invasive of the lung parenchyma and often disseminate systemically.

Convalescent-phase plasma therapy has been proposed as a treatment option for the acute respiratory diseases, like MERS, that do not have specific antimicrobial treatments available (*15*,*16*). Our data indicate that plasma with high MERS-CoV antibody titers are only likely to be available from patients who have recovered from severe MERS disease, and these titers substantially wane within the first 6 months of illness, although lower levels of MERS-CoV antibodies are maintained over longer periods. To use convalescent-phase patient plasma for treatment, it will be necessary to assess the antibody titer of potential donors before collection to ensure good antibody titer. A neutralization test is likely to be the optimal assay for assessing plasma used for therapy, but because our data indicated that the S1 ELISA correlates well with neutralization titers, the S1 ELISA might be a suitable screening test for selecting persons for plasma donation.

One limitation of this work is the virus used for neutralization tests. The clade A virus MERS-CoV EMC was used for assays, and the virus the patients were infected with and that caused the outbreak in South Korea was a clade B virus (the dominant virus clade currently circulating in the Arabian Peninsula). It has been shown that clade A and B viruses do not differ antigenically at the neutralization epitope, although they are genetically distinct (*17*,*18*). Using a subset of serum samples from this study, we confirmed that neutralizing antibody titers obtained with the clade A EMC virus were similar to those obtained with a clade B virus strain from the outbreak in South Korea.

In conclusion, our findings support and extend the research of others. We suggest that serologic tests for MERS-CoV antibodies can only identify some of the patients who have had MERS-CoV infections. Serologic responses to this virus are variable, not robust, and often undetectable when disease is mild. Thus, MERS-CoV seroepidemiologic studies will only detect a fraction of infections that are occurring in a population and will probably markedly underestimate the extent of mild infection that is taking place. Our findings also show that the MERS S1 ELISA is as good as neutralization tests at detecting antibodies a year after infection, but positive ELISA results do require confirmation with neutralization tests if false positives are to be avoided in seroepidemiologic assays (M. Peiris, unpub. data). Convalescent-phase plasma can be harvested for many months to a year after disease from patients surviving MERS-CoV infection, but plasma with a high antibody titer is only likely to be obtained during the first few months of convalescence from persons who had severe disease. Because patients during this time frame are likely to be frail, this approach will be challenging.

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Competence of *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* Mosquitoes as Zika Virus Vectors, China

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In China, the prevention and control of Zika virus disease has been a public health threat since the first imported case was reported in February 2016. To determine the vector competence of potential vector mosquito species, we experimentally infected *Aedes aegypti, Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes and determined infection rates, dissemination rates, and transmission rates. We found the highest vector competence for the imported Zika virus in *Ae. aegypti* mosquitoes, some susceptibility of *Ae. albopictus* mosquitoes, but no transmission ability for *Cx. quinquefasciatus* mosquitoes. Considering that, in China, *Ae. albopictus* mosquitoes are widely distributed but *Ae. aegypti* mosquito distribution is limited, *Ae. albopictus* mosquitoes are a potential primary vector for Zika virus and should be targeted in vector control strategies.

Zika virus is a mosquitoborne flavivirus that poses a serious threat worldwide (*1*). Because cases of Zika virus disease in humans have been sporadic and symptoms mild, Zika virus has been neglected since its discovery in 1947 (*2*,*3*). The first major Zika virus outbreak was reported on Yap Island in Micronesia in 2007 (*4*). However, the Zika virus disease outbreak in French Polynesia during 2012–2014 surprised the public health communities because of the high prevalence of Guillain-Barré syndrome (*5*). In addition, the ongoing Zika virus epidemic in the Americas since 2015 was associated with congenital infection and an unprecedented number of infants born with microcephaly (*6*,*7*). In 2015, the Zika virus epidemic spread from Brazil to 60 other countries and territories; active local virus transmission (*8*) and cases of imported Zika virus disease are occurring all over the world (*9*,*10*). In view of the seriousness of the epidemic, the World Health Organization declared the clusters of microcephaly and Guillian-Barré syndrome a Public Health Emergency of International Concern (*11*).

Experimental studies have confirmed that *Aedes* mosquitoes, including *Ae. aegypti*, *Ae. albopictus*, *Ae. vittatus*, and *Ae. luteocephalus*, serve as vectors of Zika virus (*12*–*15*). However, vector competence (ability for infection, dissemination, and transmission of virus) differs among mosquitoes of different species and among virus strains. *Ae. aegypti* mosquitoes collected from Singapore are susceptible and could potentially transmit Zika virus after 5 days of infection; however, no Zika virus genome has been detected in saliva of *Ae. aegypti* mosquitoes in Senegal after 15 days of infection (*12*,*14*). *Ae. albopictus* mosquitoes are a secondary vector for Zika virus transmission (*16*). In Italy, the population transmission rate is lower and the extrinsic incubation period is longer in *Ae. albopictus* than in *Ae. aegypti* mosquitoes (*17*). Transmission of Zika virus may also involve mosquitoes of other species such as those of the genera *Anopheles* and *Culex;* the virus had been detected in *An. coustani* and *Cx. perfuscus* mosquitoes from Senegal (*18*,*19*).

In February 2016, China recorded its first case of Zika virus infection in Jiangxi Province; the case was confirmed to have been caused by virus imported from Venezuela (*20*). Since then, 13 cases caused by imported Zika virus have been reported from several provinces (*21*); no evidence of autochthonous transmission has been found. In China, *Ae. aegypti* mosquitoes are found only in small areas of southern China, including Hainan Province and small portions of Yunnan and Guangdong Provinces (*22*). The predominant mosquitoes across China, especially in cities, are *Ae. albopictus* and *Cx. quinquefasciatus* (*23*,*24*); *Ae. albopictus* mosquitoes are the primary vector of dengue virus (family *Flaviviridae*) (*25*). *Cx. quinquefasciatus* mosquitoes are the primary vector for the causative organisms of St. Louis encephalitis, Rift Valley fever, lymphatic filariasis, and West Nile fever (*26*). The potential for *Cx. pipiens* mosquitoes to be Zika virus vectors (*27*) needs further confirmation. Because cases of Zika virus disease caused by imported virus have been reported in China, we investigated the potential vectors.

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Materials and Methods

Mosquitoes

The Guangdong Provincial Center for Disease Control and Prevention collected *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes from different sites in the cities of Foshan (in 1981) and Guangzhou (in 1993) in Guangdong Province. In 2005, the China Center for Disease Control and Prevention collected *Ae. aegypti* mosquitoes from the city of Haikou in Hainan Province. All mosquitoes were maintained under standard insectary conditions of 27 ± 1°C, 70%–80% relative humidity, and a light:dark cycle of 16 h:8 h. To obtain enough individuals for the experiments, we collected eggs from mosquitoes of all 3 species and hatched them in dechlorinated water in stainless steel trays. The larvae (150–200/L water) were reared and fed daily with yeast and turtle food. Pupae were put into 250-mL cups and placed in the microcosm (20 cm × 20 cm × 35 cm cage covered with nylon mesh) until they emerged. Adults were kept in the microcosms and given 10% glucose solution ad libitum.

Zika Virus

Zika virus (GenBank accession no. KU820899.2), provided by the Guangdong Provincial Center for Disease Control and Prevention, was originally isolated from a patient in China in February 2016 and classified as the Asian lineage (*28*,*29*). The virus had been passaged once via intracranial inoculation of suckling mice and twice in C6/36 cells. In the laboratory at Southern Medical University (Guangzhou, China), C6/36 cells were infected by virus stocks with a multiplicity of infection of 1 and left to grow at 28°C for 5–7 days. The cells were suspended and separated into an aliquot and stored at –80°C. The frozen virus stock (3.28 ± 0.15 $\log_{10}$ copies/μL) was passaged once through C6/36 cells before the mosquitoes were infected. The fresh virus suspension (5.45 ± 0.38 $\log_{10}$ copies/μL) was used to prepare the blood meal.

Infection of Mosquitoes

We transferred 5–7-day-old female *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes to 500-mL cylindrical cardboard containers covered with mesh, where they were starved for 24–48 h. The infectious blood meal was prepared by mixing defibrinated sheep blood (Solarbio, Beijing, China) with fresh virus suspension at a ratio of 1:2. The blood meal was warmed to 37°C and transferred into a Hemotek blood reservoir unit (Discovery Workshops, Lancashire, UK). Mosquitoes were then fed by using the Hemotek blood feeding system. Quantitative reverse transcription PCR (qRT-PCR) was used to detect the virus concentration (copy level) in the blood meal before and after feeding. After 30 min of exposure to the infectious blood meal, mosquitoes were anesthetized with diethyl ether. Fully engorged females were transferred to 250-mL paper cups covered with net (10–15 mosquitoes/cup). The infected mosquitoes were provided with 10% glucose and maintained in an HP400GS incubator (Ruihua, Wuhai, China) at 28°C, 80% relative humidity, and a light:dark cycle of 16 h:8 h. The experiments were conducted according to standard procedures in a Biosafety Level 2 laboratory.

Zika Virus Infection in Whole Mosquitoes

To determine Zika virus infections in *Ae. aegypti*, *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes, we selected 18–30 mosquitoes at postinfection days (dpi) 0 (same day as blood meal), 4, 7, 10, and 14. Each mosquito was placed in 50 μL TRIzol (Ambion, Life Technologies, Carlsbad, CA, USA) and homogenized in a tissue grinder (Kontes, Vineland, NJ, USA). Total RNA was extracted according to the manufacturer's protocol of TRIzol reagent and dissolved in 20 μL RNase-free water.

Zika virus cDNA synthesis reaction was performed by using the GoScript Reverse Transcription System (Promega, Madison, WI, USA). Total RNA and 10 μM Zika virus reverse primer (3′ untranslated region: 5′-ACCATTCCATTTTCTGGC-3′) were incubated, and cDNA was synthesized according to the procedure.

The nonstructural protein 1 (NS1) primers of Zika virus were designed by NCBI/Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome), which was selective for 296-bp nucleotide (forward: 5′-ACCCAAGTCTTTAGCTGGGC-3′; and reverse: 5′-CTGGTTCTTTCCTGGGCCTT-3′). The following PCR conditions were used: 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 72°C for 7 min. The PCR products were examined by use of 1% agarose gel electrophoresis. The target fragment was cloned into pMD18-T vector (Takara, Dalian, China) and sequenced.

Quantification of Zika Virus in Mosquitoes

The viral genome in the Zika virus–positive mosquitoes was determined by using absolute qRT-PCR. First, we constructed the standard. A 141-bp fragment across capsid and propeptide regions of Zika virus was amplified by specific primers (forward: 5′-GGAGAAG AAGAGACGAGGCG-3′; and reverse: 5′-GATATGGCCTCCCCAGCATC-3′) and cloned into pMD18-T vector. After sequencing, the recombinant plasmid was linearized by *EcoR* I. The concentration of plasmid was detected by NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A standard curve (linear curve slope –3.447, Y intercept 38.312, R^2 1, amplification efficiency 95.029) was generated from a range of serial 10-fold dilutions of the plasmid (6.23×10^2–6.23×10^7 copies/μL).

Each 20 μL of qRT-PCR was amplified by a 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA,

USA) under the following conditions: 1 cycle at 50°C for 2 min, 95°C for 2 min; 40 cycles at 95°C for 15 s, 60°C for 15 s, and 72°C for 1 min. Zika virus RNA copies from each sample were quantified by comparing cycle threshold value with the standard curve. The efficiency of this qRT-PCR system was evaluated by using blank control, uninfected C6/36 cells, C6/36 cells infected with Zika virus or DENV, and mosquitoes infected with Zika virus or DENV; the result displayed that its minimum detecting amount is 6.23 copies/μL of Zika virus and that the specificity is 100%.

Zika Virus Infection in Mosquito Tissues

To further analyze Zika virus tropisms and vector competence in *Ae. aegypti*, *Ae. albopictus*, and *Cx. quinquefasciatus* mosquitoes, we infected another batch of mosquitoes with Zika virus and then dissected the midgut, head, and salivary glands of each mosquito at dpi 0, 4, 7, 10, and 14 by using 18–30 mosquitoes per time point. The legs and wings of mosquitoes were removed and placed into cold phosphate-buffered saline. Each tissue was dissected and washed 3 times in phosphate-buffered saline and transferred to 50 μL TRIzol (*30*). Following the above-mentioned procedure, we extracted total RNA, and the NS1 region of Zika virus from samples was detected by RT-PCR. The viral RNA copies from the positive samples were quantified by qRT-PCR. For those mosquitoes with Zika virus–negative midguts by RT-PCR and qRT-PCR, which we considered to be uninfected, we did not further analyze the heads and salivary glands. Vector competence of mosquitoes of 3 species was evaluated by calculating infection rate (no. infected midguts/no. tested midguts), dissemination rate (no. infected heads/no. infected midguts), transmission rate (no. infected salivary glands/no. infected midguts), and population transmission rate (no. infected salivary glands/no. tested mosquitoes).

Statistical Analyses

All statistical analyses were performed by using SPSS version 20.0 (IBM, Chicago, IL, USA). Logistic regression was used to compare the infection, dissemination, and transmission rates for different mosquito species at the same time or for the same species of mosquito at different times. p value significance was corrected by Bonferroni adjustments. The Zika virus RNA copy levels were log-transformed and then compared among mosquitoes of different species at the same time or among mosquitoes of the same species at different times by using post hoc Tukey honest significant difference tests.

Results

Zika Virus Infection of and Replication in Mosquitoes

After the 414 mosquitoes (138 of each of the 3 species) had ingested the infectious blood meal (dpi 0), RT-PCR indicated that all were Zika virus positive (Figure 1, panel A). The high proportion of mosquitoes infected with Zika virus was observed for *Ae. aegypti* and *Ae. albopictus* mosquitoes at dpi 4, 7, 10, and 14 (Figure 1, panel A). Infection rates were similar for *Ae. aegypti* and *Ae. albopictus* mosquitoes at all time points ($z = 1.169, -0.277, 0.0001, 0.333$; $p>0.05$) (Figure 1, panel A). However, at dpi 4 and 7, the infection rate for *Cx. quinquefasciatus* mosquitoes was significantly lower than that for *Ae. aegypti* mosquitoes ($z = -4.924, -4.186$; $p<0.01$) and *Ae. albopictus* mosquitoes ($z = -1.169, -4.007$; $p<0.01$) (Figure 1, panel A). After dpi 7, no Zika virus was detected in the midgut of *Cx. quinquefasciatus* mosquitoes (Figure 1, panel A).

The amount of Zika virus from the mosquitoes with midgut infection was further tested by qRT-PCR. The trend for mean Zika virus copies in *Ae. aegypti* and *Ae. albopictus* mosquitoes was an increase with time after infection, but that for *Cx. quinquefasciatus* mosquitoes was a decrease (Figure 1, panel B). For *Ae. aegypti* mosquitoes, Zika virus copies increased quickly from dpi 0 to 4 ($p<0.05$ by Tukey honest significant difference test), then increased gradually. For *Ae. albopictus* mosquitoes, the trend for copy levels of Zika virus was similar to that for *Ae. aegypti* mosquitoes, but levels were slightly lower before dpi 7 ($p<0.05$). However,

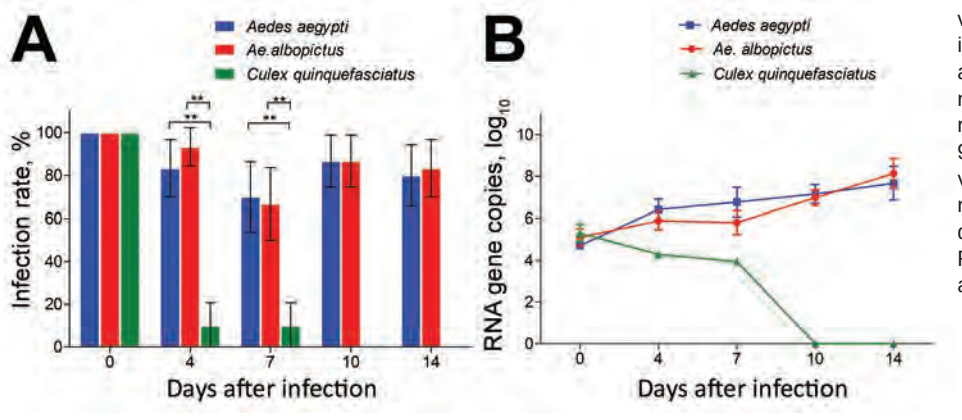

Figure 1. Infection rates and virus reproduction for Zika virus in *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes in China. A) Infection rate. Error bars represent 95% CIs. **, $p<0.01$. B) Zika virus RNA titers in the whole mosquito bodies was detected by quantitative reverse transcription PCR. The results are expressed as mean ± SD.

the copy levels were the same for mosquitoes of the 2 species at dpi 10 and 14 (p>0.05). For *Cx. quinquefasciatus* mosquitoes, the virus copy levels were low before dpi 7 and totally diminished afterward (Figure 1, panel B).

Vector Competence of Mosquitoes after Oral Challenge

The infection, dissemination, and transmission rates for Zika virus were assessed by detecting infection status of mosquito midguts, heads, and salivary glands. Another 414 mosquitoes (138 from mosquitoes of each species) were infected by Zika virus, and the midguts were measured; the overall infection rates were 89.86% for *Ae. aegypti*, 87.68% for *Ae. albopictus,* and 15.94% for *Cx. quinquefasciatus* mosquitoes (Table). At dpi 0, 100% of midguts were infected because of the undigested blood meal containing the virus, while no virus appeared in other tissues. High infection rates were maintained in *Ae. aegypti* and *Ae. albopictus* mosquitoes during the experimental period; no significant difference between *Ae. aegypti* and *Ae. albopictus* mosquitoes was found at dpi 4, 7, and 10 (z = 1.706, 1.777, 0.401; p>0.05) (Figure 2, panel A). At dpi 14, the infection rate for *Ae. albopictus* was higher than that for *Ae. aegypti* mosquitoes (z = 1.971; p = 0.04873). Compared with the infection rates for *Ae. aegypti* and *Ae. albopictus* mosquitoes, that for *Cx. quinquefasciatus* mosquitoes was significantly lower at dpi 4 (z = –5.081, –4.539; p<0.01) and 7 (z = –4.682, –4.264; p<0.01), and no midguts were positive for Zika virus at dpi 10 and 14 (Figure 2, panel A).

The dissemination of Zika virus in the heads of *Ae. aegypti* mosquitoes started from dpi 4 and increased rapidly up to 100% after dpi 7 (Figure 2, panel B). The spread of Zika virus in the heads of *Ae. albopictus* mosquitoes was first detected at dpi 7, and the rate was lower than that for *Ae. aegypti* mosquitoes at the same time point (z = –3.832; p<0.05) (Figure 2, panel B). Peak dissemination occurred during dpi 4–7 for *Ae. aegypti* (z = 4.344; p<0.001) and 7–10 for *Ae. albopictus* (z = 3.543; p<0.001) mosquitoes. Overall, Zika virus infection was disseminated in 73.39% of midgut-infected *Ae. aegypti* mosquitoes but only 42.15% of midgut-infected *Ae. albopictus* mosquitoes (Table). Zika virus was not detected in the head tissues of *Cx. quinquefasciatus* mosquitoes.

For *Ae. aegypti* mosquitoes, the detection of Zika virus in salivary glands was consistent with that in heads (Figure 2, panel C). Transmission of Zika virus by *Ae. albopictus* mosquitoes (which began at dpi 10 and increased to 68.97% at dpi 14) was lower at dpi 14 than that for *Ae. aegypti* mosquitoes at the same time (z = –3.561, –2.550; p<0.05) (Figure 2, panel C). A significant difference in transmission was detected during dpi 4–7 (z = 4.847; p<0.001) for *Ae. aegypti* and dpi 10–14 (z = 4.847; p = 0.0116) for *Ae. albopictus* mosquitoes. Zika virus was detected in the salivary glands of 78 (62.90%) midgut-infected *Ae. aegypti* and 29 (23.97%) midgut-infected *Ae. albopictus* mosquitoes. Furthermore, the population transmission rates of Zika virus were 56.52% for *Ae. aegypti*, 21.01% for *Ae. albopictus,* and 0% for *Cx. quinquefasciatus* mosquitoes (Table).

The amount of Zika virus in mosquito midguts, heads, and salivary glands was measured by qRT-PCR. The Zika virus copies ($\log_{10}$) in midguts of *Ae. aegypti, Ae. albopictus,* and *Cx. quinquefasciatus* mosquitoes did not differ significantly at dpi 0 (p>0.05). For *Ae. aegypti* mosquitoes, the Zika virus copies ($\log_{10}$) of midguts at dpi 4 were rapidly raised to 5.96 ± 0.92, which was higher than that at dpi 0 (5.00 ± 0.34) (p<0.05). Levels then increased continuously over time and reached 6.82 ± 0.47 at dpi 14 (Figure 3, panel A). For *Ae. albopictus* mosquitoes, the trend of increasing mean Zika virus copies was slow before dpi 7 and significantly lower than that for *Ae. aegypti* at the same time (p<0.05). After that, the growth of Zika virus became rapid and the Zika virus copies ($\log_{10}$) at dpi 14 reached 7.20 ± 0.48, which exceeded that in *Ae. aegypti* mosquitoes (p<0.05) (Figure 3, panel A). However, the amount of Zika virus continued to decrease in *Cx. quinquefasciatus* mosquito midguts after infection (Figure 3, panel A).

The number of Zika virus RNA copies ($\log_{10}$) in heads of *Ae. aegypti* mosquitoes continually increased from dpi 4 (4.97 ± 0.45) to 14 (6.19 ± 0.46) but remained stable for *Ae. albopictus* mosquitoes from dpi 7 (4.82 ± 0.43) to 10 (4.82 ± 0.64) and then reached 6.95 ± 0.81 at dpi 14 (Figure 3, panel B). At dpi 10, the number of virus copies in *Ae. aegypti* mosquitoes was higher than that in *Ae. albopictus* mosquitoes, whereas levels inverted at dpi 14 (p<0.05) (Figure 3, panel B). The trend of Zika virus in salivary glands was similar to that in the heads of *Ae. aegypti* mosquitoes. Compared with the Zika virus copies ($\log_{10}$) at dpi 0 (0), the value (5.54 ± 0.52) was apparently higher at dpi

Table. Rates of Zika virus infection, dissemination, transmission, and population transmission for *Aedes aegypti, Ae. albopictus, and Culex quinquefasciatus* mosquitoes, China

Rate	Mosquito species		
	Ae. aegypti	*Ae. albopictus*	*Cx. quinquefasciatus*
Infection*	124/138 (89.86)	121/138 (87.68)	22/138 (15.94)
Dissemination†	91/124 (73.39)	51/121 (42.15)	0/22 (0)
Transmission‡	78/124 (62.90)	29/121 (23.97)	0/22 (0)
Population transmission§	78/138 (56.52)	29/138 (21.01)	0/138 (0)

*No. infected midguts/no. tested midguts (%).
†No. infected heads/no. infected midguts (%).
‡No. infected salivary glands/no. infected midguts (%).
§No. infected salivary glands/no. infected mosquitoes (%).

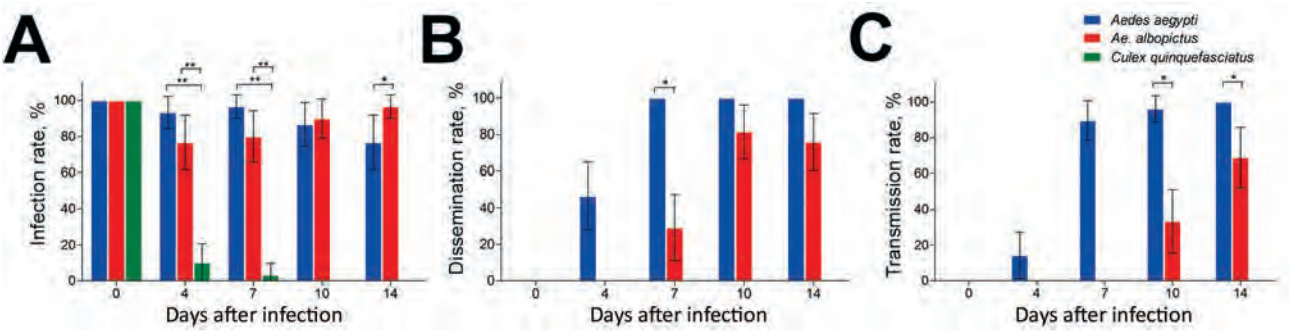

Figure 2. Vector competence of Zika virus in *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes in China. The midguts, heads, and salivary glands from mosquitoes of the 3 species were dissected at 0, 4, 7, 10, and 14 days after infection, and Zika virus was detected by reverse transcription PCR. A) Infection rate (no. positive midguts/total no. midguts). B) Dissemination rate (no. positive heads/no. positive midguts). C) Transmission rate (no. positive salivary glands/no. positive midguts). Error bars indicate 95% CIs. *p<0.05; **p<0.01.

14 (p<0.05) (Figure 3, panel C). Zika virus was detected in the salivary glands of *Ae. albopictus* mosquitoes during dpi 10–14, and the value at dpi 14 (6.19 ± 1.10) was higher than that at dpi 10 (4.92 ± 0.85). Furthermore, the number of virus copies in *Ae. albopictus* mosquitoes was higher than that in *Ae. aegypti* mosquitoes at dpi 14 (p<0.05) (Figure 3, panel C).

Discussion

Because of the absence of vaccines and specific treatment, the major approach to prevention and control of Zika virus disease is vector control (*31*). Identification of the mosquito species that could transmit Zika virus and determination of the extrinsic incubation period of Zika virus will provide a guide for vector control. In this study, we demonstrated experimentally that *Ae. aegypti* and *Ae. albopictus* mosquitoes in China possess the ability to transmit Zika virus, whereas *Cx. quinquefasciatus* mosquitoes were not able to transmit the virus under our laboratory conditions.

Our results demonstrate that *Ae. aegypti* mosquitoes could serve as vectors to spread Zika virus in China and that *Ae. aegypti* mosquitoes were better vectors than *Ae. albopictus* mosquitoes because transmission rate was higher and extrinsic incubation period was shorter for the former. The strong vector competence of *Ae. aegypti* mosquitoes could be associated with Zika virus rapid reproduction in the midgut during dpi 0–4, which enabled the viral particles to easily overcome the midgut barrier and be released into the hemolymph cavity and invade the salivary gland (*32*). Our findings are consistent with those for *Ae. aegypti* mosquitoes from Singapore and Italy (*12*,*17*). Although the distribution of *Ae. aegypti* mosquitoes is very limited in southern China, ranging from latitude 22°N to 25°N (*33*), the higher susceptibility of *Ae. aegypti* mosquitoes for Zika virus required the authorities in China to pay close attention to local epidemics of Zika virus in these regions.

Under the same experimental conditions, the whole-mosquito infection rates and midgut infection rates for *Ae. albopictus* and *Ae. aegypti* mosquitoes were similar, but the replication of Zika virus in midgut was slower for *Ae. albopictus* mosquitoes. The dissemination and transmission of Asian genotype Zika virus by *Ae. albopictus* mosquitoes in China started on 7 and 10 dpi, respectively, which indicated lower vector competence than that for *Ae. albopictus*

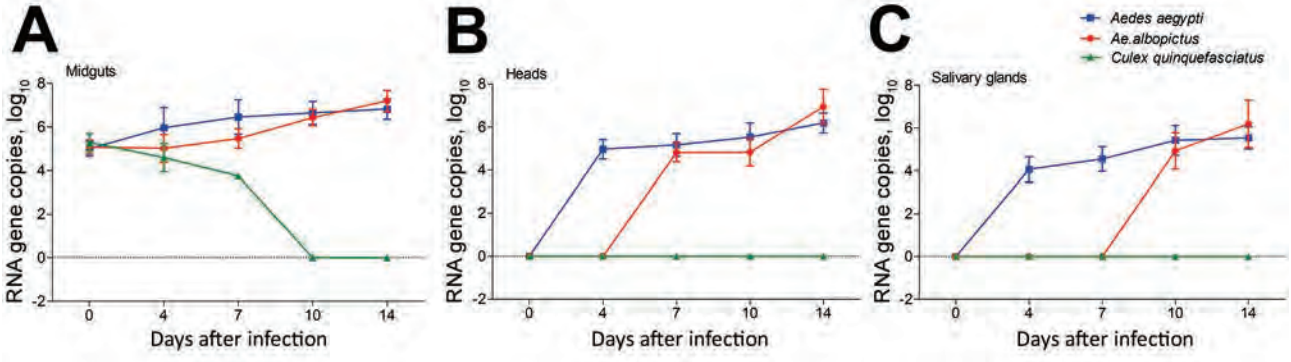

Figure 3. Zika virus RNA copies in infected midguts (A), heads (B), and salivary glands (C) of *Aedes aegypti*, *Ae. albopictus*, and *Culex quinquefasciatus* mosquitoes in China. Results are expressed as means ± SD. Dotted lines indicate the level below which minimum value could not fall. Error bars indicate SDs.

mosquitoes from Singapore infected with East African genotype Zika virus from Uganda but higher than that for *Ae. albopictus* mosquitoes from the Americas infected with Asian genotype Zika virus from New Caledonia (*13*,*34*). Although the extrinsic incubation period was longer for *Ae. albopictus* than for *Ae. aegypti* mosquitoes, *Ae. albopictus* mosquitoes are widely distributed in China, especially in Guangdong Province, where dengue was often epidemic (*35*). Moreover, *Ae. albopictus* mosquito density and survival time has increased with urbanization (*36*,*37*). Taken together, these findings indicate that *Ae. albopictus* mosquitoes can potentially become the primary vector for Zika virus in China and need attention in the vector control strategy.

Cx. quinquefasciatus are common blood-sucking mosquitoes in China, especially in southern cities, and are the vector of Western equine encephalitis virus (*38*). However, in this study, at dpi 0, all *Cx. quinquefasciatus* mosquitoes had ingested the virus, but the infection rate and Zika virus copies gradually decreased and no virus was detected in any tissues after dpi 7. The few positive midgut samples before dpi 7 could have resulted from an undigested blood meal because *Cx. quinquefasciatus* mosquitoes are larger and might take more blood than *Aedes* mosquitoes. Our results illustrate that *Cx. quinquefasciatus* mosquitoes in China are not able to transmit Zika virus, a finding that is consistent with the Zika virus susceptibility of *Cx. pipiens* mosquitoes from Iowa, USA, and *Cx. quinquefasciatus* mosquitoes from Rio de Janeiro, Brazil (*15*,*39*). However, our results contradict those of Guo et al., which indicated that *Cx. p. quinquefasciatus* mosquitoes are potential Zika virus vectors in China (*40*). These contradictory results might come from different experimental conditions, virus strains, or mosquito species and need more study.

In our study, Zika virus from C6/36 cells or infected mosquitoes was sensitively and specifically identified by qRT-PCR. We used qRT-PCR to detect virus copies because the Zika virus strain isolated from the patient who imported the virus into China can infect C6/36, Aag2, and Vero cells but did not show obvious cytopathic effect, which could be associated with the patient's mild clinical signs. Furthermore, previous research proved that the viral copies calculated by qPCR were consistent with the PFU detected by plaque assay (*41*). Although passage of the Zika virus we used in C6/36 cells was relatively low, the preliminary result demonstrated the highest virus reproduction in C6/36 cells compared with Aag2 and Vero cells.

In conclusion, our findings indicate that in China, *Ae. aegypti* and *Ae. albopictus* mosquitoes are susceptible to Zika virus, whereas *Cx. quinquefasciatus* mosquitoes are not able to transmit the imported Zika virus. Comparatively, the vector competence of *Ae. albopictus* mosquitoes is inferior to that of *Ae. aegypti* mosquitoes, but considering their wide distribution, *Ae. albopictus* mosquitoes might become the primary vector for Zika virus in China. These updated findings can be used for Zika virus disease prevention and vector control strategy.

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Clonal Clusters and Virulence Factors of Group C and G *Streptococcus* Causing Severe Infections, Manitoba, Canada, 2012–2014

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Learning Objectives

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

- Identify clinical features of patients with group C and G *Streptococcus* (GCGS) bacteremia, based on a retrospective study in Manitoba, Canada.
- Assess outcomes in patients with GCGS bacteremia.
- Determine genetic determinants of GCGS bacteremia.

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The incidence of group C and G *Streptococcus* (GCGS) bacteremia, which is associated with severe disease and death, is increasing. We characterized clinical features, outcomes, and genetic determinants of GCGS bacteremia for 89 patients in Winnipeg, Manitoba, Canada, who had GCGS bacteremia during 2012–2014. Of the 89 patients, 51% had bacteremia from skin and soft tissue, 70% had severe disease features, and 20% died. Whole-genome sequencing analysis was performed on isolates derived from 89 blood samples and 33 respiratory sample controls: 5 closely related genetic lineages were identified as being more likely to cause invasive disease than nonclade isolates (83% vs. 57%, p = 0.002). Virulence factors *cbp*, *fbp*, *speG*, *sicG*, *gfbA*, and *bca* clustered clonally into these clades. A clonal distribution of virulence factors may account for severe and fatal cases of bacteremia caused by invasive GCGS.

Group C and G *Streptococcus* (GCGS) are quickly becoming a major public health concern as the incidence of invasive infection and severe disease is increasing (*1*–*6*). In Manitoba, Canada, the incidence of GCGS bacteremia continues to increase, whereas the incidence of other invasive β-hemolytic streptococcal infections remains constant (*1*), similar to trends observed in Finland, Denmark, and Israel (*3*–*5*,*7*). These invasive infections cause severe illness, and up to 25% of patients die (*2*,*3*,*7*–*9*), yet the factors contributing to disease severity and death remain unclear.

Streptococcus dysgalactiae subsp. *equisimilis* (SDSE) is responsible for most cases of GCGS infections in humans (*10*,*11*). Historically considered nonpathogenic commensal flora, SDSE is now implicated in skin and soft tissue infections, pharyngitis, bacteremia, endocarditis, sepsis, toxic shock, and other invasive infections (*3*,*5*,*9*,*12*–*14*) that extensively overlap with the clinical presentations of *S. pyogenes* (group A *Streptococcus* [GAS]) infections. Similar to *S. pyogenes*, SDSE form large β-hemolytic colonies on sheep blood agar with hyaluronic acid capsules but express Lancefield group C or G carbohydrate (*15*) and possess M protein, which is vital in inhibiting complement pathway activation and resisting phagocytic killing (*16*). SDSE is genetically closely related to *S. pyogenes*, sharing 61%–72% sequence homology (*11*,*17*). These pathogens can exchange genes through bacterial phages and other mechanisms (*11*).

Approximately 71 virulence factor genes from *S. pyogenes* have been identified in SDSE, including hemolysin, streptolysin, exotoxin, proteinase, adhesin, streptokinase, and hyaluronic acid genes (*11*,*18*). *S. pyogenes* and SDSE carry streptolysin O (*slo*), which is required for invasive human infection (*11*,*19*), and streptolysin S (*sagA*), which has been linked to necrotizing soft tissue infections (*20*). Furthermore, the superantigen alleles *speA, C, G, H, I, K, L, M, N, O,* and *P*, which have been identified in *S. pyogenes*, have infrequently been identified in SDSE, but *speJ* and *ssa* are unique to GAS, and *szeN*, *szeP*, and *szeF* are unique to GCGS (*21*). The only commonly reported superantigen of SDSE is *speG* (*11*,*22*,*23*). Other commonly found virulence factors in SDSE are *lmb*, *gapC*, *sagA*, *hylB*, *slo*, *scpA*, and *ska*, whereas the presence of *cbp*, *fbp*, and *sicG* is variable and found only in a minority of strains (*22*). A conclusive association between virulence profile and disease propensity or site of isolation has not been demonstrated (*18*,*22*,*24*).

The monitoring of emerging pathogens requires phenotypic and molecular-based typing methodologies. Multilocus sequence typing (MLST) can be useful in tracking short-chain transmission of infections, but application of whole-genome sequencing for comparative studies provides higher resolution through a genomic epidemiology approach to investigate strain relatedness and dynamics. To uncover factors that may contribute to increased GCGS pathogenesis, we describe the clinical features of 89 GCGS bloodstream infections and the distribution of sequence types (STs) and virulence factors by whole-genome sequencing of 122 invasive and noninvasive isolates. We conducted this study in accordance with the ethical principles at the University of Manitoba after obtaining approval from the Health Research Ethics Board and Research Impact Committee.

Materials and Methods

Using the records of 2 large laboratories, we retrospectively identified GCGS bacteremia cases that occurred during January 2012–December 2014 in Winnipeg, Manitoba, Canada. We identified 89 bacteremic events (defined as ≥1 blood culture positive for GCGS during a single hospital admission) among a total of 84 patients. We reviewed charts to obtain patient characteristics and clinical parameters for each bacteremic event. During September–December 2014, within the same geographic location as the study cohort, community physicians collected control pharyngeal swab samples from outpatients with signs or symptoms of pharyngitis. The samples, which were obtained at the physicians' discretion, were cultured for identification of pyogenic streptococci: 33 noninvasive GCGS isolates were detected. These GCGS isolates were recovered from patients with symptomatic pharyngitis, but their symptoms were not severe and not necessarily attributable to GCGS. Although these control isolates were not from asymptomatic volunteers, the clinical differences between invasive bloodstream isolates and noninvasive respiratory isolates were sufficient to compare genetic differences.

Disease Severity

We considered patients with ≥1 of the following to have severe GCGS disease: in-hospital death, admission to intensive care unit, need for vasopressor or ventilatory support, diagnosis of streptococcal toxic shock syndrome (STSS) or infectious endocarditis, or a high-risk Simple Clinical Score

≥8 or Rapid Emergency Medicine Score ≥10. We defined STSS according to guidelines of the Working Group on Severe Streptococcal Infections (*25*). We calculated Simple Clinical Scores and Rapid Emergency Medicine Scores primarily by using patient vital signs and other clinical features; high-risk scores are associated with a 9.0%–10.3% risk for death by 30 days after admission (*26*–*28*).

Collection and Identification of Bacteria

At the discretion of the healthcare provider, patient blood samples were collected at symptom onset into BacT/Alert bottles (bioMérieux, Saint-Laurent, QC, Canada) according to institutional protocol and incubated using the BacT/Alert blood culture instrument (bioMérieux). Isolates were stored in frozen stocks in skim milk at −70°C and later retrieved by subculture for further analysis.

A total of 92 GCGS isolates were recorded during the study period; 90 were retrieved, 2 were lost in storage, and 1 was identified as *S. equi* subsp. *zooepidemicus* by 16S rRNA sequence similarity and excluded from the study. We plated the 89 remaining isolates onto sheep blood agar (Oxoid, Nepean, ON, Canada) and aerobically incubated them for 24 h at 37°C in the presence of 5% CO_2. We confirmed isolate identification by using MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry with the MALDI BioTyper system (Bruker, Boston, MA, USA) according to the manufacturer's protocol. To confirm isolates with ambiguous MALDI-TOF mass spectrometry identifications, we used latex agglutination to Lancefield antigens C and G and the Vitek2 system (bioMérieux) for biochemical identification. All isolates were identified as *S. dysgalactiae*.

Whole-Genome Sequencing

We extracted DNA from cultures, created multiplexed libraries, assembled reads, and performed core nucleotide variation phylogenetic analyses (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/7/16-1259-Techapp1.pdf). In brief, we generated paired-end, 300-bp indexed reads on the Illumina MiSeq platform (Illumina, San Diego, CA, USA); the average yield was 1,015,107 reads/genome, and the average genomic coverage was 145×. Read quality was assessed by using FastQC version 0.11.4 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/), assembled with SPAdes version 3.6.2 (http://cab.spbu.ru/software/spades/), and annotated with Prokka version 1.11 (http://www.vicbioinformatics.com/software.prokka.shtml), yielding an average contig length of 39,313 bp and an average N50 contig length of 82,867 bp (*29*–*31*). The high-quality reads were then mapped to the publicly available reference genome, *S. dysgalactiae* subsp. *equisimilis* AC-2713 (GenBank accession no. NC_019042.1), by using SMALT version 0.7.5 (http://www.sanger.ac.uk/science/tools/smalt-0). Single-nucleotide variations (SNVs) were called using FreeBayes version 0.9.20 (https://github.com/ekg/freebayes) and SAMtools mpileup (http://www.htslib.org/) (*32*). The percentage of bases in the core was 82.8%, and 21,746 sites were used to generate the phylogeny.

We constructed a maximum-likelihood phylogenetic tree of informative SNV positions by using PhyML version 3.0 (http://www.atgc-montpellier.fr/phyml/) (*33*) and visualized the tree by using FigTree version 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) (*34*). We determined phylogenetic clades by cluster analysis on the full dataset of blood and respiratory isolates (n = 122) and on isolates from blood only (n = 89) by using ClusterPicker version 1.2.4 (http://hiv.bio.ed.ac.uk/software.html) with the following settings: initial and main support thresholds = 0.9, genetic distance threshold = 4.5, and the large cluster threshold = 10 (*34*). We submitted whole-genome sequencing read data to the NCBI Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra/) under BioProject accession no. PRJNA325743.

Molecular Typing

We used the whole-genome sequencing data for in silico determination of MLST STs; virulence factors (*lmb*, *gapC*, *cba*, *cbp*, *fbp*, *sagA*, *slo*, *hylB*, *spegg*, *sicG*, *fbsA*, *pavA*, *fnbA*, *fnbB*, *gfbA*, *scpA*, *scpB*, *bca*, *cylE*, *ska*, *skc* and *skg*) (*22*,*35*); and superantigens (*speA*, *speB*, *speC*, *speF*, *spegg*, *speH*, *speI*, *speJ*, *speL*, *mf-2*, *mf-3*, and *smeZ*) (*21*,*23*). We determined Lancefield serogroups from sequences annotated with Prokka and confirmed them by serologic testing using commercial latex antisera (SSI Diagnostica, Hillerød, Denmark). We submitted MLST allelic profiles to the *Streptococcus dysgalactiae* MLST database (https://pubmlst.org/sdysgalactiae/). We used allelic profiles to compute a goeBURST (global optimal eBurst; http://www.phyloviz.net/goeburst/) full minimum spanning tree using PHYLOViZ (http://www.phyloviz.net/) (*36*); groups were assigned by a single-locus variation from a founding ST. All strains were confirmed to belong to *S. dysgalactiae* subsp. *equisimilis* by BLASTn (*37*) alignment of 16S rRNA sequences to reference genomes of *S. dysgalactiae* subsp. *dysgalactiae* ATCC27957 and *S. dysgalactiae* subsp. *equisimilis* ATCC12394 (PubMed accession nos. NZ_CM001076.1 and NC_017567.1, respectively).

Statistical Methods

We used descriptive statistics, χ^2 test, Kruskal-Wallis test, and Fisher exact test to compare demographics between clusters of SDSE to determine whether they differed with respect to key risk factors. We used Fisher exact test to compare risk of death and other disease severity markers between ST clusters and clades. No observations were censored, so survival analysis techniques were not necessary.

Results

Patient Characteristics and Disease Severity

We investigated 89 GCGS bacteremic events in 84 patients in Winnipeg during 2012–2014. Most patients (63%) were male, and the mean age was 61 years (SD ± 18.4 years). Many patients had co-existing conditions, predominantly cardiovascular disease (47%) and diabetes mellitus (43%). The most common source of bacteremia was from skin and soft tissue infections (51%), and 37% of patients had primary bacteremia. Infectious endocarditis was confirmed or suspected in 7% of patients. No patients had necrotizing fasciitis or pharyngitis (Table 1).

In 70% of the cases, bacteremia was associated with markers of severe disease, including admission to an intensive care unit (26%) and the need for vasopressor (19%) or ventilatory (17%) support. Seventeen percent of patients had a diagnosis of STSS, and 35%–61% of patients had high-risk disease severity scores. Twenty percent of patients with GCGS bacteremia died while in the hospital (Table 2).

Table 1. Demographic and other variables among patients with group C and G *Streptococcus* bacteremia causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014*

Patient variable	Value
Demographic characteristic	
Median age, y ± SD	61 ± 18.4 (0–99)
Age groups, y	
<18	1/89 (1)
18–64	52/89 (58)
≥65	36/89 (40)
Sex	
M	56/89 (63)
F	33/89 (37)
Medical history†	
Active alcohol abuse	12/88 (14)
Active malignancy	16/88 (18)
Active smoker	17/88 (19)
Asthma or COPD	12/88 (14)
Cardiovascular disease	41/88 (47)
Chronic kidney disease	25/88 (28)
Diabetes mellitus	38/88 (43)
Dialysis dependent	10/88 (11)
History of intravenous drug use	3/88 (3)
Immunosuppressive drug use	11/88 (13)
Total parental nutrition	3/88 (3)
No predisposing conditions	8/88 (9)
Clinical source of bacteremia‡	
Skin and soft tissue infection	43/84 (51)
Intraabdominal or gastrointestinal infection	3/84 (4)
Pharyngitis	0/84
Osteomyelitis and discitis	1/84 (1)
Meningitis	1/84 (1)
Septic arthritis	2/84 (2)
Infectious endocarditis	6/84 (7)
Primary bacteremia without source	31/84 (37)
Clinical characteristic§	
Temperature ≥38°C	48/83 (58)
Mean arterial pressure ≤80 mm Hg	50/82 (61)
Heart rate ≥90 beats/min	63/83 (76)
Glasgow Coma Scale <15	36/84 (43)

*Values are no. patients in category/total no. patients with data available (%) except as indicated. COPD, chronic obstructive pulmonary disease.
†Data missing for 1 patient.
‡Data missing for 5 patients.
§Data missing or partially missing for 7 patients.

SDSE Isolate Characteristics

SDSE isolates from blood represented 89 (73%) of 122 total isolates; 33 (37%) of the 89 isolates were from female patients and 56 (63%) were from male patients. These isolates were classified as Lancefield groups G (63%) and C (37%). Respiratory isolates represented 27% (33/122) of the isolates; information regarding the number from female and male patients was not available. These isolates also were classified as Lancefield groups G (52%) and C (48%).

Core Single-Nucleotide Variation Phylogenetic Analysis

Phylogenetic analysis of all 122 isolates showed no association between infection type and patient sex, age, or disease severity (online Technical Appendix 1 Figure). Compared with the heterogeneous nonclade isolates, those that clustered into clades A–E represented a higher proportion of blood isolates (25/45 [57%] vs. 64/77 [83%], respectively; p = 0.002). In addition, compared with the other clades combined, clade A was represented by significantly fewer blood isolates (36/38 [95%] vs. 28/39 [72%], respectively; p = 0.017). In silico molecular determinants (MLST, Lancefield serogroups, and virulence factors) were clustered in a clonal distribution (online Technical Appendix 1 Figure). However, we found no significant associations when comparing blood and respiratory isolates.

Cluster analysis of the 89 blood isolates yielded 5 clades, A–E (n = 64); the other 25 heterogeneous isolates were outside these lineages. Clade A isolates were Lancefield serogroup C, clades B–E were serogroup G, and the heterogeneous nonclade isolates were serogroups C (n = 5) and G (n = 20) (Figure 1). Isolate numbers 35, 49, 26, 40, 47, 45, and 51 were most genetically distant from the other blood isolates, averaging 3,897–3,987 SNVs.

Table 2. Death and markers of disease severity among patients with group C and G *Streptococcus* bacteremia causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014*

Disease severity marker	Value
Death	18/89 (20)
Severe disease	62/89 (70)
Streptococcal toxic shock syndrome†	14/82 (17)
Rapid Emergency Medicine Score ≥10†	29/82 (35)
High-risk Simple Clinical Score ≥8†	50/82 (61)
Vasopressor support required†	16/84 (19)
Ventilatory support required†	14/84 (17)
Admission to intensive care unit required†	22/84 (26)

*Values are no. patients in category/total no. patients with data available (%) except as indicated.
†Data missing or partially missing for 5–7 patients. A Rapid Emergency Medicine Score ≥10 and Simple Clinical Score ≥8 is considered high risk and associated with a 9.0%–10.3% risk of death.

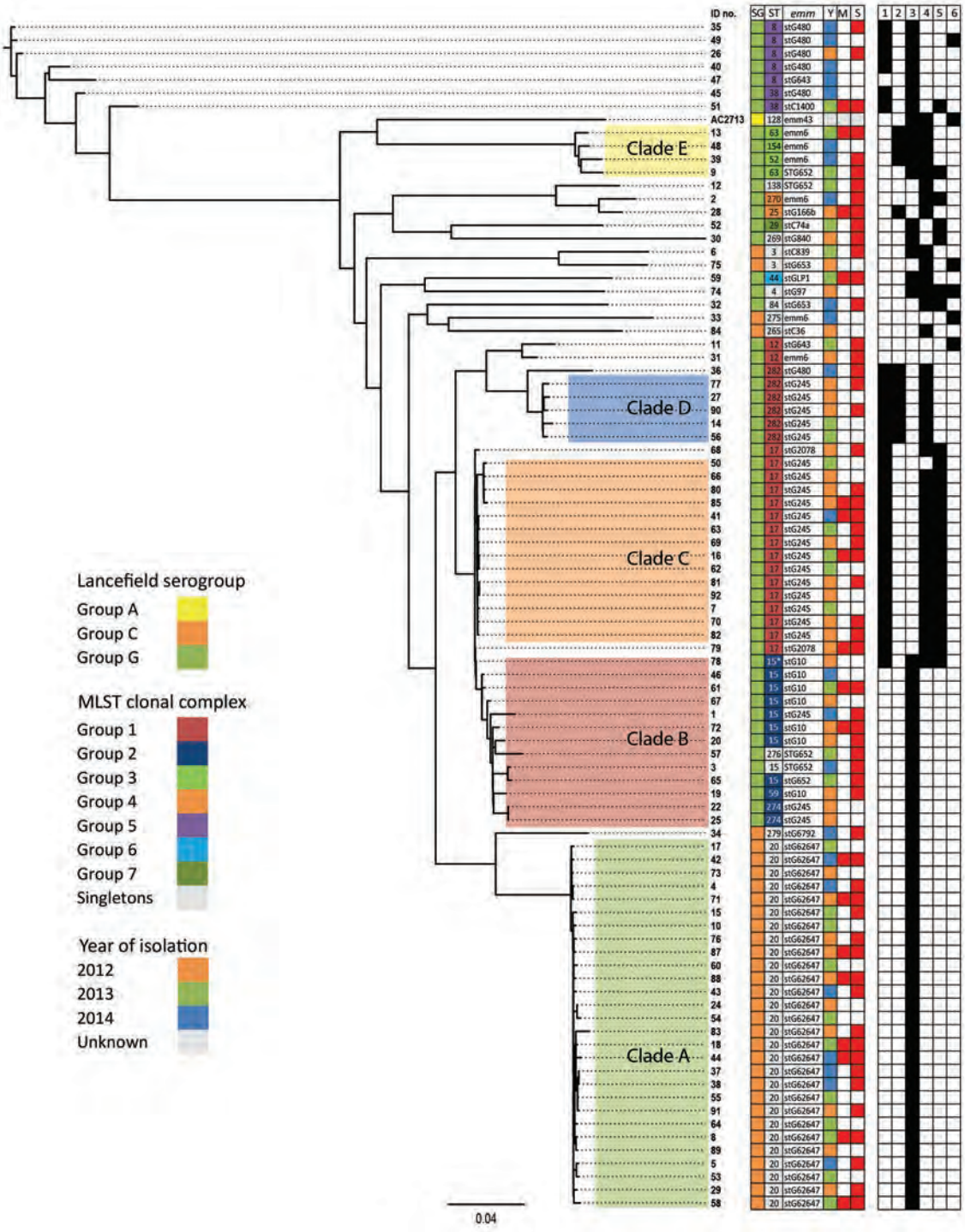

Figure 1. Maximum-likelihood whole-genome, core single-nucleotide variation (SNV) phylogenetic tree of 89 *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from the blood of patients with group C and G *Streptococcus* causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014. Multilocus sequence typing clonal complex relatedness groups were determined by using goeBURST (global optimal eBurst; http://www.phyloviz.net). In the mortality column, red and white squares indicate patient death and survival, respectively. In the severity column, red and white squares represent manifestation of severe and nonsevere disease, respectively. Black and white squares indicate the presence and absence of virulence factor genes, respectively. Scale bar indicates estimated evolutionary divergence between isolates, based on the average genetic distance between strains (estimated substitutions in sample/total high-quality SNVs). MLST, multilocus sequence type; SG, serogroup; ST, MLST; Y, year; M, mortality; S, severity; 1, *cbp*; 2, *fbp*; 3, *speG*; 4, *sicG*; 5, *gfbA*; 6, *bca*.

The greatest difference was 5,110 SNVs between isolate numbers 30 and 51 (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/23/7/16-1259-Techapp2.xlsx). Clade C was the most genetically homogenous, showing a maximum of 138 SNVs between isolates in the clade. Clade B was the most diverse, showing a maximum difference of 600 SNVs between isolates (online Technical Appendix 1 Table 1).

MLST

STs for all 122 isolates generally correlated with specific phylogenetic clades and subclades (Figure 1; online Technical Appendix 1 Figure). The most common STs were ST20 (n = 28), followed by ST17 (n = 16) and ST15 (n = 9) (Figure 2). Clade A (n = 28) consisted entirely of ST20 isolates belonging to a singleton MLST relatedness group. Clade B (n = 13) belonged to MLST clonal complex (CC) 2, in which ST15 (n = 9), ST69 (n = 1), and ST274 (n = 2) isolates grouped into subclades. An isolate with ST276 (a double-locus variant of ST15) also clustered into clade B. Clades C (n = 14) and D (n = 5) belonged to MLST CC1; clade C consisted of ST17 isolates, and clade D consisted of ST282 isolates. Clade E (n = 4) belonged to MLST CC3, in which ST63 (n = 2), ST52 (n = 1), and ST164 (n = 1) isolates grouped into subclades. Although SNV phylogenetic analysis showed that ST17 (clade C) and ST15 (clade B) isolates were closely related, large variations in MLST separated them into distinct clonal clusters (Figure 2).

A total of 18 STs were unique to blood isolates: STs 4, 8, 38, 44, 52, 59, 63, 84, 138, 154, 265, 269, 270, 274, 275, 276, 279, and 282. A total of 8 STs were unique to respiratory isolates: STs 49, 68, 206, 266, 273, 277, 280, and 283 (Figure 3).

Invasive Polymicrobial Infections

Polymicrobial bacteremia with organisms other than GCGS alone was present in 18% (16/89) of patients. In 4 patients with non-GCGS organisms plus GCGS isolates (i.e., isolate nos. 3 and 57, which clustered in clade B; and nonclade nos. 12 and 74), the non-GCGS organisms were believed to represent 1) contaminants at the time of sample collection or 2) the nonprimary pathogen. *Staphylococcus aureus* co-infection was seen in 6 patients. Four patients had GCGS isolates that clustered into clade C (nos. 41, 70, 82, 85), and the isolates were all associated with severe disease features (online Technical Appendix Table 2). Two of the 4 patients died.

Distribution of Virulence Factors

All 122 isolates carried virulence factors *gapC*, *hylB*, *lmb*, *sagA*, *scpA*, *scpB*, *ska*, *skc*, *skg*, and *slo*; however, virulence factors *cba*, *cfb*, *cylE*, *fbsA*, *fnbA*, and *pavA* were

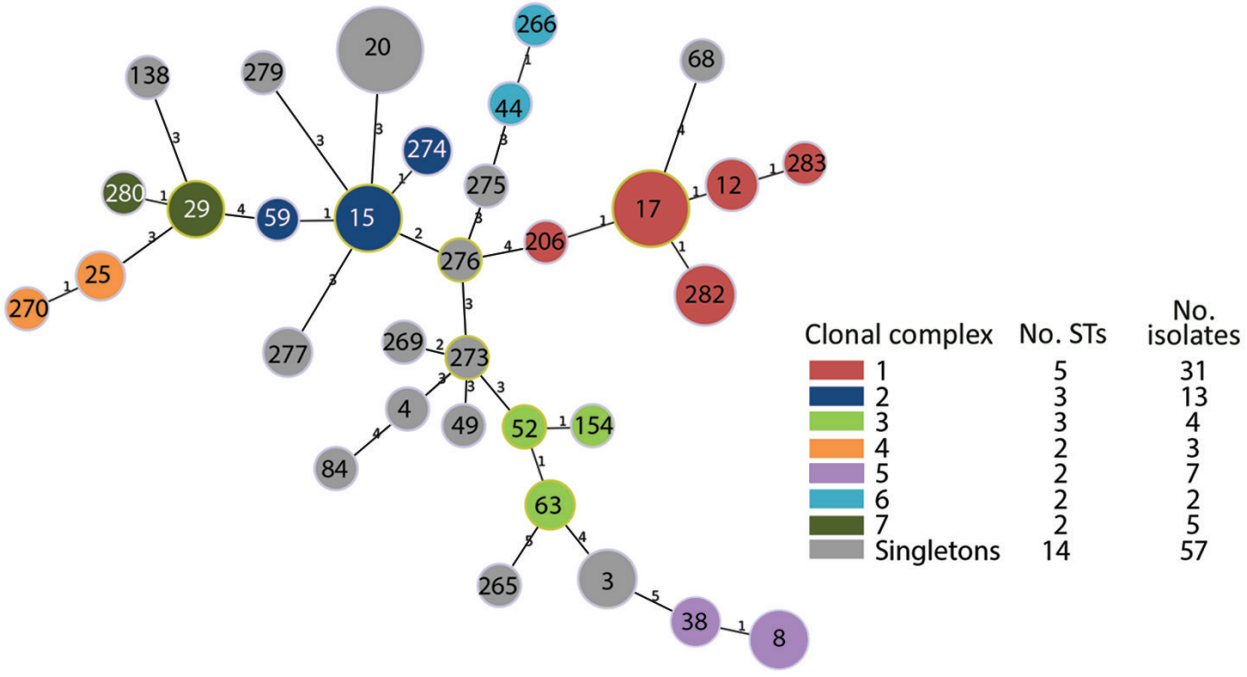

Figure 2. Minimum spanning tree representing the genetic relatedness of multilocus sequence types (MLSTs) of Streptococcus dysgalactiae subsp. equisimilis isolates from patients with group C and G Streptococcus causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014. Genetic relatedness was determined by full goeBURST (global optimal eBurst; http://www.phyloviz.net) analysis using Streptococcus dysgalactiae MLST allelic profiles of 7 housekeeping genes. Numbers on nodes correspond to individual sequence types (STs) and colored nodes correspond to clonal cluster relatedness groups defined by a single-locus variation from a founding ST. Number labels on branches indicate the number of allelic variations between STs; branch lengths are not to scale.

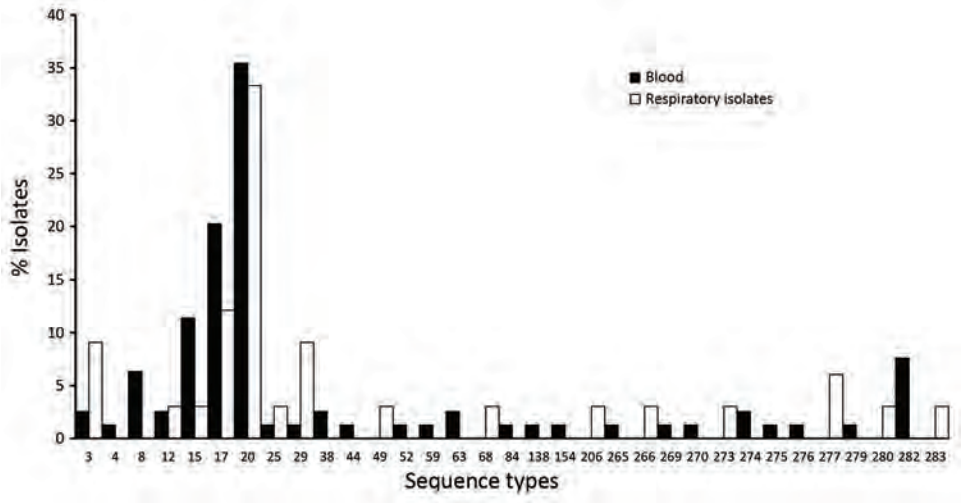

Figure 3. Prevalence of sequence types, as characterized by multilocus sequence typing, among blood and respiratory isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* from patients with group C and G *Streptococcus* causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014.

universally absent. Other factors were variably present (Table 3). Factors *cbp*, *fbp*, *speG*, *sicG*, *gfbA*, and *bca* clustered clonally into the phylogeny (Figure 1). All clade A and B isolates contained only *speG*, with the exception of 1 clade B isolate that also contained *cbp*, *sicG*, and *gfbA*. Clade C consisted of isolates with *cbp*, *sicG*, and *gfbA*; clade D isolates had *cbp*, *fbp*, and *sicG*; and clade E isolates had *fbp*, *speG*, and *sicG*. The virulence factor *fbp* was present in clades D and E and in 1 nonclade isolate (no. 28). Virulence factor *bca* was found variably in 5 nonclade isolates (nos. 49, 75, 74, 33, and 11) and in the reference isolate, AC-2713, which also contained genes *speG* and *sicG*. No association was discovered between the presence of these virulence factors and disease severity.

Clinical Outcomes within the Phylogeny

Severe disease features were present in a similar proportion of patients with GCGS disease caused by clade A–E isolates (63%, 40/64 patients) and heterogeneous nonclade isolates (68%, 17/25 patients). There was an observed trend toward increased mortality in patients with isolates from clades A–E (14 deaths) compared with patients with nonclade isolates (4 deaths), although the difference was not statistically significant ($p = 0.7698$). The number of deaths resulting from GCGS bacteremia caused by the most common clades, A–C (13/55 [24%]), was not significantly different than the number caused by other clades (5/34 [15%]; $p = 0.4179$). The death rate was also higher among patients with ST15, ST20, and ST17 (26% [14/53 patients]) than among patients with other STs (11% [4/36 patients]), but the difference was not significant ($p = 0.1075$).

Discussion

Our findings from this large study of the genomic epidemiology and molecular determinants of invasive GCGS bacteremia in association with the clinical features and outcomes of disease contribute to an evolving understanding of the changing epidemiology of β-hemolytic streptococcal infections. Similar to the findings of others (*10*), our findings showed that invasive infection is more common among older persons with underlying medical conditions. Although host factors probably contribute to changing epidemiology, enhanced GCGS virulence should be considered a contributor to the rising incidence of GCGS bacteremia. We observed rates of severe disease (70%), ICU admission (26%), and toxic shock syndrome (17%) that were higher than those from previous reports, suggesting increased GCGS virulence (*8*). Death occurred among 17 (20%) of the 84 patients with invasive GCGS bacteremia, a finding consistent with those in other reports (*7–10*).

As expected, skin and soft tissue infections served as the main portal of entry in more than half the cases of invasive GCGS bacteremia; however, primary bacteremia without alternate sources of infection was seen in a higher proportion (37%) of cases than seen in other reports (*3,5,14*). Infections without a source of bacteria entry could represent more effective bacterial penetration of skin and mucosal barriers and evasion of the host immune response due to enhanced pathogenic mechanisms.

Organisms in clades B–E were entirely Lancefield group G and had higher rates of invasive infections, possibly suggesting acquired genetic determinants are contributing to increased virulence and evolutionary selection of these clades. However, in this study, no single genetic determinant could account for an organism's ability to cause invasive infection. Although respiratory tract isolates in our study served as noninvasive controls, they were collected from persons with symptomatic pharyngitis, in whom host defenses might prevent severe infection and invasion into the blood stream. Host defenses may have obscured recognition of a shared invasion factor that could not be detected in our comparisons.

Table 3. Distribution of virulence factor genes in blood and respiratory isolates of *Streptococcus dysgalactiae* subsp. *equisimilis* from patients with group C and G *Streptococcus* bacteremia causing severe infections, Winnipeg, Manitoba, Canada, 2012–2014

Gene	Gene product	No. isolates positive for virulence factor/no. tested (%)			Reference
		Total isolates	Blood isolate	Respiratory isolate	
Adhesins					
gapC	Glyceraldehyde 3-P dehydrogenase	122/122 (100)	89/89 (100)	33/33 (100)	(*22*)
Lmb	Laminin-binding surface protein	122/122 (100)	89/89 (100)	33/33 (100)	(*22*)
fnbB	Fibronectin-binding protein	120/122 (98.4)	89/89 (100)	31/33 (94)	(*35*)
fnB	Fibronectin-binding protein	120/122 (98.4)	89/89 (100)	31/33 (94)	(*35*)
cbp	Collagen-binding protein	34/122 (27.9)	29/89 (33)	5/33 (15)	(*22*)
gfbA	Fibronectin-binding protein	32/122 (26.2)	24/89 (30)	8/33 (24)	(*35*)
fbp	Fibronectin-binding protein	11/122 (9.0)	10/89 (11)	1/33 (3)	(*22*)
fbsA	Fibrinogen-binding protein	0/122	0/89	0/33	(*35*)
pavA	Adherence and virulence protein A	0/122	0/89	0/33	(*35*)
fnbA	Fibronectin-binding protein	0/122	0/89	0/33	(*35*)
Antiphagocytosis					
cba	C protein β antigen	0/122	0/89	0/33	(*35*)
Complement protease					
scpA	C5a peptidase	122/122 (100)	89/89 (100)	33/33 (100)	(*22*,*35*)
scpB	C5a peptidase	122/122 (100)	89/89 (100)	33/33 (100)	(*35*)
Exoenzyme					
hylB	Hyaluronidase	122/122 (100)	89/89 (100)	33/33 (100)	(*22*,*35*)
Invasion					
bca	C protein α antigen	11/122 (9.0)	5/89 (6)	6/33 (18)	(*35*)
Streptokinases					
ska	Streptokinase	122/122 (100)	89/89 (100)	33/33 (100)	(*22*)
skc	Streptokinase	122/122 (100)	89/89 (100)	33/33 (100)	(*35*)
skg	Streptokinase	122/122 (100)	89/89 (100)	33/33 (100)	(*35*)
Toxins					
sagA	Streptolysin S	122/122 (100)	89/89 (100)	33/33 (100)	(*22*)
slo	Streptolysin O	122/122 (100)	89/89 (100)	33/33 (100)	(*22*)
speG	Streptococcus pyrogenic exotoxin G	81/122 (66.4)	58/89 (65)	23/33 (70)	(*22*)
cylE	β hemolysin/cytolysin	0/122	0/89	0/33	(*35*)
Cfb	CAMP factor	0/122	0/89	0/33	(*35*)
Other					
sicG	Streptococcal inhibitor of a complement	47/122 (38.5)	35/89 (39)	12/33 (36)	(*22*)

The virulence factor profiles we described were similar to those previously reported (*11*,*18*,*22*,*23*,*35*). However, *sicG* was present in a substantially higher proportion of isolates in our study (38.5%) than in another study (9.0%) (*18*), and it was primarily within clades C–E. The gene for *bca*, which has only rarely been described in SDSE, was present in a minority of our isolates (9.0%). The superantigen *speG* gene was found to cluster in Lancefield groups C and G, belonging to clades A and B, respectively, and was present in a proportion of isolates similar to that described in other reports (*18*,*22*). The reference isolate, AC-2713, also possessed all 3 of these virulence factors. All other superantigens found in GAS were absent from the isolates in our study.

The toxin gene *sagA* was present in all invasive and noninvasive isolates in our study. Although this toxin has previously been implicated in necrotizing skin and soft tissue infections (*20*), we did not confirm these findings in our study. No cases of necrotizing fasciitis were present in the study cohort; however, skin and soft tissue infections were common and severe, requiring surgical intervention in 17 (19%) of the 89 patients with bacteremia.

A specific cluster within clade C organisms was associated with polymicrobial bacteremia with *S. aureus*. All 4 patients co-infected with *S. aureus* and clade C GCGS organisms had severe infections: 2 patients, 1 of whom died, required renal replacement therapy; 1 was an intravenous drug user with endocarditis; and 1 was a 60-year-old man with diabetes who sought medical care for STSS from an unknown source and subsequently died. All isolates had *cbp*, *sicG*, and *gfbA* virulence factors. Three of the 4 patients had risk factors for endovascular infection; however, the clustering of these organisms may suggest a synergistic effect of co-infection and invasion with *S. aureus*.

Overall, the rising incidence and severity of invasive GCGS infections are probably associated with several evolving bacterial virulence factors. These factors probably take advantage of aging hosts with complex chronic diseases, susceptibilities, and co-existing conditions. Although our findings did not show a single virulence factor to account for emerging virulence, clonal clustering of factors within clades causing invasive infection suggests a survival and invasion advantage over clades without similar virulence clusters. Antimicrobial pressure may lead to accelerated transfer of genetic material, leading to acquisition of virulence factors. Furthermore, it is possible that newly acquired or novel virulence

factors not previously described in other β-hemolytic streptococci are present.

In conclusion, the frequency of invasive GCGS infections is surpassing that of GAS infections in patients in Manitoba, Canada, and these infections are associated with severe disease and death. Related strains that cluster clonally are more likely than others to cause invasive disease. The clonal distribution of virulence factors, in combination with host factors, is probably contributing to the emergence of invasive GCGS.

Acknowledgments

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Y.K. conceived and supervised this project. S.A.L. wrote the proposal for the study; obtained ethics approval for the research; performed retrospective chart reviews and analysis of clinical data; obtained, cultured, and identified the clinical isolates; and was the primary author of the manuscript. P.L.-W. oversaw the laboratory operations at St-Boniface hospital and provided the isolates. I.M. is section head of the Streptococcus and STI Unit at NML where Lancefield testing and whole-genome sequencing of isolates was performed. W.D. performed bioinformatic analyses. M.M. oversaw the laboratory operations at NML. B.D. performed statistical analyses. S.A.L. prepared the manuscript with significant contribution from W.D.

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Novel Retinal Lesion in Ebola Survivors, Sierra Leone, 2016

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We conducted a case–control study in Freetown, Sierra Leone, to investigate ocular signs in Ebola virus disease (EVD) survivors. A total of 82 EVD survivors with ocular symptoms and 105 controls from asymptomatic civilian and military personnel and symptomatic eye clinic attendees underwent ophthalmic examination, including widefield retinal imaging. Snellen visual acuity was ≤6/7.5 in 75.6% (97.5% CI 63%–85.7%) of EVD survivors and 75.5% (97.5% CI 59.1%–87.9%) of controls. Unilateral white cataracts were present in 7.4% (97.5% CI 2.4%–16.7%) of EVD survivors and no controls. Aqueous humor from 2 EVD survivors with cataract but no anterior chamber inflammation were PCR-negative for Zaire Ebola virus, permitting cataract surgery. A novel retinal lesion following the anatomic distribution of the optic nerve axons occurred in 14.6% (97.5% CI 7.1%–25.6%) of EVD survivors and no controls, suggesting neuronal transmission as a route of ocular entry.

The most recent Ebola virus disease (EVD) outbreak in West Africa is the largest outbreak in history. As of March 27, 2016, an estimated 3,956 persons in Sierra Leone had died from EVD, and 10,168 had survived (*1*). The scale of this epidemic has enabled the study of large numbers of survivors, facilitating the characterization of post-Ebola syndrome. Ocular symptoms have been reported, with incidence among survivors ranging from 14% to 60% (*2*–*4*). Evidence of acute uveitis on ophthalmic examination ranges from 18% to 58% (*4*–*7*). Classification of uveitis also varies and has been reported as 36%–62% anterior, 3% intermediate, 26%–36% posterior, and 18%–25% panuveitis (*4*,*8*). However, little is known regarding the medium- to long-term visual outcome of survivors or the rates of background uveitis and chorioretinal lesions within the local population.

Two published cases (*9*–*11*) and 2 case series (*7*,*12*) included fundus imaging, which attribute a range of retinal lesions to Ebola uveitis. Fourteen weeks after EVD discharge, a unilateral anterior hypertensive uveitis developed in 1 survivor and soon progressed into an aggressive anterior scleritis and intermediate uveitis. Viable Zaire Ebola virus (EBOV) was detected from the aqueous humor 9 weeks after the clearance of viremia (*9*). The duration of EBOV ocular persistence remains unknown, although repeated aqueous humor testing in the same survivor was negative for EBOV by quantitative reverse transcription PCR (qRT-PCR) 1 year later (*10*). Recurrences up to 13 months after EVD discharge have been reported, but confirmation of Ebola etiology through aqueous humor analysis was not conducted (*7*). Because of the unknown prevalence and duration of EBOV persistence in aqueous humor, survivors' access to cataract surgery is still restricted. Our study aimed to detect if any specific retinal signs can be attributed to past EVD in survivors, to describe the implications for visual acuity, and to assess for EBOV persistence in survivors with cataracts amenable to cataract surgery where no intraocular inflammation was present.

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Methods

Study Design

We conducted a case–control prospective study comparing ophthalmic findings between EVD survivors and a control group during January–June 2016. Reporting of the findings is in accordance with guidelines set forth in the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement (*13*).

Study Population

We searched a database of EVD survivors from the 2014–2016 EVD epidemic who had attended the EVD survivors clinic at 34th Regiment Military Hospital in Freetown, Sierra Leone, for patients who had reported ophthalmic complaints at any of their follow-up appointments (*2*). Patients

were contacted by telephone and invited to attend the ophthalmology clinic for review. EVD survivors from other medical facilities in the region who had reported ophthalmic complaints also attended the clinic through word of mouth and electronic social media networking from other survivors. EVD survivor status was verified by the possession of a valid discharge certificate from an Ebola treatment center. Date of acute admission, date of discharge, and location of the Ebola treatment center were recorded from each discharge certificate.

Controls were recruited from ophthalmically symptomatic and asymptomatic local military personnel, their local family members, and symptomatic civilians. Survivors and controls were invited to participate in English or Krio, as preferred, with local ophthalmic nurses acting as interpreters. Consent was confirmed by fingerprint or signature.

Ocular Examination

Data were collected on first visit. The onset and nature of ocular complaint, and any systemic complaints were recorded on a standardized form before examination. Patients underwent visual acuity testing with either Snellen or Illiterate E-chart acuity methods. Snellen visual acuity was grouped into visual acuity ranges according to the International Classification of Diseases, Ninth Revision, Clinical Modification, and reported as patient's best eye vision.

Ocular anterior chamber assessment was conducted with a table-mounted slit lamp by 3 local ophthalmic clinical officers. The initial 35% of anterior chamber examinations were supervised and verified by an ophthalmologist from the United Kingdom. Patient examinations thereafter were conducted by local clinical officers alone with a telecommunication link for advice if required. Assessment of anterior chamber inflammation was graded according to the Standardization of Uveitis Nomenclature criteria (*14*). Intraocular pressures were measured by automated pneumatic tonometry (Canon TX-F; Melville, NY, USA); if out of reference range, this measure was repeated by using Goldmann applanation tonometry.

Widefield retinal images were obtained from patients with the use of a nonmydriatic Daytona Scanning Laser Ophthalmoscope (fundus camera; Optos, Dunfermline, UK). Optical coherence tomography was undertaken with the use of a Topcon DRI Triton swept source optical coherence tomography (Topcon Corporation, Tokyo, Japan). Posterior subcapsular and cortical cataract were graded from a comparison of standard images used in the Lens Opacities Classification System III (*15*) and applied to acquired fundus images. White cataracts were identified during patient examination, and fundus imaging was not possible. Presence of signs in the vitreous indicative of intermediate uveitis were also recorded from scanning laser ophthalmoscope imaging.

All clinical and artifactual signs present on scanning laser ophthalmoscopic imaging and corresponding autofluorescent imaging were recorded, grouped, and incorporated into an original classification form with associated standard images and descriptions (online Technical Appendix 1, https://wwwnc.cdc.gov/EID/article/23/7/16-1608-Techapp1.pdf). All images were graded for these features by 2 independent, masked ophthalmologists from the United Kingdom with specialist interests in medical retina. Certainty of positive findings were quantified as "yes, definitely," defined as >90% certainty, or "yes, questionably," defined as >50% certainty. Mutual agreements of definite or probable certainty were counted. Where discordance existed between findings, a third independent consultant ophthalmologist made final arbitration.

Paracentesis of the anterior chamber was performed at a slit lamp with a sterile 30-gauge needle while the clinician was wearing personal protective equipment. After informed consent was obtained, the procedure was conducted on 2 patients with white cataracts but no clinical signs of anterior chamber inflammation. At the time of sampling, the 2 survivors were 430 and 482 days postdischarge from their respective Ebola treatment centers. By using an anterior chamber tap procedure protocol (online Technical Appendix 2, https://wwwnc.cdc.gov/EID/article/23/7/16-1608-Techapp2.pdf), 0.1 mL of aqueous humor was obtained in both cases. Both specimens were delivered to the Public Health England laboratory (Makeni, Sierra Leone) for analysis for EBOV RNA on qRT-PCR assay. Testing was performed with the use of the standard institutional operating protocols by clinical laboratory technologists who were trained in the safe handling of infectious pathogens.

Statistical Methods

We reported results per patient and grouped by subject by using IBM SPSS version 22 (http://www-01.ibm.com/support/docview.wss?uid=swg27038407). Where data were missing, we reduced the denominator for each variable. We double-checked 10% of data entry and found 0% transcription errors. We calculated 97.5% CIs by using the exact binomial (Clopper-Pearson) method (*16*); no overlap between CIs indicates a statistically significant result. Fisher exact statistical value was calculated for significant results.

The study was approved by the Sierra Leone Ethics and Scientific Review Committee on January 29, 2016. In addition, the study was authorized by the Pharmacy Board of Sierra Leone.

Results

The numbers of patients recruited and examined at 34th Regiment Military Hospital were 82 EVD survivors (161 eyes; 2 missing retina images and 1 prosthetic eye) and 105 never-infected controls (208 eyes; 2 missing retinal images).

Male-to-female ratio was 1:1.48 of EVD survivors and 1:0.64 of controls. Median age at time of ophthalmic examination was 28 years (interquartile range [IQR] 22–38 years) for EVD survivors and 41 years (IQR 30–48 years) for controls. Median time from Ebola treatment unit discharge to ophthalmic examination for survivors was 411 days (n = 70) (IQR 368–470 days). Ophthalmic examination findings were summarized for survivors and controls (Table).

We subclassified pigmented and nonpigmented retinal lesions into 10 discrete groups (online Technical Appendix 1) and noted frequency of each lesion type (Figure 1). We found no occurrences of the retinal lesion documented in a previous case report (*9*) in this EVD survivor cohort. Only the type 6 subcategory of retinal lesion was observed exclusively in EVD survivors, occurring in 12/82 (14.6% [97.5% CI 7.1%–25.6%]) EVD survivors and 0/105 controls (0%

Table. Ophthalmic examination findings in a case–control study of ocular signs in Ebola virus disease survivors, Sierra Leone, 2016*

	Survivors		Controls	
Finding	No.	% (97.5% CI)†	No.	% (97.5% CI)†
Best eye visual acuity‡				
Missing data	3	–	56	–
Normal	59	74.7 (62.1–84.9)	37	75.5 (59.1–87.9)
Near normal	18	22.8 (13.1–35.1)	8	16.3 (6.4–31.6)
Moderate	1	1.3 (0–7.8)	3	6.1 (1–18.6)
Severe	1	1.3 (0–7.8)	0	0 (0–8.6)
Profound	0	0 (0–5.5)	1	2 (0–12.3)
Near total	0	0 (0–5.5)	0	0 (0–8.6)
Total	0	0 (0–5.5)	0	0 (0–8.6)
Intraocular pressure, mmHg				
Missing data	35	–	74	–
Hypotonous (≤5)	5	10.6 (3–25)	0	0 (0–13.2)
Reduced (6–10)	5	10.6 (3–25)	3	9.7 (1.6–28.2)
Within normal range (11–21)	35	74.5 (57.6–87.3)	26	83.9 (63.8–95.4)
Elevated (22–29)	1	2.1 (0–12.8)	2	6.5 (0.5–23.7)
High (≥30)	1	2.1 (0–12.8)	0	0 (0–13.2)
Worst eye cup:disc ratio§				
Bilateral ungradable	1	–	0	–
Unilateral ungradable	11	–	8	–
Normal (0.1–0.6)	73	90 (80.1–96.2)	79	75.2 (64.5–84.1)
Moderate (0.7–0.8)	7	8.6 (3.1–18.3)	23	21.9 (13.5–32.3)
Advanced (≥0.9)	1	1.2 (0–7.6)	3	2.9 (0.5–9)
Cataract				
All cataract	19	23.2 (13.6–35.3)	18	17 (9.7–27)
White cataract	6	7.3 (2.3–16.5)	0	0 (0–4.1)
White cataract with hypotony, IOP ≤5 mm Hg¶	4	80 (23.6–99.7)	NA	NA
Active anterior uveitis				
Missing data	13	–	67	–
Anterior chamber cells present	5	7.3 (2–17.4)	4	10.5 (2.4–27)
Previous anterior uveitis				
Missing data	12	–	65	–
Signs of previous anterior uveitis#	7	10 (3.6–21)	0	0 (0–10.4)
Vitreous signs**				
Signs suggestive of active or past intermediate uveitis	8 (9.8)	9.8 (3.8–19.6)	14	13.3 (6.9–22.5)
Retinal signs**				
Retinal hemorrhages	0	0 (0–5.2)	2	1.9 (0.2–7.5)
Retinal neovascularization	0	0 (0–5.2)	1	1 (0–5.9)
Papilledema	0	0 (0–5.2)	0	0 (0–4.1)
Retinal vasculitis	0	0 (0–5.2)	4	3.8 (0.8–10.4)
Macula hole	0	0 (0–5.2)	1	1 (0–5.9)
Retinal tears	1	1.2 (0–7.5)	1	1 (0–5.9)
Retinal detachment	0	0 (0–5.2)	2	1.9 (0.2–7.5)
Asteriod hyalosis	0	0 (0–5.2)	1	1 (0–5.9)
Myelinated nerve fibers	0	0 (0–5.2)	1	1 (0–5.9)
Benign flecked retina	1	1.2 (0–7.5)	0	0 (0–4.1)
Geographic retinal darkening and variants	16	19.5 (10.7–31.2)	13	12.4 (6.2–21.4)
White without pressure	18	22 (12.6–34)	20	19 (11.2–29.2)

*IOP, intraocular pressure; NA, not available; –, not applicable.
†Calculated by using exact binomial Clopper-Pearson method.
‡Grading based on International Classification of Diseases, Ninth Revision, Clinical Modification (true Snellen fractions).
§When only 1 cup:disc ratio was gradable, only that ratio was used for analysis.
¶Missing data on 2 patients.
#Posterior synechiae and/or pigment on anterior lens capsule, keratic precipitates but no anterior chamber inflammation, or both.
**Graded based on widefield retinal image.

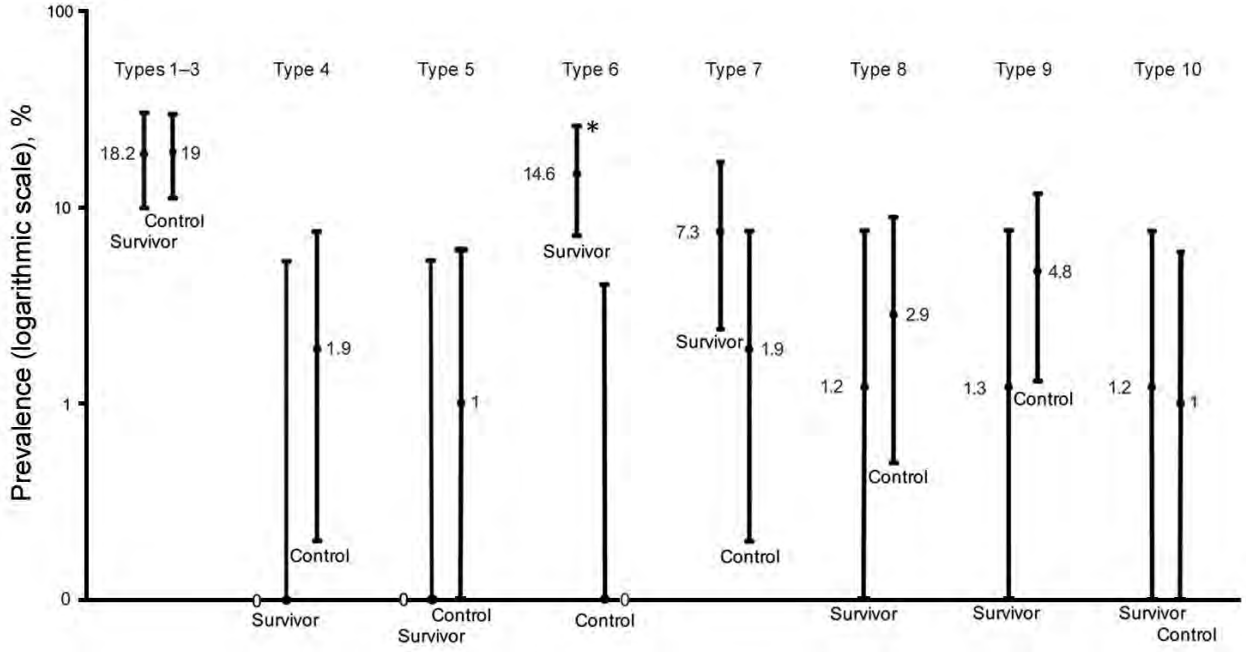

Figure 1. Prevalence of retinal scar lesion types in a case–control study of ocular signs in Ebola virus disease survivors, Sierra Leone, 2016. Type 1, uniform pigmented lesion; type 2, uniform pigmented lesion with gray halo; type 3, uniform pigmented lesion with lacunae; type 4, pigmented lesion with deep surrounding atrophy; type 5, previously described lesion attributed to Ebola (*8*); type 6, angulated lesions (peripapillary and/or peripheral); type 7, indistinct small pigmented lesions; type 8, irregularly pigmented vascular projection lesion; type 9, pigmented curvilinear peripheral bands; type 10, optic disc projection to macula lesion. Error bars indicate 97.5% CI. Asterisk indicates statistical significance ($p<0.01$) based on Fisher exact statistic value (2.7×10^5).

[97.5% CI 0%–4.1%]) ($p<0.01$). In 50% of EVD survivors, this type of lesion was observed bilaterally.

Two fundal distributions of type 6 lesions were evident: isolated or multifocal lesions in the peripheral retina or peripapillary lesions observed emanating from the optic disc (Figure 2). Each lesion shape was variable but often exhibited characteristic sharp angulations, resembling a diamond or wedge (Figure 3). Surrounding these lesions was a well-demarcated area of darkened retina in comparison with the adjacent retina. Presence of any retinal lesions of types 1–10, excluding type 6, were observed in 21/82 (25.6% [97.5% CI 15.5%–38%]) EVD survivors and 25/105 (23.8% [97.5% CI 15.1%–34.4%]) controls.

The aqueous humor of 2 EVD survivors with white cataract and no anterior chamber inflammation was negative for EBOV RNA on qRT-PCR assay. Postprocedure conjunctival swabs also were negative. The aqueous humor sampling procedure was uncomplicated and well-tolerated. No complications were reported on follow up.

Discussion

This case–control study identified a novel retinal sign that appears to be specific to EVD survivors. This sign occurred among a local population with a high rate of background chorioretinal disease. Uveitis after EVD has been reported (*3*,*8*), and a recent case report included a published fundus image from a survivor with a chorioretinal lesion attributed to EVD (*9*). That patient went on to have panuveitis.

The retinal lesions specific to EVD survivors were located either adjacent to the optic disc or in the fundus periphery. In the 8 cases in which lesions appear adjacent to the optic disc, their curvilinear projections from the disc margin appear to align with the anatomic pathways of the retinal ganglion cell axons that constitute the optic nerve. This distribution suggests a neurotrophic spread into the eye from the optic nerve and along the retinal ganglion cell axons. The other possible mode of entry into the eye is hematologic. Although the retinal ganglion cell axons often have parallel curvatures around the retinal arcade vessels, the lesions clearly follow the nerve fiber distribution in the absence of major vessels (Figure 1, panels A and C). Furthermore, we have not found any signs suggestive of associated vascular involvement, such as vasculitis, vascular occlusions, retinal ischemia, or secondary neovascularization, to support a hematologic spread. Neurotrophic properties are increasingly being recognized in EBOV (*18*). West Nile virus disease, caused by a known neurotropic virus, is associated with retinal lesions that follow a similar pattern of distribution to the pattern we have observed in our study (*19*).

Each Ebola lesion shape is variable, but a characteristic angulated appearance often resembling a diamond or wedge shape appears unique (Figure 2). As far as we are

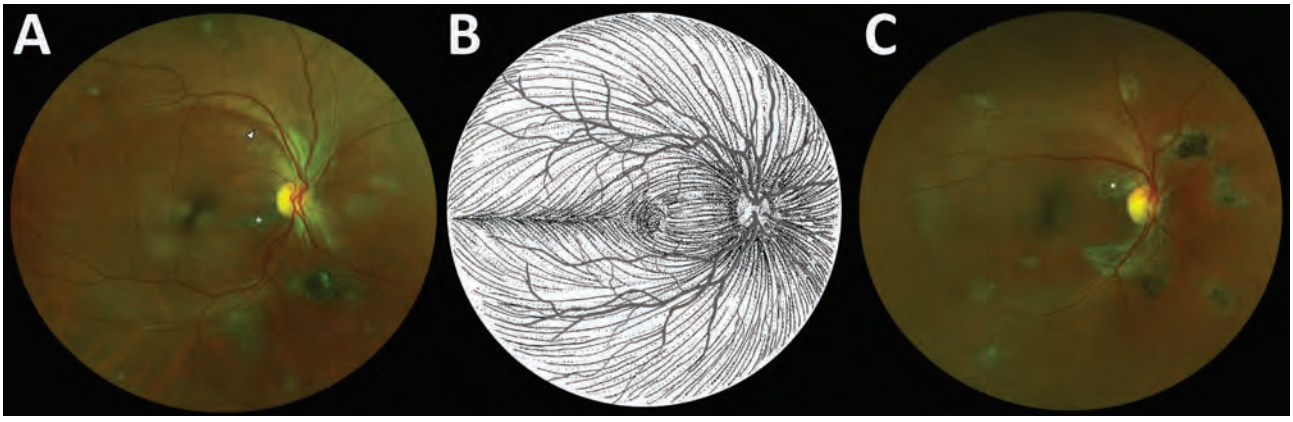

Figure 2. Composite scanning laser ophthalmoscope retinal images showing type 6 Ebola peripapillary and peripheral lesions, observed following the anatomic distribution of the ganglion cell axons (retinal nerve fiber layer), in a case–control study of ocular signs in Ebola virus disease survivors, Sierra Leone, 2016. A) Example 1, right eye. B) Illustration of the ganglion cell axon anatomic distribution. Courtesy of W.L.M. Alward. C) Example 2, right eye. Asterisks indicate curvilinear lesions distinct from the retinal vasculature. White arrowhead indicates retinal nerve fiber wedge defect.

aware, the appearance of these lesions is not characteristic of any other retinal disease. The reason for the sharp angulated appearance of these lesions might be explained by the tight triangular packing of the retinal cone mosaic (Figure 3, panel D) (*17*,*20*). The regular pattern of the photoreceptor triangular mosaic is disrupted by larger blue cones (*17*) and diminishes with eccentricity (*20*), which might explain the variability in shape. Optical coherence tomography indicates that these lesions are limited to the retina (Figure 3, panel B), and the resemblance of the lesion shape to the photoreceptor mosaic suggests that the ganglion cell axons act merely as a means of transportation to the photoreceptor end target.

Despite the proximity of the lesions to the optic nerve head, we observed no optic nerve head swelling or pallor in our study. This fact is in contrast to the 10% of optic nerve swelling reported in 1 abstract (*5*), although the time from acute infection to ophthalmic examination in that case was not stated. This difference might be attributable to varying durations since acute infection, allowing for any potential disc swelling to resolve in our cohort, for whom the median time since discharge was 411 days. Further optic nerve functional assessment, such as visual field analysis or color vision testing, has yet to be conducted.

The Ebola retinal lesions did not affect visual acuity. Overall, no difference was observed in uncorrected visual acuity between EVD survivors and controls. The most common cause of visual impairment in EVD survivors was white cataract (7.3%), which was accompanied by hypotony (low intraocular pressure) in 80% of EVD survivors. Hypotony suggests inadequate aqueous humor production and can limit the visual potential of an eye through complications such as retinal folds at the macula (i.e., hypotensive maculopathy).

Concern exists about the safety of cataract surgery in EVD survivors in Sierra Leone because of the unknown duration of EBOV ocular persistence. A sample size of 2 negative aqueous humor samples in this study is too small to make any definitive conclusions but shows that EBOV does not necessarily persist in aqueous humor in those with cataract but no ongoing intraocular inflammation. This finding suggests that cataract surgery can be conducted safely, providing an opportunity to restore vision and remove the stigma of EVD survivor status associated with having a visible white cataract. At present, we would recommend that anterior chamber sampling with EBOV PCR and a negative result should precede cataract surgery. However, cataract surgery might be challenging and visual outcomes disappointing in cases of secondary hypotony, which occurred in 80% of EVD survivors.

Before this study, only 1 aqueous humor sample had been obtained in an EVD survivor (*9*), enabling the detection of viable EBOV in aqueous humor during acute uveitis 9 weeks after discharge from hospital (*9*). Virus persistence in aqueous humor has also been observed in uveitis after Marburg virus infection (*21*), becoming negative on being repeated at 10 weeks (*22*). In EVD and Marburg virus–associated uveitis, intraocular pressure was markedly elevated (*9*,*21*). Although Ebola-related acute uveitis has been reported to be associated with high intraocular pressure, we did not find any evidence of persistently high intraocular pressure in survivors with Ebola retinal lesions.

Uveitis accounts for 24% of blindness in Sierra Leone and is second only to cataracts as the leading cause (*23*). A proportion of those cataracts might be a consequence of intraocular inflammation, especially in younger patients. Given the high endemic rates of parasitic, viral, and fungal

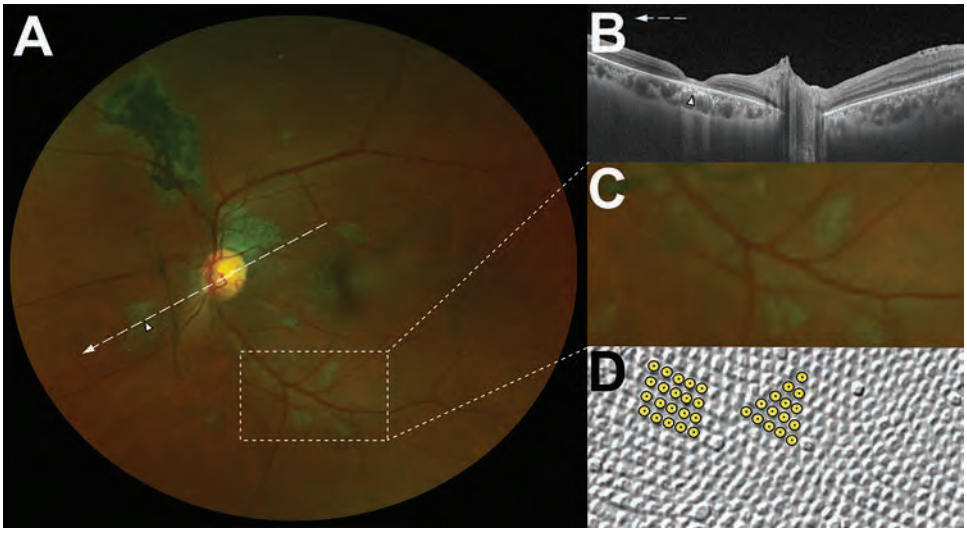

Figure 3. Characteristic features of lesions observed in a case–control study of ocular signs in Ebola virus disease survivors, Sierra Leone, 2016. A) Composite scanning laser ophthalmoscope retinal image, left eye. Arrow indicates direction of the optical coherence tomography scan. B) Optical coherence tomography. White, long, dashed line indicates cross-sectional plane; white arrowhead indicates Ebola lesion limited to the retinal layers with an intact retinal pigment epithelium. C) Examples of straight-edged, sharp angulated lesions (magnified from panel A 1.5×). D) Example of tangential section through the human fovea with illustrative highlighting of a triangular photoreceptor matrix corresponding to Ebola lesional shape. Courtesy of Ahnelt et al. (*17*).

disease in the region, infectious uveitis is likely to have a higher prevalence than in Western populations (*24*). Nevertheless, the proportion of controls with chorioretinal lesions and retinal vasculitis was surprising. Pigmented and atrophic chorioretinal scars not in keeping with the Ebola retinal lesions were no more common in EVD survivors than controls, and it is important not to attribute these findings to EBOV infection in survivors documented in case series (*7*,*12*).

The leading cause of uveitis in Sierra Leone is onchocerciasis, but this disease is in decline because of the systematic distribution of ivermectin to affected areas (*25*,*26*). The rate of other uveitis-associated blindness appears to be increasing in Sierra Leone (*23*). This study was conducted in Freetown, where the incidence of onchocerciasis is lower than in rural regions, and other causes are probably responsible. Toxoplasmosis accounted for 43% of symptomatic cases of posterior uveitis in 1 study (*27*), and it was probably a common cause among the patients in our study, although no serologic testing for toxoplasmosis was available. HIV prevalence in persons ≥15 years of age in Sierra Leone was estimated to be 1.25% in 2015 (*28*). The Ebola outbreak disrupted the fragile health system, including HIV reporting mechanisms and AIDS response (*29*). This HIV rate is still relatively low compared with many other African nations. Further diagnostic investigation is required to attempt to attribute causation to the various chorioretinal lesions observed in this study. Geographic areas of retinal whitening (white without pressure) are thought to be normal variants (*30*,*31*). Areas of retinal darkening (dark without pressure) have previously been attributed to sickle cell disease (*32*).

Our study is subject to 1 limitation with regard to the control group, who were selected opportunistically with unmatched cases and controls, and differences in age and sex ratios between the groups. This fact reflects the difficulties and limitations of conducting research in the post-Ebola setting in Freetown in 2016. The study was conducted in a military hospital, which housed the Ebola treatment unit and the continuing EVD survivors clinic. The hospital also serves the local civilian community and a military barracks community. The use of a non-EVD control group, even without matching, allowed a comparison in the fundus findings between post-EVD and control groups. We found a higher prevalence of retinal disease in the symptomatic clinic-attending control group than in the asymptomatic population control group; both groups included some military members of staff and families. This comparison allows us to be more positive about the specificity of the Ebola retinal lesion. Given our aim to compare EVD with non-EVD fundus findings, an age- and sex-matched population control group probably would not change the study conclusions.

EVD survivors were identified by the possession of an Ebola treatment center discharge certificate. Forgery of these certificates has been known given the free access to healthcare it confers. IgG confirmation of previous EBOV infection is planned for ongoing follow-up studies. Our study provides information on the medium-term ocular sequelae of EVD survivors with a median time of 411 days since hospital discharge. Our study does not provide data on acute uveitis and ocular disease in the immediate aftermath of EVD as reported elsewhere (*2*,*4*,*6*).

Although we can reasonably conclude the retinal lesions described in our study are sequelae of EVD, no

pre-EVD retinal imaging was available to conclusively identify the timing of acquisition of the lesions. Our control group demonstrates the common retinal signs and pathologies that are present in the population before Ebola exposure.

We have documented a novel retinal abnormality in EVD survivors that appears to be specific to EVD, although the proportion in the cohort with the condition is small. The background prevalence of chorioretinal abnormalities, including scarring with pigmentation, in the population is high and should not be attributed to EVD. Although further studies with larger sample sizes are required, EBOV does not necessarily persist in the aqueous humor of those with cataracts and no ongoing intraocular inflammation. These initial results raise the possibility of safe cataract surgery for EVD survivors with no signs of ongoing intraocular inflammation.

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P.J.S. had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. P.J.S., J.T.S., N.A.V.B., and M.G.S. conceived and designed the study. P.J.S., J.T.S., and M.G.S. wrote the ethics submission. P.J.S., J.T.S., and M.G.S. wrote the specimen collection protocols. P.J.S., J.T.S., and M.G.S. wrote the consent forms, and F.M., A.D.F., and P.K. translated them into Krio. P.J.S. trained and initially supervised staff in ophthalmic examination and imaging. M.G.S. supervised consent and aqueous humor specimen collection/transport and maintained quality assurance over procedures. P.J.S. and F.M. undertook aqueous humor collection. P.J.S., F.M., A.D.F., and P.K. were responsible for data collection and data storage. P.J.S., M.G.S., and N.A.V.B. developed the classification of image abnormalities and the data analysis plan. P.J.S., C.K.P., R.D., J.M.B., and N.A.V.B. performed image grading and the data analysis. N.A.V.B. provided the final arbitration of image grading. P.J.S., J.T.S., and G.C. wrote the statistical analysis plan. J.R. performed aqueous humor laboratory analysis. P.J.S. and N.A.V.B. drafted the manuscript. P.J.S. and M.G.S. drafted the paper, and all other authors reviewed and approved the final version.

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Effects of Zika Virus Strain and *Aedes* Mosquito Species on Vector Competence

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In the Western Hemisphere, Zika virus is thought to be transmitted primarily by *Aedes aegypti* mosquitoes. To determine the extent to which *Ae. albopictus* mosquitoes from the United States are capable of transmitting Zika virus and the influence of virus dose, virus strain, and mosquito species on vector competence, we evaluated multiple doses of representative Zika virus strains in *Ae. aegypti* and *Ae. albopictus* mosquitoes. Virus preparation (fresh vs. frozen) significantly affected virus infectivity in mosquitoes. We calculated 50% infectious doses to be 6.1–7.5 $\log_{10}$ PFU/mL; minimum infective dose was 4.2 $\log_{10}$ PFU/mL. *Ae. albopictus* mosquitoes were more susceptible to infection than *Ae. aegypti* mosquitoes, but transmission efficiency was higher for *Ae. aegypti* mosquitoes, indicating a transmission barrier in *Ae. albopictus* mosquitoes. Results suggest that, although Zika virus transmission is relatively inefficient overall and dependent on virus strain and mosquito species, *Ae. albopictus* mosquitoes could become major vectors in the Americas.

Zika virus (family *Flaviviridae*, genus *Flavivirus*) is the latest in a series of arboviruses to successfully invade the Americas; cases locally acquired in the Western Hemisphere were first identified in Brazil in May 2015 (*1*), and invasion subsequently expanded throughout Latin America and into the United States. To date, >460,000 suspected cases of autochthonous transmission have occurred in at least 45 Western countries (http://www.paho.org). Zika virus was first isolated in Uganda in 1947 (*2*) but was not implicated in a major epidemic until an explosive outbreak occurred on the island of Yap in Micronesia in 2007 (*3*). Subsequent outbreaks occurred in Cambodia in 2010, French Polynesia in 2013, and surrounding South Pacific islands in 2014 (*4*). Phylogenetic studies have suggested that the South Pacific islands are probably the source of the current outbreak in the Americas (*5*).

Although Zika virus infection had generally been thought to be asymptomatic or to result in a mild febrile illness (*6*), the 2013 outbreak marked the first time the virus was implicated as a causative agent of Guillain-Barré syndrome (*7*). It has also been confirmed that Zika virus can have teratogenic effects (*8*), causing a spectrum of neurologic problems, referred to as Zika congenital syndrome, in developing fetuses (*9*), particularly in women infected during their first trimester of pregnancy (*10*). Although the primary route of transmission is through blood feeding by an infected mosquito, efficient sexual transmission (*11*) and long-term persistence in male reproductive tissues and fluids have been well documented (*12*).

Epidemiologic and laboratory studies have implicated various *Aedes* spp. mosquitoes as Zika virus vectors (*3*,*13*–*16*). In the Americas, *Ae. aegypti* mosquitoes are the primary vector for Zika virus, as they are for dengue and chikungunya viruses (*17*). *Ae. albopictus* mosquitoes potentially act as a secondary or supplemental vector (*18*). In the laboratory, mosquitoes of both species have been shown to be efficient vectors (*14*,*18*–*20*). However, few Zika virus isolates have been obtained from mosquitoes in the Americas and few experiments have assessed competence with currently circulating strains and representative mosquito populations. In addition, because previous experimental studies generally used individual blood meal doses with virus titers rarely achieved in nature, the relationship between viremia levels and vector competence is largely uncharacterized, making determination of the duration and likelihood of host transmissibility difficult.

We conducted comparative studies of recent Zika virus isolates from the Americas and an isolate from the 2010 outbreak in Cambodia (*21*). Genetic differences identified among these strains translated to modest variability in replicative kinetics in vitro in mosquito (C6/36) and mammalian (Vero) cells. In addition, we characterized the dose response for vector competence in *Ae. aegypti* mosquitoes and a recently colonized *Ae. albopictus* mosquito population from New York, USA.

Methods

Viruses

The New York State Department of Health (NYSDOH) Arbovirus Laboratory isolated Zika virus HND (2016–19563,

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GenBank accession no. KX906952) from serum from a patient who had traveled to Honduras in early 2016. Amplification was obtained by inoculating 100 μL of serum into shell vials (ViroMed Laboratories, Burlington, NC, USA) confluent with Vero cells (ATCC, Manassas, VA, USA), followed by centrifugation at 700 × *g* for 40 min at 37°C and an additional 4 days of growth (*22*). Zika virus CAM (strain FSS130325, GenBank accession no. JN860885; kindly provided by C. Pager, State University of New York at Albany, NY, USA) was originally isolated in 2010 from human serum in Cambodia and passaged 3 times on Vero cell culture and 1 time on C6/36 cell culture. Zika virus PR (kindly provided by the Centers for Disease Control and Prevention, Fort Collins, CO, USA; strain PRCABC59, GenBank accession no. KU5012 15), used for preliminary experiments, was initially obtained from serum of a patient who had traveled to Puerto Rico in 2015 and was passaged 3 times on Vero cell culture and 1 time on C6/36 cell culture. Nucleotide and amino acid sequence alignments were created with Zika virus coding regions by using the MegAlign module of the DNAStar software package (http://www.dnastar.com).

In Vitro Growth Kinetics

We inoculated confluent monolayers of Vero and C6/36 cells with Zika virus strains in duplicate at a multiplicity of infection of 0.01 PFU/cell. After a 1-hour absorption period at 37°C (Vero) or 28°C (C6/36), the inoculum was removed and cells were washed twice with appropriate maintenance media. Cultures were maintained in 6-well plates with 3 mL of maintenance media (Eagle minimum essential medium with 2% fetal bovine serum) and incubated at 37°C (Vero) or 28°C (C6/36). Samples of 100 μL supernatant were harvested at days 1–4 (Vero) or 1–7 (C6/36) after infection, diluted 1:10 in media containing 20% fetal bovine serum, and stored at –80°C. Titrations were performed in duplicate, by plaque assay on Vero cells (*23*); mean titers for each time point were calculated and compared by *t*-test. Growth kinetics were compared by using repeated measured analysis of variance (ANOVA) and Tukey post hoc tests (GraphPad Prism version 5.0; GraphPad Software, La Jolla, CA, USA).

Experimental Infections and Mosquito Competence

Ae. albopictus mosquitoes (kindly provided by Illia Rochlin, Suffolk County Health Department, Yaphank, NY, USA) were originally collected in Suffolk County in 2014 and subsequently colonized in the NYSDOH Arbovirus Laboratory. F5–F7 female mosquitoes from New York were used for experimental feedings. *Ae. aegypti* mosquitoes used for preliminary experiments were collected by C. Mangudo in Salta, Argentina, in 2014 and initially colonized by V. Micieli and L.D. Kramer at the Centro de Estudios de Parasitología y Vectores (La Plata, Argentina) before being shipped to the NYSDOH Arbovirus Laboratory for maintenance. F4–F5 females from Argentina were used for experimental feedings. *Ae. aegypti* mosquitoes (kindly provided by G.D. Ebel, Colorado State University, Fort Collins, CO, USA) were originally collected in Poza Rica, Mexico. F7–F8 females from Mexico were used for experimental feedings. For preliminary blood feeding experiments, *Ae. aegypti* mosquitoes from Argentina were fed Zika virus PR stock virus diluted 1:1, 1:5, or 1:20 in defibrinated sheep blood (Colorado Serum Co., Denver, CO, USA) with 2.5% sucrose. For feedings with freshly propagated virus, supernatant from infected C6/36 cultures was harvested at 96 h after infection (multiplicity of infection ≈1.0) and diluted 1:1 with blood-sucrose mixture without freezing. Female mosquitoes, 4–7 days of age, were deprived of sucrose for 18–24 h and offered blood meal mixtures by use of a Hemotek membrane feeding system (Discovery Workshops, Acrington, UK) with a porcine sausage casing membrane. For all subsequent experiments assessing dose-dependent vector competence, similarly prepared fresh C6/36 cultures of Zika virus HND and Zika virus CAM were used to feed *Ae. aegypti* mosquitoes from Mexico and *Ae. albopictus* mosquitoes from New York. In addition to undiluted supernatant, 1:20, 1:400, and 1:8,000 dilutions were made in C6/36 maintenance media before being mixed with blood.

For all blood feeding experiments, mosquitoes were sedated with CO_2 after 1 h of feeding, and fully engorged mosquitoes were transferred to 0.6-L cartons and maintained at 27°C for experimental testing. Infection, dissemination, and transmission rates were determined as previously described (*24*) on day 14 or 21 after feeding. After the mosquitoes were sedated, the legs were removed from 12–30 mosquitoes and placed in 1 mL mosquito diluent (20% heat-inactivated fetal bovine serum in Dulbecco phosphate-buffered saline plus 50 μg/mL penicillin/streptomycin, 50 μg/mL gentamicin, and 2 μg/mL Fungizone [Sigma Aldrich, St. Louis, MO, USA]). For 30 minutes, mosquitoes were allowed to expectorate into capillary tubes containing ≈20 μL fetal bovine serum plus 50% sucrose (1:1), at which time the mixture was ejected into 250 μL mosquito diluent. Mosquito bodies were then placed in individual tubes with mosquito diluent. All samples were held at –80°C until tested. To test for infection, dissemination, and transmission, we processed and screened bodies, legs, and salivary secretions, respectively, by Zika virus–specific quantitative reverse transcription PCR (*25*). Zika virus body titers were calculated from standard curves based on infectious particle standards created from matched virus stocks. Data were analyzed by using GraphPad Prism version 4.0. Rates were compared by using Fisher exact tests, and dose dependence was evaluated and compared by using linear regression analyses.

Results

In Vitro Characterization

Sequencing analysis of these strains revealed 1.7% divergence and 16 aa differences distributed throughout the genome (Table 1). These differences include 8 aa in capsid; premembrane; envelope; and nonstructural 1, 3, and 5 genes, which differ from Zika virus CAM and are shared among the 2 isolates from the Americas. Peak virus titers were ≈3-fold higher on mosquito cells than on mammalian cells. Although in vitro kinetics were similar among strains (Figure 1), Zika virus CAM replicated to modestly higher titers (mean difference 3.0-fold by repeated measures ANOVA; $p<0.05$ by Tukey multiple comparison test) relative to Zika virus PR and HND and a significantly higher peak titer (mean 5.3-fold; $p<0.05$ by *t*-test). Zika virus HND was also modestly attenuated in mammalian cell culture, replicating to titers 2–3.3-fold lower than the titers achieved by Zika virus PR and Zika virus CAM, respectively (repeated measures ANOVA, by Tukey multiple comparison test). Peak titer for Zika virus CAM was statistically higher than that for Zika virus HND ($p = 0.04$ by *t*-test), yet Zika virus PR replicated to an intermediate value and was statistically equivalent to both Zika virus HND and CAM.

Infectivity and Vector Competence

Initial experiments that used previously amplified Zika virus PR stock frozen at –80°C failed to achieve high levels of infection in *Ae. aegypti* mosquitoes. No infection was identified at 14 days after feeding for mosquitoes fed 6.0 $\log_{10}$ PFU/mL, and only 3 (10%) of 30 mosquitoes were Zika virus positive when the dose was increased to 7.4 $\log_{10}$ PFU/mL (Figure 2). In an effort to achieve higher infectivity, we freshly harvested supernatant from mosquito cells after virus propagation and immediately used it for blood meal preparation. Blood meal titers for this experiment were high, 9.1 $\log_{10}$ PFU/mL, as were rates of infection and dissemination. At day 14 after feeding, 24 (96%) of 25 mosquitoes were Zika virus positive. Of the 24 positive mosquitoes, 22 (91.6%) had disseminated infections and 13 (54.2%) had Zika virus–positive saliva. To clarify the extent to which differences in infectivity were the result of virus titer or preparation (freshly propagated vs. frozen virus stocks), we fed a subset of mosquitoes the same blood meal (titer 9.1 $\log_{10}$ PFU/mL) after freezing at –80°C for 2 weeks. Although feeding rates were poor and survival was low for this cohort (n = 12), only 2 of the mosquitoes surviving to day 14 after feeding were Zika virus positive, which equated to a significantly lower infection rate than that obtained with freshly propagated virus ($p<0.001$ by Fisher exact test; Figure 2). All subsequent experiments were therefore completed with C6/36-derived Zika virus–positive supernatant before freezing.

After isolation, Zika virus HND was used as the representative Western Hemisphere strain and compared with Zika virus CAM for all experiments assessing the relationships between dose, vector competence, virus strain, and mosquito species. To maximize transmission potential, we waited until day 21 after mosquito feeding to conduct these studies. For *Ae. aegypti* mosquito feedings, the

Table 1. Amino acid differences among Zika virus isolates used in study of species-specific Zika virus vector competence of *Aedes* mosquitoes*

Position	Gene	Zika virus strain, amino acids		
		CAM	HND	PR
80	C	I	I	T
106	C	T	A	A
123	prM	V	A	A
130	prM	N	S	S
151	prM	M	L	L
620	E	V	V	L
763	E	V	M	M
894	NS1	G	A	G
982	NS1	A	V	V
1274	NS2A	P	L	L
1795	NS3	S	A	S
2074	NS3	M	L	M
2086	NS3	Y	H	H
2611	NS5	A	A	V
2634	NS5	M	V	V
3045	NS5	R	C	R

*C, capsid; E, envelope; NS, nonstructural; prM, premembrane.

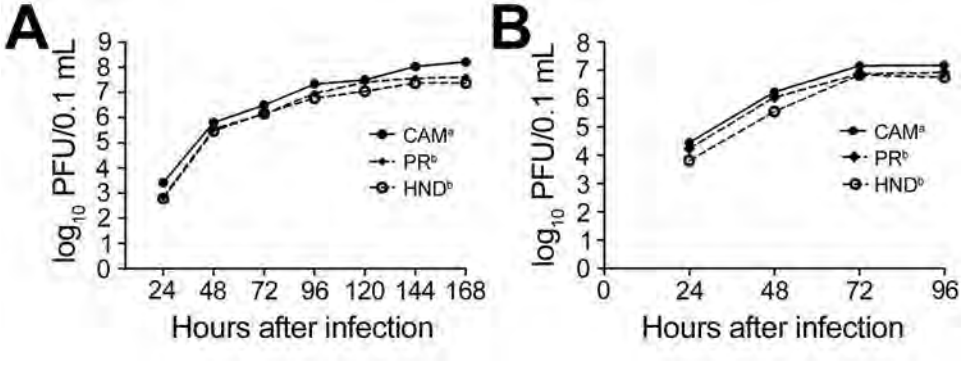

Figure 1. Growth kinetics of Zika virus in A) mosquito (C6/36) and B) mammalian (Vero) cells. Cells were infected in duplicate with Zika virus strain CAM, PR, or HND, at a multiplicity of infection of 0.1. Concentration of Zika virus in supernatant was determined by plaque titration for 4 (Vero) or 7 (C6/36) days after infection. Values represent geometric means ± SD, and different superscript letters represent statistically different growth kinetics (repeated measures analysis of variance; $p<0.05$ by Tukey post hoc test).

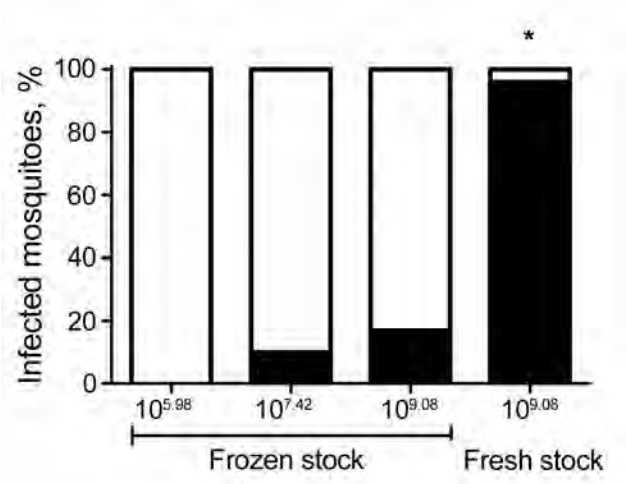

Figure 2. Relationship between dose, infectivity, and preparation of Zika virus for *Aedes aegypti* mosquitoes. Quantitative reverse transcription PCR was used to test 12–25 processed *Ae. aegypti* mosquitoes for Zika virus 14 days after exposure to infectious blood meals containing various doses of Zika virus PR. Frozen stocks had been stored at –80°C and thawed before blood meal preparation, and fresh stocks were used directly after propagation without freezing. The difference in proportion infected when fresh and frozen stock at equivalent titers were compared was highly significant. *p<0.0001 by Fisher exact test.

highest doses achieved for Zika virus HND and CAM were 8.9 and 8.7 $\log_{10}$ PFU/mL, respectively. Similar titers of 8.9 (Zika virus HND) and 8.6 (Zika virus CAM) $\log_{10}$ PFU/mL were used for *Ae. albopictus* feedings (Table 2). For Zika virus HND, significantly higher viral loads were measured in *Ae. aegypti* relative to *Ae. albopictus* mosquitoes at both the highest dose and the 1:20 dilution (≈7.5 $\log_{10}$ PFU/mL; Figure 3; p<0.01 by *t*-test). Although differences were also measured at the lower doses, deviation is higher and statistical power is constrained by the smaller sample sizes. Viral loads among mosquitoes of each species were similar for Zika virus CAM, yet significantly higher than Zika virus HND in *Ae. albopictus* mosquitoes (Figure 3; p<0.001 by *t*-test), indicating an influence of mosquito species and of virus strain on Zika virus replication.

Infection rates for high-dose feedings were 80%–100% (Table 2). Susceptibility, particularly for Zika virus HND, was generally higher among *Ae. albopictus* mosquitoes. This difference was highly significant for the Zika virus HND 1:20 dilution (≈7.5 $\log_{10}$ PFU/mL), for which 93.3% of *Ae. albopictus* mosquitoes were infected compared with 46.7% of *Ae. aegypti* mosquitoes (p<0.001 by Fisher exact test). Although the higher infection rate measured in *Ae. albopictus* mosquitoes was not significant at the 1:400 dilution (33.3% vs. 16.7%; p = 0.233 by Fisher exact test), it is notable that the input titer was ≈5-fold lower for the *Ae. albopictus* mosquito feeding (6.6 vs. 5.9 $\log_{10}$ PFU/mL), consistent with the increased infectiousness of Zika virus HND in this species.

To measure and compare dose dependence and competence among species and strains, we completed linear regression analyses of each individual feeding and used best-fit lines to calculate doses at which 50% of mosquitoes were infected, had disseminated infections, and were capable of transmission ($ID_{50,}$ $DD_{50,}$ $TD_{50,}$ respectively; Figure 4). Although the relationship between dose and infection (as measured by slope) was similar among strains and species, infectiousness was higher in *Ae. albopictus* relative to *Ae. aegypti* mosquitoes for both strains. Infectiousness was determined both by statistical comparison of Y-intercepts by linear regression analyses (p<0.05) and comparisons of calculated ID_{50s}. The ID_{50} of Zika virus HND was found to be greater than a log lower in *Ae. albopictus* compared with *Ae. aegypti* mosquitoes (6.1 vs.

Table 2. Zika virus vector competence of *Aedes aegypti* and *Ae. albopictus* mosquitoes at 21 days after infection*

	Zika virus		Mosquitoes		
Mosquito species	Strain	Dose, $\log_{10}$ PFU/mL	Infected, % (no. tested)	Infected and disseminating, %	% Infected and transmitting
Ae. aegypti	HND	8.9	90.9 (22)	95.0	80.0†↑
		7.7	46.7 (30)†↓	85.7	78.0†↑
		6.6	16.7 (30)	40.0	40.0
		4.6	3.3 (30)	0	0
Ae. aegypti	CAM	8.7	80.0 (30)	100.0	75.0
		7.2	44.4 (26)	91.7†↑	75.0†↑
		5.6	10.0 (30)	66.7	33.3
		4.3	7.0 (30)	100	50.0
Ae. albopictus	HND	8.9	100.0 (30)	93.3	33.3S↓
		7.5	93.3 (30)†↑‡↑	75.0‡↑	21.4†↓
		5.9	33.3 (30)	40.0	10.0
		4.1	10.0 (30)	66.7	0
Ae. albopictus	CAM	8.6	95.2 (21)	95.0	55.0
		6.6	40.0 (30)‡↓	25.0†↓‡↓	25.0†↓
		5.3	23.3 (30)	85.7	14.3
		4.2	6.0 (16)	0	0

*Up and down arrows indicate value is significantly above or below the comparison value (p<0.05 by Fisher exact test).
†Significant differences between different species at the same dose of the same virus strain.
‡Differences between viral strains at the same dose, within species.

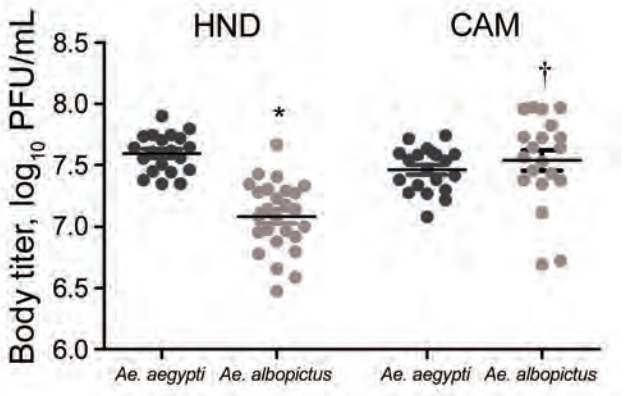

Figure 3. Viral load of Zika virus in *Aedes* mosquito bodies at day 21 after infection. Zika viral load (PFU equivalents) was determined in whole mosquitoes by using Zika virus–specific quantitative reverse transcription PCR and strain-specific standards. The graph shows titers in individual mosquitoes after feeding on the highest dose (8.6–8.9 $\log_{10}$ PFU/mL). Significant differences (*t*-test, $p<0.05$) were identified between mosquito species (*) and virus strains (†). Horizontal lines indicate means ± SD.

7.5 $\log_{10}$ PFU/mL) and ≈5-fold lower for Zika virus CAM (6.6 vs. 7.3 $\log_{10}$ PFU/mL; Figure 4).

The increased infectivity of *Ae. albopictus* mosquitoes did not translate to increased rates of dissemination and transmission (Table 2; Figure 4) and is consistent with the fact that similar (Zika virus CAM) or lower (Zika virus HND) viral loads were measured in mosquitoes of this species (Figure 3). Dissemination rates for infected individuals were generally statistically equivalent among strains and species. Significant differences were achieved only when compared with the *Ae. albopictus* mosquito 1:20 Zika virus CAM feeding, yet the input titer for this feeding was ≈1 $\log_{10}$ lower than that for the other 1:20 feedings, which probably contributed to this difference. The linear regression analysis, for which the exact dose is considered, demonstrates again that the relationship of dissemination rate and dose is similar among virus strain and mosquito species (Figure 4). Although DD_{50} values for both strains were lower in *Ae. albopictus* mosquitoes, differences were significant only for Zika virus HND and can be wholly explained by differences in infectivity.

Transmission rates for *Ae. aegypti* mosquitoes were consistently higher, particularly for Zika virus HND (Table 2). Although no transmission occurred in mosquitoes of either species at the lowest dose, an average of 45% more Zika virus HND–infected *Ae. aegypti* than *Ae. albopictus* mosquitoes transmitted virus. These differences were highly significant at the undiluted and 1:20 doses (≈8.8 and 7.5 $\log_{10}$ PFU/mL; $p<0.001$ by Fisher exact test; Table 2). Although transmission of Zika virus CAM was also higher among *Ae. aegypti* mosquitoes, differences were smaller and only significant at the 1:20 dose ($p<0.001$ by Fisher exact test; Table 2). It is notable that the only instance for which transmission was measured at the lowest dose (1:8,000, 4.3 $\log_{10}$ PFU/mL) was with Zika virus CAM in *Ae. aegypti* mosquitoes. Despite increased infection rates for *Ae. albopictus* mosquitoes, dose dependence for Zika virus HND transmission was

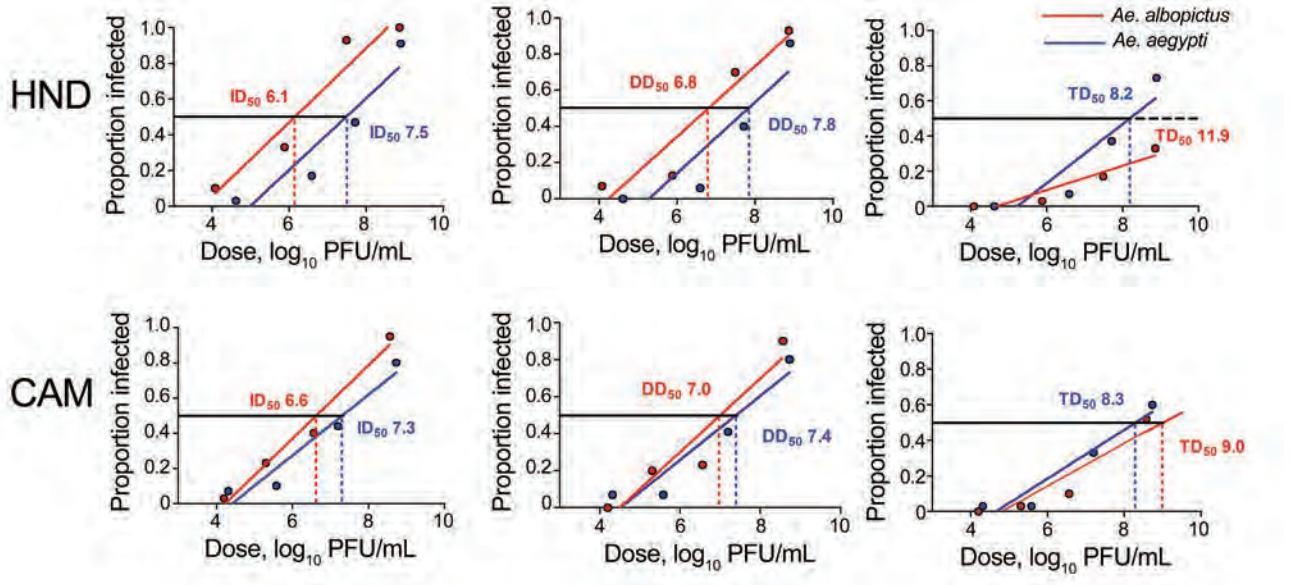

Figure 4. Relationship between dose and competence of *Aedes aegypti* and *Ae. albopictus* mosquitoes for Zika virus HND and CAM. Graphs show proportion of blood-engorged mosquitoes infected, with disseminated infections, and transmitting. Lines depict the best-fit linear relationships as determined by linear regression analyses. All relationships are linear and correlative ($r^2 = 0.82–0.97$). Doses at which 50% of mosquitoes are infected, have disseminated infections, and are transmitting (ID_{50}, DD_{50}, and TD_{50}, respectively) were calculated by using best-fit lines.

significantly lower than that for *Ae. aegypti* mosquitoes ($p = 0.035$ by linear regression analysis; Figure 4), resulting in a TD_{50} ≈4 log_{10} higher for *Ae. albopictus* mosquitoes. Overall transmission efficiency was highly strain dependent for *Ae. albopictus* mosquitoes; the TD_{50} of Zika virus CAM was ≈3 log_{10} lower than that for Zika virus HND. Unlike slopes and intercepts for Zika virus HND, those for Zika virus CAM TD_{50} were similar among species (Figure 4).

Discussion

Reports of autochthonous Zika virus transmission in Florida demonstrate the capacity of Zika virus to continue to expand in the Americas (*26*), yet a comprehensive assessment of the current and future threat requires experimental assessment of the transmission potential of various mosquito populations and circulating strains. Our initial attempts to infect large numbers of *Ae. aegypti* mosquitoes by using previously frozen stocks of Zika virus were largely unsuccessful, even at unnaturally high doses (>9.0 log_{10} PFU/mL). Previous studies have noted differences in arbovirus infectivity and vector competence when use of artificial feeding was compared with feeding on experimentally infected hosts (*27*) or previously frozen to freshly propagated stocks (*28–30*). These studies almost exclusively identified significant differences in vector competence with lower titer blood meals, yet our results clearly demonstrate that freezing/thawing of Zika virus significantly impairs infectivity to mosquitoes at a range of doses. Although the mechanistic basis of this difference has not been adequately studied and plaque assays did not indicate a decline in Zika virus infectious particles on Vero cell culture after freeze/thaw, differences in competence may be attributed to structural perturbations of the virion that inhibit efficient particle binding in vivo (*31*). Future studies characterizing Zika virus structure and binding could help elucidate the unique sensitivity of this virus to the negative effects of freeze/thaw.

Although information about Zika virus kinetics and tropism in humans remains limited, current estimates of mean viremia range from 4.4 to 4.7 log_{10} copies/mL, probably equating to <2.5 log_{10} PFU/mL (*25*,*32*). Symptomatic persons may at times have higher levels of viremia (*32*,*33*), and these estimates are probably low because sample acquisition generally occurs after symptom onset (well past peak viremia) and because titers may be higher in whole blood than in serum (*34*). Despite these caveats, current data still suggest that peak and mean levels for dengue and chikungunya viruses are substantially higher than those for Zika virus (*32*). Of course, transmission efficiency of host to vector is dependent on both host viremia and vector susceptibility. Calculations of ID_{50} for dengue and chikungunya viruses in *Aedes* spp. mosquitoes are variable but have generally been estimated to be $\leq 10^5$ PFU/mL (*35–37*). We estimated Zika virus ID_{50} to be 6.1–7.5 log_{10} PFU/mL, with a low threshold for infection of 4.2 log_{10} PFU/mL. The recent success of Zika virus in the Western Hemisphere unequivocally demonstrates the capacity for widespread transmission, yet the combination of lower host viremia levels and mosquito susceptibility suggests that the intensity of vector-to-host transmission could be less efficient than has been observed with previous epidemics of dengue and chikungunya virus infection. It is feasible that efficient sexual transmission could supplement current levels of mosquito transmission (*38*) or that vertical transmission among particular mosquito populations could play a larger role than is documented for other flaviviruses (*39–41*). In addition, as demonstrated here and in previous studies (*20*), Zika virus vector competence can vary by virus strain and population, so particular vector/virus combinations may be more efficient at maintaining transmission. Highly variable vector competence that is specific for population and virus strain is well documented for other flaviviruses (*42*,*43*), and specific mosquito/virus genotype-by-genotype interactions have been well described in the *Aedes* mosquito/dengue virus system (*42*,*44–46*). Our analysis reveals 13 aa differences between the Zika virus 2010 CAM and 2016 HND strains and additional base changes that could be associated with phenotypically relevant changes to RNA structure. Although the CAM strain is ancestral to the America strains, it is notable that the 2 America strains used in this study (HND and PR) possess 8 aa differences. More comprehensive genetic studies demonstrate a range of mutations among strains currently circulating in the Western Hemisphere (*5*), all of which could feasibly translate to variability in virus fitness and vector competence.

We have demonstrated that US populations of *Ae. albopictus* mosquitoes exposed to a Zika virus strain currently circulating in the Americas are competent vectors that may be capable of maintaining virus transmission. Although these mosquitoes have been colonized for >1 year and may not be fully representative of current populations, this population was derived from a location (Suffolk County, NY) adjacent to New York City, which is among the largest centers for the movement of Zika virus–exposed travelers. Indeed, the highest number of confirmed travel-associated cases of Zika virus infection in the United States are reported from New York state (http://www.cdc.gov/zika/geo/united-states.html). The combination of the recent success of *Ae. albopictus* mosquitoes in the region (*47*) and the influx of viremic patients is probably what led to the first documented locally acquired case of dengue virus infection in the state (Suffolk County, 2013; http://diseasemaps.usgs.gov). Despite this potential vulnerability, transmission intensity is dependent on more than host and vector competence and it is the frequent and highly anthropophilic feeding of *Ae. aegypti* mosquitoes that often results in their relative success as vectors, even in the absence of high

competence (*48*). In addition, the increased infectivity of Zika virus in *Ae. albopictus* mosquitoes does not translate to increased overall competence; transmission efficiency was significantly higher for *Ae. aegypti* mosquitoes. These data are consistent with a midgut escape barrier and, more significantly, with a salivary gland infection or transmission barrier. Although it is well documented that these barriers are capable of preventing transmission (*49*), increased infectivity in *Ae. albopictus* mosquitoes is notable because the increased infectivity we measured in *Ae. albopictus* mosquitoes translates to more opportunity for adaptive events that could increase fitness or competence over time. The potential epidemiologic consequences for adaptation to *Ae. albopictus* mosquitoes are well documented for chikungunya virus, for which primary and secondary epistatic mutations increased competence in mosquitoes of this species and facilitated the explosive outbreaks in the islands of the Indian Ocean and beyond (*50*). The combination of additional laboratory studies assessing the adaptive potential of Zika virus in various *Ae. albopictus* mosquito populations, together with continued genetic and phenotypic monitoring of circulating strains, will help elucidate the potential for similar adaptive events enabling increased transmission efficiency of Zika virus in the Americas.

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Concurrent Infection with Hepatitis C Virus and *Streptococcus pneumoniae*

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Little is known about concurrent infection with hepatitis C virus (HCV) and *Streptococcus pneumoniae*, which causes invasive pneumococcal disease (IPD). We hypothesized that co-infection with HCV and *S. pneumoniae* would increase risk for death and complications. We captured sociodemographic and serologic data for adults with IPD in a population-based cohort study in northern Alberta, Canada, during 2000–2014. IPD patients infected with HCV were compared with IPD patients not infected with HCV for risk of in-hospital deaths and complications by using multivariable logistic regression. A total of 355 of 3,251 patients with IPD were co-infected with HCV. The in-hospital mortality rate was higher for IPD patients infected with HCV. Prevalence of most IPD-related complications (e.g., cellulitis, acute kidney injury, mechanical ventilation) was also higher in HCV-infected patients. Infection with HCV is common in patients with IPD, and HCV is independently associated with an increased risk for serious illness and death.

Hepatitis C virus (HCV) is a flavivirus that can cause a chronic hepatic infection in 75%–85% of persons initially infected (*1*). This virus is present worldwide, infects 130–175 million persons, and causes sequelae that include cirrhosis, end-stage liver disease, and hepatocellular cancer (*1*). In some patients infected with HCV, bacterial infections are more common and more severe than in patients not infected with HCV (*2*–*6*). Co-infection with HIV is also common among HCV-infected patients and can lead to higher mortality rates from sepsis than infection with either virus alone (*4*). Bacteremia has also been shown to be higher in hemodialysis patients infected with HCV (38.3%) than those not infected (21.8%), and mortality rates are higher for bacteremic patients infected with HCV than in those not infected (*3*).

Streptococcus pneumoniae is a gram-positive bacterium found in the nasopharynx and is a common cause of bacteremia. This microorganism can cause a variety of diseases ranging from noninvasive disease, such as pneumonia without bacteremia, to invasive pneumococcal disease (IPD), such as pneumonia with bacteremia, primarily in young or elderly persons (*5*,*6*). The virulence of this bacterium is driven by its wide assortment (currently 97) of polysaccharide capsule types (serotypes) (*7*).

To improve understanding of IPD and the effects of the use of pneumococcal protein conjugate vaccines introduced into Alberta, Canada, in 2002 and 2010, we began a 15-year longitudinal study of IPD in 2000. We took advantage of this prospective cohort to examine the prevalence, correlates, and outcomes of IPD patients co-infected with HCV. We hypothesized that IPD patients co-infected with HCV would have higher mortality rates and more complications than IPD patients not infected with HCV.

Materials and Methods

Definitions

In Canada, the national case definition for IPD is isolation of *S. pneumoniae* from a normally sterile site, such as blood, cerebrospinal fluid, pleural fluid, biopsy tissue, joint aspiration, pericardial fluid, or peritoneal fluid (*8*). IPD is a provincially reportable disease in Alberta. Therefore, all invasive pneumococcal isolates are submitted to the Provincial Laboratory for Public Health (Edmonton, Alberta, Canada) for further characterization. Access to these isolates enabled us to prospectively identify all cases of IPD in northern Alberta. However, the study was limited to persons ≥17 years of age. HCV infection was defined as a positive antibody test result for this virus documented in the chart of a patient as part of usual care (i.e., we did not specifically test each patient for HCV for purposes of this study).

Clinical Data Collection

Research nurses collected sociodemographic, clinical, functional, and laboratory data from the medical record of each patient by using a standardized case report form (Tables 1, 2). These nurses received training on data

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Table 1. Characteristics of 3,251 IPD patients ≥17 years of age by HCV status, Alberta, Canada, 2000–2014*

Characteristic	HCV-positive	HCV-negative	p value
Total no.	355 (10.9)	2,896 (89.1)	
Sex			
M	203 (57.2)	1,635 (56.5)	0.810
F	152 (42.8)	1,261 (43.5)	NA
Mean (SD) age, y	45.2 (9)	55.7 (18.4)	<0.001
Aboriginal	95 (26.8)	355 (11.6)	<0.001
Residence at time of admission			
Home	220 (62.0)	2,408 (83.3)	NA
Homeless	88 (24.8)	148 (5.0)	<0.001
Functional status			
Walking independently	264 (74.4)	2,107 (72.8)	0.520
Concurrent condition			
HIV infection	81 (22.8)	55 (1.9)	<0.001
Cancer in past 5 y	19 (5.4)	385 (13.4)	<0.001
Diabetes	17 (4.8)	179 (6.1)	0.300
Coronary heart disease	16 (4.5)	437 (15.1)	<0.001
Chronic obstructive pulmonary disease	32 (9.0)	532 (18.4)	<0.001
Cirrhosis	86 (24.2)	91 (3.1)	<0.001
≥2 other chronic conditions	160 (45.1)	1,354 (46.8)	0.550
Lifestyle factors			
Current smoker	245 (69)	1,222 (42.2)	<0.001
Illicit drug use	236 (66.5)	323 (11.1)	<0.001
Alcoholism	217 (61.1)	568 (19.6)	<0.001

*Values are no. (%) unless otherwise indicated. HCV, hepatitis C virus; IPD, invasive pneumococcal disease; NA, not applicable.

collection before the start of the study. In addition to the case report form, standard operating procedures documents, definitions, drug classification, and underlying illness categorization were part of working documents. With respect to concurrent illnesses, if the attending physician recorded such an illness, it was accepted. Complete data were available for sociodemographic and functional status. If there was no record of a concurrent illness, it was assumed that the illness was not present. Additional details of the methods used in the study have been reported (*9*). This study was approved by the institutional research review committees of the Alberta Health Regions and the ethics review board of the University of Alberta.

Identification and Serotyping of *S. pneumoniae* Isolates

S. pneumoniae isolates were received at the Provincial Laboratory for Public Health from acute diagnostic laboratories in Alberta as per requirements of provincial notifiable disease regulations. *S. pneumoniae* isolates were confirmed as *S. pneumoniae* on the basis of characteristic morphology and optochin susceptibility before serotyping (*10*). All pneumococcal isolates that showed a positive Quellung reaction with commercial type-specific antisera (Statens Serum Institute, Copenhagen, Denmark) were assigned a serotype designation (*11*). Strains that were susceptible to optochin but that could not be serotyped by the Quellung assay were assayed by using the AccuProbe *S. pneumoniae* Culture Identification Test (Gen-Probe, San Diego, CA, USA) to confirm species identification.

Exposure and Outcomes

Our independent exposure of interest was a dichotomous variable representing the presence or absence of concurrent HCV infection. Our primary outcome was in-hospital deaths as recorded in patient records. Secondary outcomes included prespecified complications of IPD, defined as the presence of ≥1 of the following features: cellulitis, osteomyelitis, septic arthritis, endocarditis, empyema, meningitis, acute kidney injury requiring dialysis, liver failure, peritonitis, and need for mechanical ventilation.

Table 2. Manifestations and complications of IPD patients stratified by HCV infection, Alberta, Canada, 2000–2014*

Characteristic	HCV-positive, no. (%)	HCV-negative, no. (%)	Fully adjusted OR (95% CI)	p value
In-hospital death	57 (16.1)	429 (14.8)	1.71 (1.15–2.54)	0.008
Pneumonia	304 (85.6)	2,388 (82.5)	0.82 (0.57–1.20)	0.310
Cellulitis	24 (6.8)	59 (2)	3.40 (1.81–6.39)	<0.001
Septic arthritis	5 (1.4)	46 (1.6)	1.00 (0.35–2.86)	1.000
Endocarditis	4 (1.1)	24 (0.8)	0.93 (0.27–3.21)	0.910
Empyema	30 (8.4)	188 (6.5)	1.11 (0.69–1.80)	0.660
Renal failure requiring dialysis	26 (7.3)	94 (3.2)	2.14 (1.23–3.72)	0.007
Liver failure	21 (5.9)	49 (1.7)	2.70 (1.41–5.18)	0.003
Mechanical ventilation	110 (31)	575 (19.9)	1.44 (1.07–1.94)	0.017
Peritonitis	16 (4.5)	29 (1.0)	3.79 (1.75–8.17)	0.001
Meningitis	6 (1.7)	154 (5.3)	0.38 (0.16–0.92)	0.001

*HCV, hepatitis C virus; IPD, invasive pneumococcal disease.

Statistical Analysis

Descriptive data are shown as mean (SD) for continuous variables and number (%) for categorical variables. Differences were evaluated by using the χ^2 test for categorical variables and the *t*-test for continuous variables. Pneumococcal serotypes according to HCV status were summarized descriptively.

For our main outcome, we completed a series of multivariate logistic regression analyses to evaluate the association between HCV and in-hospital deaths after accounting for increasing degrees of clinical and prognostic data known to be associated with HCV infection or IPD-related outcomes in an effort to examine risk factor stacking. First, we calculated unadjusted estimates of death for HCV-positive patients compared with estimates for HCV-negative patients (reference group). Second, we adjusted for sociodemographic information (i.e., age, sex, Aboriginal status, and residence at time of admission). Third, we adjusted for the following concurrent conditions: solid organ cancer, HIV, diabetes, coronary heart disease, and chronic obstructive pulmonary disease. In addition, we included a marker for the presence of ≥2 other chronic conditions as a measure of additional illnesses as reported (*12*,*13*). Fourth, we included functional status in our model (independently walking versus not walking before hospital admission) because this factor has better prognostic value than age and disease severity and acts as a proxy for frailty (*14*). Fifth, we included several health behaviors associated with increased risk for HCV infection, including current smoking status, illicit drug use, and alcoholism.

In addition to our primary analysis, we conducted analyses for patients with a history of cirrhosis and for those infected with HIV. Infection with HIV is associated with cirrhosis, and both factors are associated with increased risk for IPD.

We report adjusted odds ratios (ORs) from logistic regression models with their respective 95% CIs and associated p values. All analyses were conducted by using Stata version 14 (StataCorp LLC, College Station, TX, USA).

Results

During the 15 years of the study, 3,251 adults ≥17 years of age were identified as having cases of IPD. Of these adults, 355 (11%) had HCV infections. We obtained sociodemographic characteristics of the 2 population groups, those infected with HCV and those not infected (Table 1). The population with HCV infections had a mean age that was 10 years younger than that for persons without HCV infections (p<0.001). Persons with HCV infections were significantly (all p<0.001) more likely to be Aboriginal, homeless, have concomitant HIV infection and cirrhosis of the liver, have alcoholism, and be current smokers and illicit drug users (Table 1).

Inpatient Mortality Rates and Complications

Overall, 486 (15%) patients died in a hospital: higher mortality rates were observed for those with concurrent HCV infections (57, 16%) versus no HCV infections (429, 15%). This absolute increased risk for death of 1% was statistically significant in models sequentially adjusted for sociodemographic variables, concurrent conditions, and lifestyle factors (fully adjusted OR [aOR] 1.71, 95% CI 1.15–2.54; p<0.001) (Table 2; Figure). For sequential models, age had the largest effect on the association between HCV and death (OR 1.10 for unadjusted analyses, OR 1.93 when age was added to model). The risk for death by infection with HCV was similar between age groups (persons <65 years of age, OR 1.76 and persons ≥65 years of age, OR 1.45; p for interaction = 0.86).

Other variables had minor influence on the point estimate for HCV by sequential analyses. Additional variables independently associated with death in a hospital in the final fully adjusted model included increasing age, history of cancer, functional status, smoking status, and alcoholism (p<0.05). Furthermore, patients infected with HCV alone (aOR 1.60) and those infected with HCV who had cirrhosis (aOR 2.85) had an increased risk for death compared with patients who were not infected with HCV and did not have cirrhosis (p for interaction >0.05). Patients infected with HIV alone (aOR 1.71) and those infected with HCV and HIV (aOR 1.46) also had an increased risk for death compared with patients not infected with HCV or HIV (p for interaction >0.05).

In addition to an increased mortality rate, the prevalence of most IPD-related complications, including cellulitis, acute kidney injury, liver failure, peritonitis, and requirement for mechanical ventilation, were higher in HCV-positive patients, but meningitis was significantly lower in HCV-positive patients (p<0.05 for all comparisons) (Table 2).

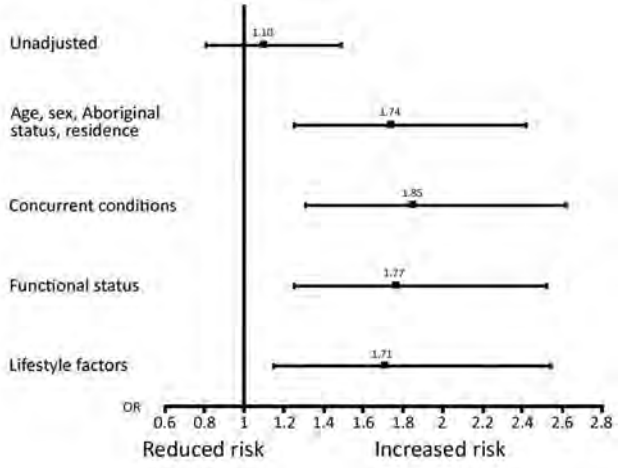

Figure. Sequential adjustment for in-hospital deaths, by hepatitis C virus status, Alberta, Canada, 2000–2014. Squares indicate ORs (values shown), and error bars indicate 95% CIs. OR, odds ratio.

Pneumococcal Serotypes

We identified the 10 most common pneumococcal serotypes causing IPD in the 2 study groups (Table 3). There were notable differences between the 2 groups. Serotype 5 caused 19% of infections in HCV-positive patients and 7% of infections in HCV-negative patients. Serotypes 12F, 20, and 9N were approximately twice as common in HCV-positive patients, and 22F was twice as common in HCV-negative patients (Table 3). Overall, 40 serotypes were found in HCV-positive patients and 51 serotypes in HCV-negative patients. Serotypes in 7-valent pneumococcal conjugate vaccine (PCV7), 13-valent pneumococcal conjugate vaccine (PCV13), and 23-valent pneumococcal polysaccharide vaccine caused 18%, 47%, and 71% of infections, respectively, in HCV-positive patients and 24%, 48%, and 70% of infections, respectively, in HCV-negative patients.

Table 3. Ten most common *Streptococcus pneumoniae* serotypes causing IPD in HCV-positive and HCV-negative patients, Alberta, Canada, 2000–2014*

Rank	Serotype	HCV-positive, no. (%)	HCV-negative, no. (%)
1	5	67 (18.9)	210 (7.3)
2	4	38 (10.7)	268 (9.3)
3	8	25 (7.0)	236 (8.1)
4	3	22 (6.2)	197 (6.8)
5	12F	20 (5.6)	83 (2.9)
6	20	17 (4.8)	84 (2.9)
7	9N	17 (4.8)	80 (2.8)
8	11A	11 (3.1)	86 (3.0)
9	22F	11 (3.1)	209 (7.2)
10	9V	7 (2.0)	87 (3.0)
Total	NA	235 (66.2)	1, 540 (53.3)

*HCV, hepatitis C virus; IPD, invasive pneumococcal disease; NA, not applicable.

Discussion

In a prospective cohort of patients with IPD, we found that concurrent infection with HCV was common, as were risk factors for acquisition of HCV, such as Aboriginal status, alcoholism, and illicit drug use. Furthermore, adjusted analyses showed that HCV infection was associated with a 70% increased risk of death. In addition to an increased mortality rate, patients infected with HCV were more likely to have serious complications of IPD other than meningitis.

A recent health technology report from the University of Calgary (Calgary, Alberta, Canada) indicated a prevalence of diagnosed hepatitis C of ≈0.7% in adults in Alberta and a prevalence as high as 3% in Aboriginal persons (*15*). Thus, the overall prevalence rate for hepatitis C of 11% for those with IPD is 15-fold higher than that for the general population and 3.6-fold higher than that for the Aboriginal population. The reasons for this increased prevalence are complicated by known risk factors, such as concurrent tobacco smoking, alcoholism, and homelessness (*16–19*). Whether HCV infection only confers a higher risk for IPD requires further study.

We have shown that cirrhosis of the liver is a risk factor for death in patients with IPD (*9*). A rat model of cirrhosis showed multiple impairments of pulmonary defenses against *S. pneumoniae*, including impairment of extrapulmonary killing of the infecting organism before arrival of neutrophils (*20*). Levels of lysozyme and C3 were decreased in bronchoalveolar lavage washings, and neutrophil-mediated killing of the virulent type 3 strain of *S. pneumoniae* was also impaired (*20*).

The prevalence of peritonitis for HCV-positive patients (5%) versus that for HCV-negative patients (1%) in our study was expected, given the likely higher amount of ascites in HCV-positive patients. Empyema complicated the course of 8% of HCV-positive patients compared with 3.4% and 3.3% of HCV-positive patients in previous studies of IPD (*21–23*).

A cellulitis prevalence of 7% in HCV-positive patients was ≈3 times the prevalence of 2% for HCV-negative patients. Cellulitis caused by *S. pneumoniae* was reported in 1917 and seems to be an uncommon entity; only 45 cases had been reported as of 2006 (*23–26*). Our data indicated that cellulitis complicates IPD much more frequently than previously recognized. In our study, we detected 83 cases of cellulitis. This finding strongly suggests that HCV infection is associated with pneumococcal cellulitis, especially given the aOR of 3.4 we documented (*27*). Moreover, although HCV-positive patients were more likely to be homeless, occurrence of cellulitis was similar irrespective of underlying place of residence and could not explain these findings. A higher rate of liver failure among HCV-positive patients probably indicates that these patients had cirrhosis at time of hospitalization, and IPD triggered liver failure in patients with compromised liver function.

Renal failure was more common in IPD patients infected with HCV. There are several mechanisms whereby renal involvement occurs in HCV-infected patients, including glomerular immune complex disease, direct viral invasion of renal parenchyma, and nephrotoxicity of some drugs used to treat HCV infection (*28*).

A finding that is more difficult to explain is the lower rate of meningitis for HCV-positive than for HCV-negative patients. However, whether meningitis is less frequent in HCV-positive patients remains uncertain. In France, a reduction in pneumococcal meningitis was observed after introduction of PCV13 for children and adults (*29*). Conversely, Moore et al. found that the rate of meningitis for adults increased during the post-PCV13 era when compared with the pre-PCV13 era (*30*).

To explain why meningitis rates were different for HCV-positive and HCV-negative patients in our study cohort, rates of vaccination with PCV13 would also have to be different between HCV-positive and HCV-negative

patients. Although possible, we believe that this suggestion is unlikely. An alternative explanation that we believe is more plausible might be that HCV-positive patients are at substantially increased risk for all-cause and IPD-related deaths and that the HCV-positive patients who do not die during hospitalization end up also being at a lower risk for meningitis (e.g., a form of healthy survivor bias) and therefore appear as if this complication is less likely to develop in them. Thus, those patients infected with HCV who died during hospitalization probably also had clinically unrecognized meningitis, rather than having HCV being protective against this complication. Alternatively, it might be possible that patients infected with HCV tended to have lower platelet counts and were therefore less likely to have cerebrospinal fluid collected and a diagnosis of meningitis confirmed.

With respect to serotypes, serotype 5 was most common in the HCV group because of an outbreak of serotype 5 infections that occurred in western Canada and preferentially infected the homeless population (*31*). We also observed an outbreak of infections with serotype 20 among middle-age homeless persons in Edmonton, Alberta, starting in 2012 (G.J. Tyrrell, unpub. data). Serotypes contained in PCV7, PCV13, and 23-valent pneumococcal polysaccharide vaccines had a similar occurrence in HCV-positive and HCV-negative patients, except that HCV-positive patients were less likely to be infected with serotypes in PCV7.

The strengths of this study include the large number of patients with IPD studied over a long period and the richness of the clinical data obtained for each patient. However, our study had limitations. First, we identified HCV-positive patients on the basis of a positive antibody test result. Thus, some patients who were antibody positive but HCV RNA negative would be falsely identified as having active infection with HCV. Second, we were unable to categorize the severity of liver disease. Third, we did not have detailed information regarding the vaccination status of all patients. Fourth, we examined only patients who had a diagnosis of HCV and did not prospectively obtain serologic data for all patients admitted to a hospital with IPD.

In conclusion, we have shown that concurrent HCV infection in patients with IPD is common, and HCV infection in this population portends an increased risk for death and more serious complication of IPD, such as cellulitis and peritonitis (but not meningitis) than for patients not infected with HCV. Because many HCV-infected patients have risk factors that are indications for pneumococcal vaccination, additional studies of IPD in patients infected with HCV are needed to determine whether HCV without other risk factors is an indication for vaccination.

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T.J.M. and G.J.T. designed the study, organized data collection, and had full access to all data; D.T.E. performed analyses and had full access to all data; T.J.M., G.J.T., D.T.E., and S.R.M. wrote the article; and all authors contributed to interpretation of data, revising the article for content, and approved the final version of the article.

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Attributable Fraction of Influenza Virus Detection to Mild and Severe Respiratory Illnesses in HIV-Infected and HIV-Uninfected Patients, South Africa, 2012–2016

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The attributable fraction (AF) of influenza virus detection to illness has not been described for patients in different age groups or with different HIV infection statuses. We compared the age group–specific prevalence of influenza virus infection among patients with influenza-like illness (ILI) or severe acute or chronic respiratory illness (SARI and SCRI, respectively) with that among controls, stratified by HIV serostatus. The overall AF for influenza virus detection to illness was 92.6% for ILI, 87.4% for SARI, and 86.2% for SCRI. Among HIV-uninfected patients, the AF for all syndromes was highest among persons <1 and ≥65 years of age and lowest among persons 25–44 years of age; this trend was not observed among HIV-infected patients. Overall, influenza viruses when detected in patients with ILI, SARI, or SCRI are likely attributable to illness. This finding is particularly likely among children and the elderly irrespective of HIV serostatus and among HIV-infected persons irrespective of age.

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Influenza virus infections cause substantial illness and death worldwide, in particular among persons <5 and ≥65 years of age and among persons with underlying medical conditions, including HIV infection (*1*–*6*). In recent years, advances in the development of sensitive PCR assays have considerably expanded the ability of laboratories to detect pathogens, especially viral agents. Such assays have been used for clinical diagnostics, surveillance, and assessment of disease burden.

Nonetheless, establishing a clinical association between pathogen detection and illness remains challenging. Contradicting results have been reported about the association of influenza virus detection with illness (*7*–*11*). These differences may be due to different study designs, including the selection of controls and geographic distribution of cases. Furthermore, this association is poorly understood among different age groups and HIV-infected persons, even though children <5 years of age, the elderly, and HIV-infected persons are recommended groups for influenza immunization (*12*).

Improving the understanding of the association between influenza virus detection and disease could provide insight into the vaccine-preventable fraction of illness overall and among groups recommended for influenza immunization. In addition, a better understanding of this association could assist with the interpretation of surveillance data and improve disease burden estimates.

We assessed the attributable fraction (AF) of influenza virus detection to illness (an estimate of the proportion of patients positive for influenza virus who have symptomatic illness resulting from that virus; hereafter referred as influenza virus AF) among HIV-infected and HIV-uninfected persons in different age groups who sought medical care for influenza-like illness (ILI); severe acute respiratory illness (SARI) [i.e., syndromes recommended by the World

Health Organization (WHO) for global influenza surveillance (*13*)]; or severe chronic respiratory illness (SCRI) at selected sentinel sites in South Africa during 2012–2016. In addition, we separately assessed the AF of influenza virus types and subtypes.

Methods

Surveillance among Hospitalized Persons

During May 2012–April 2016, we conducted prospective hospital-based surveillance for SARI and SCRI at 3 public hospitals in 2 South Africa provinces: the Edendale Hospital in a periurban area of KwaZulu-Natal Province and the Klerksdorp and Tshepong Hospitals (the Klerksdorp-Tshepong Hospital Complex) in a periurban area of North West Province. We defined a case of SARI as illness in a hospitalized patient who had symptom onset within 10 days before admission and who met age-specific clinical inclusion criteria. Cases in infants 2 days to <3 months of age included any hospitalized patients in this age group with a diagnosis of suspected sepsis or physician-diagnosed acute lower respiratory tract infection, irrespective of signs and symptoms. Cases in children 3 months to <5 years of age included any hospitalized patients in this age group with physician-diagnosed acute lower respiratory tract infection (e.g., bronchitis, bronchiolitis, and pneumonia) or pleural effusion. Cases in children ≥5 years of age included any hospitalized patients in this age group with manifestation of acute lower respiratory tract infection with recorded temperature ≥38°C or history of fever and cough. A case of SCRI was defined as illness in a hospitalized person who had symptom onset >10 days before admission and who met the age-specific clinical inclusion criteria described above.

Surveillance among Persons at Outpatient Clinics

During May 2012–April 2016, we also conducted prospective surveillance for persons with ILI and for asymptomatic persons (controls) at 2 outpatient clinics located in the catchment areas of the hospitals where we conducted SARI and SCRI surveillance: the Edendale Gateway Clinic in KwaZulu-Natal Province and the Jouberton Clinic in North West Province. We defined a case of ILI as illness in an outpatient of any age who sought medical care at 1 of the sentinel clinics and had a recorded temperature ≥38°C or a history of fever and cough of ≤10 days' duration. We defined controls as persons of any age who sought medical care at 1 of the sentinel clinics for reasons other than fever or respiratory or gastrointestinal symptoms during the 14 days preceding the visit. Most controls visited the clinics for dental procedures, family planning, well baby visits, voluntary HIV counseling and testing, or acute care for nonfebrile illnesses. We aimed to enroll 1 HIV-infected and 1 HIV-uninfected control every week in each clinic within each of the following age categories: <1 year, 1–4 years, 5–24 years, 25–44 years, 45–64 years, and ≥65 years.

Study Procedures

The procedures for these surveillance programs have been previously described (*4*,*7*,*14*,*15*). In brief, study staff completed case report forms for all enrolled controls and for patients with ILI, SARI, or SCRI. Referral to hospital was recorded for all enrolled patients with ILI, and patients with ILI who were referred to a hospital were excluded from the analysis.

Laboratory Procedures

We collected respiratory specimens from all persons enrolled in the study (i.e., controls and patients with ILI, SARI, or SCRI); specimens consisted of nasopharyngeal aspirates for children <5 years of age and nasopharyngeal and oropharyngeal swabs for persons ≥5 years of age. When collected, the specimens were placed in universal transport medium, stored at 4°C–8°C, and transported within 72 hours of collection to the National Institute for Communicable Diseases of the National Health Laboratory Service in Johannesburg, South Africa, for testing. We used a multiplex real-time reverse transcription PCR assay (*16*) to test the specimens for 10 respiratory viruses: influenza A and B viruses; parainfluenza virus types 1, 2, and 3; respiratory syncytial virus; adenovirus; rhinovirus; human metapneumovirus; and enterovirus. We further subtyped influenza A–positive specimens (*17*).

Determination of HIV Status

We obtained HIV status for study participants from 2 sources: patient clinical records, when available; and, for consenting study participants, by testing dried blood spots at the National Institute for Communicable Diseases. We used an HIV ELISA to test samples from all patients ≥18 months of age, and we used PCR to test samples from children <18 months of age if the ELISA was reactive.

Statistical Analyses

We used unconditional logistic regression or exact logistic regression to estimate the AF of influenza-associated hospitalizations and outpatient consultations by comparing the influenza virus detection rate among patients with ILI, SARI, or SCRI with that among controls. The attributable fraction was estimated from the odds ratio (OR) obtained from the regression models as follows:

$$AF = \frac{OR - 1}{OR} \times 100$$

We subsequently estimated the influenza virus detection rate associated with illness among patients with ILI, SARI, or SCRI ($Infl_{DetectRateIll}$) from the observed detection rate ($Infl_{DetectRateObs}$) as follows:

$$Infl_{DetectRateIll} = Infl_{DetectRateObs} \times AF$$

We implemented this analysis overall and by HIV serostatus within the following age categories: <1 year, 1–4 years, 5–24 years, 25–44 years, 45–64 years, ≥65 years, <5 years, and ≥5 years of age. All estimates were adjusted for co-infections with the other respiratory viruses investigated in this study and underlying medical conditions known as risk factors for influenza-associated severe illness (*18*). HIV infection and age (i.e., <1 year, 1–4 years, 5–24 years, 25–44 years, 45–64 years, ≥65 years of age) were also included as covariates in the non-HIV–stratified overall model and in the models implemented among children <5 year of age (i.e., <1 and 1–4 years of age) and persons ≥5 years of age (i.e., 5–24, 25–44, 45–64 and ≥65 years of age). We also evaluated the AF of influenza virus types and subtypes overall and by HIV serostatus, using the same approach. For this analysis we also adjusted for co-circulating influenza virus types and subtypes.

In addition, we implemented the described analysis after reclassifying the SARI and SCRI cases by using a duration of symptoms cutoff of 7 days. This classification was in accordance with practices previously recommended by WHO for global influenza surveillance (*13*). We implemented this analysis to assess the influenza virus AF among SARI cases as previously defined in other Africa studies (*19*), including South Africa (*4*,*14*,*15*).

To assess trends in the magnitude of the influenza virus AF among HIV-infected and HIV-uninfected persons across age groups, we used linear regression with the inclusion of first-order (model 1, a linear model) or first-order and second-order (model 2, a quadratic model) polynomial terms for age categories treated as a continuous numerical variable. We used STATA version 14.1 (StataCorp LLP, College Station, Texas, USA) to implement the statistical analyses.

Ethics Approval

The protocol for patients with SARI and SCRI was approved by the University of the Witwatersrand Human Research Ethics Committee (HREC; protocol no. M081042) and the University of KwaZulu-Natal Human Biomedical Research Ethics Committee (BREC; protocol no. BF157/08). The protocol for controls and patients with ILI was approved by HREC (protocol no. M120133) and BREC (protocol no. BF080/12). The surveillance was deemed nonresearch by the Centers for Disease Control and Prevention.

Results

Study Population

During May 2012–April 2016, we enrolled 14,431 persons, of whom 13,335 (92.4%) had known age and available influenza and HIV results and were thus included for further analyses. Of these 13,335 persons, 2,504 (18.8%) were controls; 4,797 (36.0%) had ILI; 3,755 (28.2%) had SARI; and 2,279 (17.1%) had SCRI.

Children <5 years of age accounted for 34.9% (875/2,504) of controls; 32.6% (1,565/4,797) of patients with ILI; 53.1% (1,995/3,755) of patients with SARI; and 6.7% (153/2,279) of patients with SCRI. Overall, the HIV prevalence was 41.0% (1,026/2,504) among controls (owing to enrollment criteria for controls); 27.0% (1,295/4,797) among patients with ILI; 38.7% (1,452/3,755) among patients with SARI; and 73.1% (1,667/2,279) among patients with SCRI (p<0.001).

The HIV prevalence among patients with ILI, SARI, or SCRI was lowest among infants <1 year of age: 2.0% (11/554) among those with ILI; 9.1% (114/1,250) among those with SARI; and 20.2% (20/99) among those with SCRI. The HIV prevalence among these patients was highest among persons 25–44 years of age: 60.4% (825/1,366) among those with ILI; 89.0% (745/837) among those with SARI; and 91.6% (1,007/1,099) among those with SCRI. The median duration of illness was 2 days among patients with ILI, 3 days among patients with SARI, and 16 days among patients with SCRI.

We detected influenza viruses in 1,082 (8.1%) of 13,335 specimens. Of these, 466 (43.1%) were influenza A(H3N2) viruses; 227 (21.0%) were influenza A(H1N1) pdm09 viruses; 20 (1.8%) were influenza A viruses that were not subtyped; and 369 (34.1%) were influenza B viruses (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/7/16-1959-Techapp1.pdf).

Influenza Virus Detection Rate and AF

During the study period, we detected influenza viruses in 32 (1.3%) of the 2,504 controls; 666 (13.9%) of the 4,797 patients with ILI; 251 (6.7%) of the 3,755 patients with SARI; and 133 (5.8%) of the 2,279 patients with SCRI (p<0.001) (Table 1). Among influenza-positive patients ≥5 years of age with severe illness (SARI or SCRI), 46.6% (117/251) had symptoms for >10 days, compared with 12.0% (16/133) among children <5 years of age (p<0.001). Among controls, after we adjusted by age, the influenza virus detection rate was statistically significantly higher among HIV-uninfected compared with HIV-infected persons (adjusted OR 1.8; 95% CI 1.1–2.7) (for HIV-infected compared with HIV-uninfected persons, adjusted OR 0.5; 95% CI 0.1–0.9).

The overall influenza virus AF was 92.6% (95% CI 89.3%–94.8%) among patients with ILI; 87.4% (95% CI 81.3%–91.5%) among patients with SARI; and 86.2% (95% CI 77.7%–91.5%) among patients with SCRI (Table

Table 1. Persons positive for influenza virus infection in a study of the attributable fraction of influenza virus detection to mild and severe respiratory illnesses, Klerksdorp and Pietermaritzburg, South Africa, May 2012–April 2016*

Category	No. positive/no. total (%)		
	Total	HIV-infected	HIV-uninfected
Asymptomatic controls			
Age, y			
<1	2/363 (0.5)	0/37 (0.0)	2/326 (0.6)
1–4	9/512 (1.8)	3/223 (1.3)	6/289 (2.1)
5–24	11/729 (1.5)	3/323 (0.9)	8/406 (2.0)
25–44	5/386 (1.3)	2/238 (0.8)	3/148 (2.0)
45–64	4/357 (1.1)	2/173 (1.2)	2/184 (1.1)
≥65	1/157 (0.6)	0/32 (0.0)	1/125 (0.8)
<5	11/875 (1.3)	3/260 (1.2)	8/615 (1.3)
≥5	21/1,629 (1.3)	7/766 (0.9)	14/863 (1.6)
All	32/2,504 (1.3)	10/1,026 (1.0)	22/1,478 (1.5)
Influenza virus types/subtypes			
A	21/2,504 (0.8)	6/1,026 (0.6)	15/1,478 (1.0)
A(H3N2)	6/2,504 (0.2)	2/1,026 (0.2)	4/1,478 (0.3)
A(H1N1)pdm09	12/2,504 (0.5)	3/1,026 (0.3)	9/1,478 (0.6)
B	11/2,504 (0.4)	4/1,026 (0.4)	7/1,478 (0.5)
Outpatients with influenza-like illness			
Age, y			
<1	42/554 (7.6)	4/11 (36.4)	38/543 (7.0)
1–4	158/1,011 (15.6)	5/24 (20.8)	153/987 (15.5)
5–24	214/1,319 (16.2)	24/228 (10.5)	190/1,091 (17.4)
25–44	183/1,366 (13.4)	118/825 (14.3)	65/541 (12.0)
45–64	59/451 (13.1)	31/181 (17.1)	28/270 (10.4)
≥65	10/96 (10.4)	3/26 (11.5)	7/70 (10.0)
<5	200/1,565 (12.8)	9/35 (25.7)	191/1,530 (12.5)
≥5	466/3,232 (14.4)	176/1,260 (14.0)	290/1,972 (14.7)
All	666/4,797 (13.9)	185/1,295 (14.3)	481/3,502 (13.7)
Influenza virus types/subtypes			
A	443/4,797 (9.2)	125/1,295 (9.7)	318/3,502 (9.1)
A(H3N2)	310/4,797 (6.5)	94/1,295 (7.3)	216/3,502 (6.2)
A(H1N1)pdm09	119/4,797 (2.5)	25/1,295 (1.9)	94/3,502 (2.7)
B	223/4,797 (4.6)	60/1,295 (4.6)	163/3,502 (4.7)
Inpatients with severe acute respiratory illness			
Age, y			
<1	54/1,250 (4.3)	7/114 (6.1)	47/1,136 (4.1)
1–4	63/754 (8.4)	11/97 (11.3)	52/648 (8.0)
5–24	22/284 (7.7)	12/150 (8.0)	10/134 (7.5)
25–44	60/837 (7.2)	55/745 (7.4)	5/92 (5.4)
45–64	38/479 (7.9)	28/312 (9.0)	10/167 (6.0)
≥65	14/160 (8.7)	5/34 (14.7)	9/126 (7.1)
<5	117/1,995 (5.9)	18/211 (8.5)	99/1,784 (5.5)
≥5	134/1,760 (7.6)	100/1,241 (8.1)	34/519 (6.6)
All	251/3,755 (6.7)	118/1,452 (8.1)	133/2,303 (5.8)
Influenza virus types/subtypes			
A	170/3,755 (4.5)	74/1,452 (5.1)	96/2,303 (4.2)
A(H3N2)	98/3,755 (2.6)	42/1,452 (2.9)	56/2,303 (2.4)
A(H1N1)pdm09	70/3,755 (1.9)	32/1,452 (2.2)	38/2,303 (1.7)
B	81/3,755 (2.2)	44/1,452 (3.0)	37/2,303 (1.6)
Inpatients with severe chronic respiratory illness			
Age, y			
<1	9/99 (9.1)	2/20 (10.0)	7/79 (8.9)
1–4	7/54 (13.0)	1/16 (6.3)	6/38 (15.8)
5–24	15/202 (7.4)	9/141 (6.4)	6/61 (9.8)
25–44	53/1,099 (4.8)	48/1,007 (4.8)	5/92 (5.4)
45–64	37/661 (5.6)	22/438 (5.0)	15/223 (6.7)
>65	12/164 (7.3)	3/45 (6.7)	9/119 (7.6)
<5	16/153 (10.5)	3/36 (8.3)	13/117 (11.1)
>5	117/2,126 (5.5)	82/1,631 (5.0)	35/495 (7.1)
All	133/2,279 (5.8)	85/1,667 (5.1)	48/612 (7.8)
Influenza virus types/subtypes			
A	79/2,279 (3.5)	53/1,667 (3.2)	26/612 (4.2)
A(H3N2)	52/2,279 (2.3)	34/1,667 (2.0)	18/612 (2.9)
A(H1N1)pdm09	26/2,279 (1.1)	19/1,667 (1.1)	7/612 (1.1)
B	54/2,279 (2.4)	32/1,667 (1.9)	22/612 (3.6)

*Numbers indicate positive test result for any influenza virus. Influenza A category includes viruses that were not subtyped.

2). Our findings showed that the AF did not statistically significantly differ (overlapping CIs) across the syndromes evaluated or by age group or HIV serostatus.

Overall, for all syndromes, the influenza virus AF was highest among persons <1 and ≥65 years of age and lowest among persons 25–44 years of age (Table 2; online Technical

Table 2. AF and AF-adjusted prevalence of influenza viruses among participants in a study of the AF of influenza virus detection to mild and severe respiratory illnesses, Klerksdorp and Pietermaritzburg, South Africa, May 2012–April 2016*

	Total		HIV-infected		HIV-uninfected	
Category	AF, % (95% CI)	AF-adjusted prev, %	AF, % (95% CI)	AF-adjusted prev, %	AF, % (95% CI)	AF-adjusted prev, %
Outpatients with influenza-like illness						
Age, y†						
<1	96.8 (85.3–99.3)	7.4	98.4 (83.0–∞)‡	35.8	94.1 (74.9–98.6)	6.6
1–4	92.8 (82.2–97.0)	14.5	94.6 (72.2–98.9)	19.7	90.3 (77.7–95.8)	14.0
5–24	91.4 (81.3–96.0)	14.8	93.5 (77.5–98.1)	9.8	89.8 (78.8–95.1)	15.6
25–44	90.8 (82.7–95.1)	12.2	95.5 (81.8–98.9)	13.7	87.7 (60.2–96.2)	10.5
45–64	93.4 (81.6–97.6)	12.2	95.1 (78.8–98.8)	16.3	91.3 (62.8–98.0)	9.5
≥65	94.3 (53.2–99.3)	9.8	96.3 (21.1–∞)‡	11.1	91.8 (27.6–99.1)	9.2
<5	93.9 (87.8–97.0)	12.0	97.3 (87.8–99.4)	25.0	91.7 (82.9–96.0)	11.5
≥5	92.5 (88.2–95.2)	13.3	95.4 (88.5–97.5)	13.4	89.8 (82.4–94.1)	13.2
All	92.6 (89.3–94.8)	12.9	95.6 (91.0–97.8)	13.7	90.6 (85.5–93.9)	12.4
Influenza virus types/subtypes§						
A¶	93.3 (89.5–95.7)	8.6	96.5 (91.4–98.6)	9.4	91.5 (85.7–95.0)	8.3
A(H3N2)	97.3 (93.9–98.8)	6.3	98.6 (93.9–99.7)	7.2	96.7 (91.0–98.8)	6.0
A(H1N1)pdm09	85.4 (73.1–92.0)	2.1	91.0 (64.9–97.7)	1.7	82.9 (65.7–91.5)	2.2
B	92.7 (86.5–96.0)	4.3	94.4 (82.9–98.2)	4.3	91.6 (82.0–96.1)	4.3
Inpatients with severe acute respiratory illness						
Age (in years)†						
<1	93.5 (72.8–98.5)	4.0	94.3 (11.2–∞)‡	5.8	93.2 (71.2–98.4)	3.8
1–4	87.9 (73.7–94.5)	7.5	90.2 (61.0–97.6)	10.2	86.5 (66.4–94.6)	6.9
5–24	84.3 (59.5–93.9)	6.6	90.6 (64.6–97.5)	7.2	78.6 (13.6–95.9)	5.9
25–44	83.1 (63.3–92.3)	6.4	90.4 (60.1–97.7)	6.7	75.6 (31.8–91.3)	4.1
45–64	87.3 (63.2–95.6)	6.9	90.9 (54–9-97.7)	8.2	85.2 (29.0–96.9)	5.1
≥65	94.5 (57.7–99.3)	8.2	91.5 (18.6–∞)‡	13.5	91.0 (27.9–98.9)	6.5
<5	89.3 (79.3–94.5)	5.3	91.0 (65.4–97.7)	7.7	89.1 (76.8–94.9)	5.0
≥5	86.0 (76.9–91.5)	6.5	91.2 (80.3–96.1)	7.4	79.3 (59.6–89.4)	5.2
All	87.4 (81.3–91.5)	5.9	91.1 (82.2–95.5)	7.4	84.5 (74.7–90.5)	4.9
Influenza virus types/subtypes§						
A¶	86.7 (78.7–91.7)	3.9	91.3 (79.2–96.3)	4.7	81.9 (68.0–89.8)	3.4
A(H3N2)	93.6 (85.1–97.2)	2.4	94.6 (76.9–98.7)	2.7	92.1 (77.6–97.2)	2.2
A(H1N1)pdm09	80.7 (63.5–89.8)	1.5	88.1 (68.7–97.4)	1.9	71.7 (39.2–86.8)	1.1
B	88.4 (77.6–94.0)	1.9	90.3 (71.5–96.7)	2.7	89.0 (74.2–95.3)	1.4
Inpatients with severe chronic respiratory illness						
Age, y†						
<1	87.6 (70.7–99.4)	8.0	89. 3(9.3–∞)‡	8.9	88.1 (72.4–99.8)	7.8
1–4	85.2 (64.8–97.3)	11.1	88.3 (7.4–99.3)	5.6	83.9 (50.9–97.5)	13.3
5–24	81.3 (54.9–92.2)	6.0	85.3 (27.4–95.7)	5.5	78.8 (31.3–93.5)	7.7
25–44	78.7 (43.8–91.9)	3.8	84.6 (35.7–96.3)	4.1	69.5 (32.7–93.0)	3.8
45–64	82.9 (51.1–94.0)	4.6	83.1 (17.6–95.6)	4.2	81.6 (31.5–96.5)	5.5
≥65	88.2 (38.4–99.0)	6.4	89.7 (12.0–∞)‡	6.0	88.0 (11.7–98.7)	6.7
<5	87.1 (73.0–97.4)	9.1	88.6 (52.4–99.0)	7.4	85.1 (71.7–98.1)	9.4
≥5	82.7 (70.7–89.8)	4.5	86.9 (64.8–93.2)	4.3	80.2 (60.1–90.2)	5.7
All	86.2 (77.7–91.5)	5.0	87.4 (69.9–93.7)	4.5	82.9 (74.1–92.3)	6.5
Influenza virus types/subtypes§						
A¶	82.7 (68.6–90.5)	2.9	83.3 (55.8–93.7)	2.7	81.1 (60.5–91.0)	3.5
A(H3N2)	93.1 (81.3–97.4)	2.1	94.8 (64.1–98.6)	1.9	92.7 (75.5–97.8)	2.7
A(H1N1)pdm09	64.5 (32.6–84.6)	0.7	74.2 (17.3–93.8)	0.8	52.8 (9.7–84.5)	0.6
B	91.5 (82.1–95.9)	2.2	92.4 (80.7–97.0)	1.8	88.9 (61.9–96.8)	3.2

*AF, attributable fraction; AF-adjusted prev, prevalence attributable to illness.
†The overall influenza virus AF was obtained from models adjusted for any underlying medical conditions and for other respiratory viruses investigated in this study. Underlying medical conditions were asplenia, including asplenia or sickle cell anemia; chronic illness, including chronic lung, renal, liver, or cardiac disease, diabetes mellitus, and asthma; other immunocompromising conditions (excluding HIV), including organ transplant, primary immunodeficiency, immunotherapy, and malignancy; neurologic disorders; burns; obesity; malnutrition; and prematurity. Comorbidities were considered absent in cases for which the medical records stated that the patient had no underlying medical conditions or when there was no direct reference to that condition. Prematurity and malnutrition were assessed only in analyses for children <1 and <5 years of age, respectively. Age and HIV infection were also included as predictors in nonstratified models.
‡Estimated using exact logistic regression.
‡The influenza virus AF was obtained from models adjusted for age, HIV infection, any underlying medical conditions, and other respiratory viruses investigated in this study, including co-circulating influenza virus types and subtypes.
¶Includes influenza A viruses that were not subtyped.

Appendix Figures 2–4, panels A). The HIV-stratified analysis did not show this trend among HIV-infected persons, but it was marked among HIV-uninfected persons across syndromes (Table 2; online Technical Appendix Figures 2–4, panels B, C). On linear regression analysis, we did not detect statistically significant trends of the magnitude of the influenza virus AF across age groups among HIV-infected persons for all syndromes (Figures 1–3, panels A; online Technical Appendix Table 1). Conversely, we did detect a statistically significant U-shaped trend of the magnitude of the influenza virus AF across age groups among HIV-uninfected persons for all syndromes (Figures 1–3, panels B; online Technical Appendix Table 1).

The AF of influenza virus types and subtypes was statistically significant across syndromes and HIV serostatus (Table 2). The influenza virus AF did not statistically significantly differ (overlapping CIs) between types and subtypes for all syndromes; however a gradient of the magnitude of the influenza virus AF was observed. The AF of influenza A(H3N2) virus was the highest, followed by that of influenza B virus and then influenza A(H1N1)pdm09 virus for all syndromes and HIV serostatus (Table 2).

Overall, the influenza virus detection rate associated with illness (AF-adjusted) was 12.9% among patients with ILI, 5.9% among those with SARI, and 5.0% among those with SCRI (Table 2). Using a duration of symptom cutoff of 7 days, we showed that the overall influenza virus detection rate among patients classified as having SARI was 6.8% and the detection rate among patients classified as having SCRI was 6.1% (online Technical Appendix Table 2). We showed that the influenza virus AF was similar among patients classified as having SARI or SCRI, using a duration of symptom cutoff of 7 days (online Technical Appendix Table 3) or 10 days (Table 2).

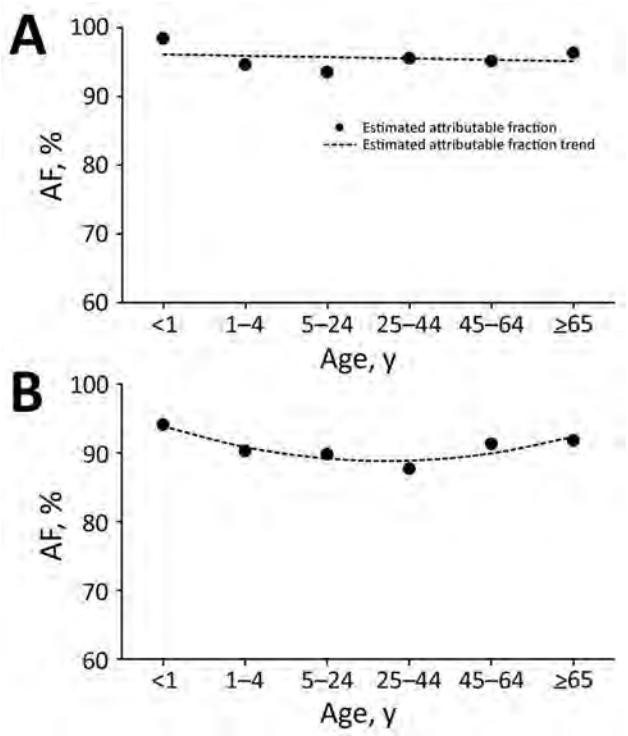

Figure 1. Estimated influenza virus attributable fraction (AF) and AF trends across age groups among outpatients with influenza-like illness, Klerksdorp and Pietermaritzburg, South Africa, May 2012–April 2016. A) HIV-infected patients (AF trends estimated using model 1, a linear model). B) HIV-uninfected patients (AF trends estimated using model 2, a quadratic model).

Discussion

We provide estimates of the influenza virus AF among HIV-infected and HIV-uninfected patients of different age groups with mild or severe respiratory illness. Overall, influenza virus detection was statistically significantly associated with illness across syndromes, age groups, and HIV serostatus. Given the elevated influenza virus AF that we found in this study, the difference between the observed influenza virus detection rate and that attributable to illness was minimal. Nonetheless, among HIV-uninfected patients, the influenza virus AF was highest among persons <1 and ≥65 years of age and lowest among persons 25–44 years of age for all syndromes. This trend was not observed among HIV-infected patients, for whom the AF was similar across age groups for all syndromes.

A statistically significant association of influenza virus detection with illness among patients with ILI has previously been reported (*7*). A statistically significant association of influenza virus detection with illness among patients with SARI has also been reported (*7*,*10*,*11*). Nonetheless, other studies reported that respiratory syncytial virus but not influenza virus detection was associated with pneumonia in children (*8*,*9*). It should be noted that the latter studies had relatively few controls, potentially resulting in lack of power to detect statistically significant disease association for pathogens with low detection rates. A lack of studies that quantify the AF of influenza virus detection with illness among patients with SCRI hindered our ability to compare the estimates obtained in this study with those of others. In our study, the influenza virus detection rate was similar among patients with SARI (6.7%) and SCRI (5.8%). The high influenza virus AF found among patients with SCRI (86.2%) suggests that using the recommended WHO SARI case definition may underestimate the burden of influenza-associated severe illness, especially in older persons, among whom the number of patients seeking medical care for illness of longer duration is elevated. In our study, 46.6% of influenza-positive patients ≥5 years of age with severe illness had symptoms for >10 days, compared with 12.0% of children <5 years of age. Chronic lung diseases are a

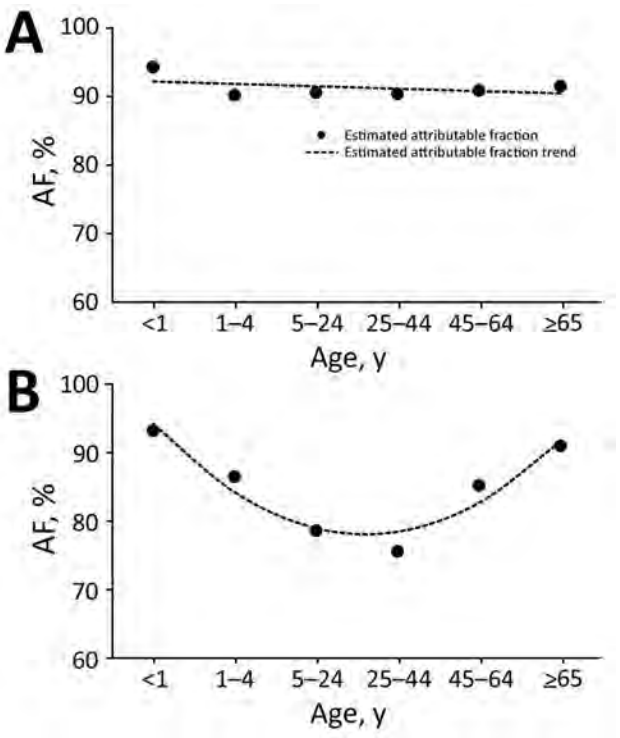

Figure 2. Estimated influenza virus attributable fraction (AF) and AF trends across age groups among inpatients with severe acute respiratory illness, Klerksdorp and Pietermaritzburg, South Africa, May 2012–April 2016. A) HIV-infected patients (AF trends estimated using model 1, a linear model). B) HIV-uninfected patients (AF trends estimated using model 2, a quadratic model).

known risk factor for influenza-associated severe illness (*18*,*20*); however, illness in patients with such conditions might not be identified as SARI (because of their prolonged underlying respiratory illness), even if the patients are hospitalized after an acute infection.

The statistically significant U-shaped trends of the magnitude of the influenza virus AF found in this study among HIV-uninfected patients across age groups suggest that symptomatic illness sufficient to warrant seeking care after influenza virus infection may be more likely to develop in infants, young children, and the elderly than in young to middle-age adults. Extremes of age are known risk factors for influenza-associated severe illness (*4–6*,*21*), a fact that was reflected in the marked U-shaped trend of the magnitude of the influenza virus AF found in this study, especially among patients with severe illness. Statistically significant influenza virus AF has been reported among children and adults in other studies (*7*,*10*,*11*), but none of those studies were powered to assess the influenza virus AF in refined age groups.

In our study, we did not find a statistically significant trend in the magnitude of the influenza virus AF among HIV-infected persons across age groups. In addition, after adjusting by age, we found that the detection rate of influenza virus was statistically significantly lower among HIV-infected compared with HIV-uninfected controls. This finding suggests that HIV-infected persons may be more likely to develop symptomatic illness sufficient to warrant seeking care following influenza virus infection irrespective of the person's age (*22*). Although HIV infection is a known risk factor for influenza-associated severe illness (*4–6*,*18*,*21*), documentation of the influenza virus AF among HIV-infected patients is lacking, hindering our ability to compare the estimates obtained in this study with those of others.

The difference in magnitude of the influenza virus AF that we observed in our study among HIV-infected and HIV-uninfected persons across age groups may affect estimates of the relative risk for influenza-associated severe illness among persons of different age and HIV serostatus. This possibility should be investigated.

We found that the AF of influenza virus types and subtypes was statistically significant for all syndromes investigated in this study. Although we did not observe a statistically significant difference of the estimated AF between influenza virus types and subtypes, the AF of influenza A(H3N2) virus was consistently the highest and that of influenza A(H1N1)pdm09 virus was consistently the lowest across syndromes and HIV serostatus. A higher severity of influenza A(H3N2) compared with influenza A(H1N1) pdm09 and influenza B infections has been found in some studies (*23*,*24*).

Our study has limitations that warrant discussion. First, in our analysis, we did not adjust for co-infections with bacterial pathogens. Nonetheless, the synergistic effect of influenza virus with bacterial pathogens, especially *Streptococcus pneumoniae*, is well described in the literature (*25–27*). Specifically, studies conducted in South Africa suggest that influenza virus detection among patients with SARI is associated with increased risk for pneumococcus colonization and elevated colonization density that increases the risk for invasive pneumococcal disease and associated death, placing influenza virus infection in the pathogenesis pathway for severe respiratory illness (*15*,*28*). Second, the small sample size in some subgroups of the age- and HIV-stratified analyses hindered our ability to obtain accurate estimates of the influenza virus AF, resulting in wide CIs. Third, we did not follow up on controls after enrollment, and the development of undetected mild or severe illness for some persons cannot be excluded. Should illness have developed in some controls, the influenza virus AF in our study would have been underestimated. Fourth, we used asymptomatic persons enrolled at selected clinics as controls. Other studies have used randomly selected persons (either symptomatic or asymptomatic) in the community as controls for severe cases (*29*,*30*). We cannot

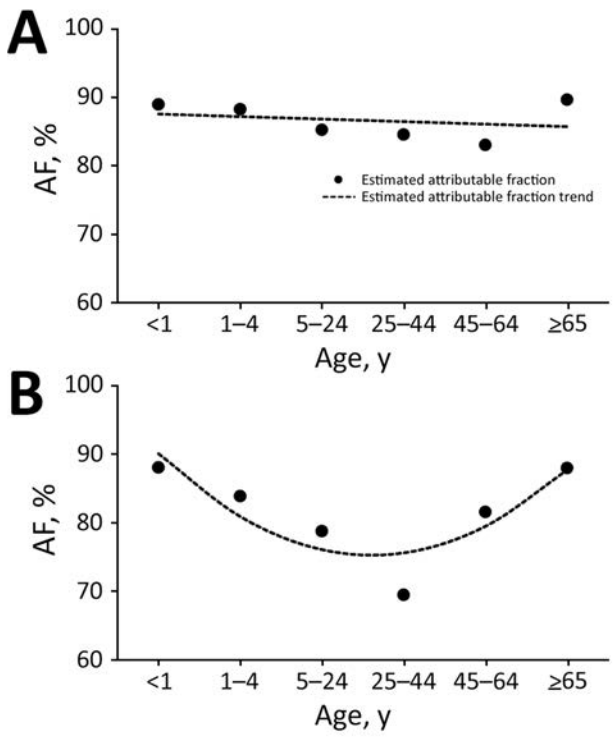

Figure 3. Estimated influenza virus attributable fraction (AF) and AF trends across age groups among inpatients with severe chronic respiratory illness, Klerksdorp and Pietermaritzburg, South Africa, May 2012–April 2016. A) HIV-infected patients (AF trends estimated using model 1, a linear model). B) HIV-uninfected patients (AF trends estimated using model 2, a quadratic model).

dismiss that our study may have included patients who had influenza-associated mild illness but were hospitalized because of infection with other pathogens. Nonetheless, given our study design, we had to use asymptomatic controls for standard comparison across syndromes, including mild illness such as ILI. In addition, the use of controls from the community versus outpatient clinics for the estimation of AFs has not been evaluated. Fifth, we did not have data on influenza B lineages or influenza A strains in different years nor complete data on the level of immunosuppression in HIV-infected persons. This lack of data hampered our ability to estimate lineage- or strain-specific AFs and to assess the effect of different levels of immunosuppression on the influenza virus AF among HIV-infected persons.

In conclusion, influenza viruses, when detected in patients with ILI, SARI, or SCRI, are probably attributable to illness overall, especially among children and the elderly, irrespective of the HIV serostatus and among HIV-infected persons irrespective of age. Compared with the reporting of influenza virus detection rates alone, the estimated influenza virus detection rate attributable to illness reflects a more accurate description of the proportion of illness caused by influenza viruses in South Africa. Such estimates can be used to better interpret surveillance data and refine disease burden estimates so as to improve understanding of the vaccine-preventable fraction of illness overall and among groups recommended for influenza vaccination (e.g., children, the elderly, and HIV-infected persons). In addition, a differential influenza virus AF may also occur among persons with conditions associated with increased risk of influenza-associated severe illness other than age and HIV infection, and this possibility should be investigated.

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Phylogeography of *Burkholderia pseudomallei* Isolates, Western Hemisphere

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The bacterium *Burkholderia pseudomallei* causes melioidosis, which is mainly associated with tropical areas. We analyzed single-nucleotide polymorphisms (SNPs) among genome sequences from isolates of *B. pseudomallei* that originated in the Western Hemisphere by comparing them with genome sequences of isolates that originated in the Eastern Hemisphere. Analysis indicated that isolates from the Western Hemisphere form a distinct clade, which supports the hypothesis that these isolates were derived from a constricted seeding event from Africa. Subclades have been resolved that are associated with specific regions within the Western Hemisphere and suggest that isolates might be correlated geographically with cases of melioidosis. One isolate associated with a former World War II prisoner of war was believed to represent illness 62 years after exposure in Southeast Asia. However, analysis suggested the isolate originated in Central or South America.

Melioidosis is caused by the bacterium *Burkholderia pseudomallei* and is mainly associated with tropical areas. Although considered endemic to northern Australia and Southeast Asia, it has increasingly been recognized in other regions, such as Central America, South America, and the Caribbean (*1*–*3*).

A study by Pearson et al. based on multilocus sequence typing (MLST) data and analysis of single-nucleotide polymorphisms (SNPs) from whole genome sequences indicated that *B. pseudomallei* originated on the Australian continent because of the high level of genetic diversity seen in isolates from this region. They proposed that *B. pseudomallei* was transferred to Southeast Asia next, and from there was disseminated to other regions of the world (*4*).

Although MLST is the most common method to subtype isolates of *B. pseudomallei*, over time it has become recognized that it lacks the resolution to firmly link an isolate to a specific geographic origin (*4*–*7*). Because of homoplasy, the same sequence type (ST) can be found in isolates from different continents that are not truly closely related and instead have different genomic content (*7*).

To improve the ability to link genetic data with geographic provenance, we previously used a typing scheme for length polymorphisms in the 16S–23S internal transcribed spacer (ITS) of *Burkholderia* spp. to characterize isolates of *B. pseudomallei* with associations in the Western Hemisphere. We found that all isolates with a clear origin in the Western Hemisphere were ITS type G (*5*,*8*). We also established that the genomes contained the *Yersinia*-like fimbrial (YLF) gene, which is associated mainly with isolates not from Australia (*5*,*9*). Because of limited diversity detected by these methods, we hypothesized that a genetic bottleneck was associated with isolates from the Western Hemisphere (*5*).

Subsequent studies by Sarovich et al. (*10*) and Chewapreecha et al. (*11*) added support to this hypothesis by inferring relatedness from whole-genome SNPs. These studies indicate that Southeast Asia was the source of isolates from Africa, and Africa then became the source for *B. pseudomallei* in the Western Hemisphere, potentially associated with transfer to the Americas by the slave trade (*10*,*11*). Recent work has also shown the utility of SNP analysis in associating isolates of *B. pseudomallei* from patients with environmental isolates to establish epidemiologic links to better understand sources of infection (*12*–*14*).

In this study, we further characterized isolates associated with the Western Hemisphere by analysis of SNPs in whole-genome sequences to infer phylogenetic relatedness. We also sought to determine if this analysis could be used to associate isolates with geographic regions in the Western Hemisphere to improve epidemiologic associations, especially in cases where the source of infection was unclear.

Materials and Methods

Genomic DNA Extraction and Sequencing

DNA was extracted from isolates by using the Maxwell 16 Cell DNA Purification Kit with the Maxwell 16MDx automated nucleic acid purification system (Promega, Madison, WI, USA) per the manufacturer's instructions. Final elution

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was performed by using PCR-grade water and RNase A. Samples were filtered by using a 0.1-μm filter. Sequencing was performed on an RSII apparatus (Pacific Biosciences, Menlo Park, CA, USA) for most isolates. DNA was sheared to 20 kb by passing it through a needle and used to generate 20-kb SMRTbell libraries by using a standard Template Preparation Kit (Pacific Biosciences). Libraries were further selected by size by using a BluePippin instrument (Sage Science, Beverly, MA, USA). The libraries were then sequenced by using the RSII apparatus (Pacific Biosciences), P6 polymerase, and C4 chemistry for 360-min movies. Library size ranged from 4 kb to 10 kb.

Sequence for MD2013 was determined from paired-end reads, which were generated by using an MiSeq apparatus (Illumina, San Diego, CA, USA). Genomic DNA was sheared to a mean size of 600 bp by using an LE220 focused ultrasonicator (Covaris Inc., Woburn, MA, USA). DNA fragments were cleaned by using Ampure (Beckman Coulter Inc., Indianapolis, IN, USA) and used to prepare single-indexed sequencing libraries by using the PrepX ILM DNA Library Preparation Kit (Wafergen Biosystems, Fremont, CA, USA), Truseq barcoding indices, and PCR primer cocktail (Illumina). Libraries were analyzed for size and concentration, pooled, and denatured for loading onto flow cells for cluster generation. Sequencing was performed by using Miseq 2 × 250-bp paired-end sequencing kits (Illumina). After completion, sequence reads were filtered for read quality, base called, and demultiplexed by using Casava version 1.8.2 software (Illumina).

Genome Assembly

De novo assembly was performed by using the Hierarchical Genome Assembly Process version 3 software (Pacific Biosciences) (*15*). Resulting consensus sequences were checked and edited for circularity either automatically by using Circlator (*16*) or manually by using Gepard (*17*). Illumina reads were cleaned by using BBDUK version 36.20 software (Joint Genome Institute, Walnut Creek, CA, USA) to remove PhiX (NC_001422.1). We used Trimmomatic version 0.36 software to clip off adapters and perform a 20-bp sliding window quality trim to a Phred 30 average (*18*). Cleaned reads were then assembled in SPAdes version 3.9.0 software by using the only-assembler option and retaining contigs >500 bp (*19*). Sequences were deposited into GenBank (accession numbers are in Table 1).

Strain Typing

We performed MLST as described by using traditional Sanger methods or genome assembly to query the *B. pseudomallei* MLST database (http://pubmlst.org/bpseudomallei/) (*5*,*6*,*20*–*23*). We used kSNP version 3.021 software to analyze SNPs for strains sequenced for this study (Table 1) and a reference panel of genomes (Table 2) (*24*). The maximum-parsimony tree from core SNPs was used to identify clades by using FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Select isolates and clades were investigated for where SNPs occurred by manual interrogation of the kSNP version 3.021 variant call format output file and SnpEff version 4.3 (*25*).

We detected the *Yersinia*-like fimbrial (YLF) gene by using CLC Genomics Workbench version 8.0.3 software, YP_110141.1, and blastn for each genome (CLCbio, Aarhus, Denmark) (*26*). ITS typing involved 3 sequence types: type C (FJ981718.1), type G (FJ981723.1), and type E (FJ981706.1) These types were queried against each target genome by using blastn version 2.2.31+ software (https://www.ncbi.nlm.nih.gov/news/06-16-2015-blast-plus-update/). Matches with 100% sequence alignment and >99% nt identity were assigned ITS types, and these blastn alignments were manually visualized/verified by using CLC Genomics Workbench version 8.0.3 software (*8*,*9*,*26*).

Results

A dendrogram based on maximum-parsimony from analysis of core SNPs showed a distinct clade for genomes with an origin in the Western Hemisphere that branches off a node in common with genomes from isolates associated with Africa (Figure, https://wwwnc.cdc.gov/EID/article/23/7/16-1978-F1.htm). BLAST analysis yielded ITS type G for isolates with a clear origin in the Western Hemisphere (Table 1). We also observed presence of the YLF gene in all isolates in our panel.

Within the Western Hemisphere clade, distinct subclades appear to be associated with geographic origin (Figure). The largest subclade is the Puerto Rico/Trinidad clade, which consists of 5 genomes (PR1982, PR1998, PR2012, PR2013a, and PR2013b) from clinical cases and environmental isolates from Puerto Rico and 1 clinical case in Florida, USA (FL2012), from a resident of Trinidad (*2*,*20*,*27*). PR2013a and PR2013b, which were isolated from the same soil sample, also had no common SNPs detected but had 9 SNPs when compared with PR2012.

The second largest subclade consists of 5 genomes (CA2009, IL2014, MX2013, MX2014, and TX2015), which are all associated with clinical cases of melioidosis in patients who resided in or visited Mexico before seeking treatment in the United States (*2*,*27*,*28*). The patient from whom isolate MX2013 was obtained also had prior military service in Vietnam.

A subclade containing 4 genomes was resolved and consisted of GA2015, RI2013a, RI2013b, and TX2004. GA2015 was obtained from a patient who sought treatment in Georgia, USA, and resided in Panama but who had also visited Peru. The 2 isolates RI2013a and RI2013b, which have different colony morphologies, were obtained from a patient who traveled from Rhode Island, USA, to Guatemala. No common SNPs were detected for RI2013a and

Table 1. Phylogeographic analysis of 26 isolates of *Burkholderia pseudomallei* from the Western Hemisphere by whole-genome sequencing*

Isolate	GenBank accession no.	Origin	Patient travel or residence	Year isolated	MLST type	ITS type
7894	CP018373,CP018374	Ecuador	Unknown	1960	11	G
724644	CP018377,CP018378,CP018379	Massachusetts, USA	Aruba	2012	698	G
CA2007	CP018418,CP018419	California, USA	Unknown	2007	518	G
CA2009	CP018380,CP018381	California, USA	Mexico	2009	95	G
CA2010	CP018382,CP018383	California, USA	Unknown	2010	550	C
CA2013a	CP018398,CP018399	California, USA	Unknown	2013	518	G
FL2012	CP018391,CP018392	Florida, USA	Trinidad	2012	297	G
GA2015	CP018416,CP018417	Georgia, USA	Panama and Peru	2015	436	G
IL2014	CP018414,CP018415	Illinois, USA	Mexico	2014	92	G
MD2013	MPSL01000000	Maryland, USA	Africa	2013	1053	C
MX2013	CP018395,CP018396,CP018397	California, USA	Mexico and Vietnam	2013	297	G
MX2014	CP018410,CP018411	California, USA	Mexico	2014	951	G
NY2010	CP018384,CP018385,CP018386	New York, USA	Aruba	2010	698	G
OH2013	CP018400,CP018401	Ohio, USA	None	2013	17	CE
PB 1007001	CP018387,CP018388	Arizona, USA	Costa Rica	2009	518	G
PR1982	CP018367,CP018368	Puerto Rico, USA	Unknown	1982	NA	G
PR1998	CP018369,CP018370	Puerto Rico, USA	Unknown	1998	92	G
PR2012	CP018393,CP018394	Puerto Rico, USA	Unknown	2012	297	G
PR2013a	CP018406,CP018407	Puerto Rico, USA, soil 1	None	2013	297	G
PR2013b	CP018408,CP018409	Puerto Rico, USA, soil 2	None	2013	297	G
RI2013a	CP018402,CP018403	Rhode Island, USA	Guatemala	2013	1038	G
RI2013b	CP018404,CP018405	Rhode Island, USA	Guatemala	2013	1038	G
Swiss2010	CP018389,CP018390	Switzerland	Martinique	2010	92	G
TX2004	CP018375,CP018376	Texas, USA	Southeast Asia	2004	297	G
TX2015	CP018412,CP018413	Texas, USA	Mexico	2015	92	G
VEN1976	CP018371,CP018372	Venezuela	Unknown	1976	12	G

*ITS, internal transcribed spacer; MLST, multilocus sequence typing; NA, not available (allele for *gltB* was not resolvable by conventional Sanger sequencing or whole-genome analysis).

RI2013b. TX2004 was obtained from a resident of Texas, USA, who had spent time in Southeast Asia during World War II as a prisoner of war (*29*).

Genomes (NY2010 and 724644) from 2 cases of melioidosis in persons from the United States who traveled to Aruba clustered together and had 22 SNP differences between each other (*2*,*27*,*30*). Two isolates from pet iguanas (*Iguana iguana*) in California, USA (CA2007 and CA2013a) also clustered together (*31*). Three SNPs differentiate CA2007 from CA2013a.

Other genomes from isolates investigated in the United States from patients with no clear history of travel to regions to which melioidosis is endemic did not cluster within the Western Hemisphere clade. CA2010 groups with isolate PB08298010 in a subcluster among reference genomes from Southeast Asia and has ITS type C. OH2013 has ITS type CE and clusters most closely to K96243, which groups with other isolates from Thailand and Southeast Asia (*32*). Isolate MD2013, which was obtained from a US military veteran who served in Asia but most recently resided in Africa, has a genome that groups between isolates from Burkina Faso and Madagascar and has ITS type C.

Discussion

Previous methods have been used to associate genetic features of *B. pseudomallei* with geographic origin. For example, it has been observed that the YLF gene cluster is predominant in strains from outside Australia, and our results are consistent with that association (*5*,*9*). We also observed that ITS type G predominates in our panel of isolates from the Western Hemisphere, which is consistent with results from our previous study (*5*). MLST has been used to associate isolates with geographic origin (*6*). However, MLST alone does not appear to be sufficient to provide geographic correlation when investigating an isolate of unknown provenance and can be confounded by homoplasy (*4*,*5*,*7*).

Analysis of SNPs in whole-genome sequences appears quite promising as a way to correlate isolates with geographic origin (*4*,*10*,*11*). The ability to link a strain with a geographic origin has been shown to be increasingly useful, especially in areas such as the US mainland, which so far does not appear to be endemic for melioidosis (*2*). This ability is particularly useful in cases in which there is no clear travel history to regions to which *B. pseudomallei* is endemic, or which may have resulted from exposure to contaminated products, such as medical supplies, from those areas (*3*). Our study supports previous observations by Sarovich et al. (*10*) and Chewapreecha et al. (*11*) that, on the basis of SNP analysis, isolates from the Americas belong to a distinct clade that branches off from strains from Africa. The larger size of our panel of isolates from the Western Hemisphere enabled a finer-scale association of some isolates on the basis of geographic

Table 2. Reference genomes of *Burkholderia pseudomallei* strains from the Western Hemisphere used for phylogeographic analysis

Strain	Origin	GenBank accession no.
576	Thailand	ACCE01000000
350105	Hainan, China	CP012093, CP012094
1026b	Thailand	CP002833, CP002834
11–1617	Madagascar	SRR3145392
11–1696	Madagascar	SRR3145393
1258a	Thailand	AHJB01000000
1710b	Thailand	CP000124, CP000125
406e	Thailand	AAMM02000000
4900CF	Brazil	ARZE01000000
A79C	Papua New Guinea	JQHQ01000000
ABCPW1	Australia	JQIJ01000001
BCC215	Brazil	ABBR01000000
BEL2013	Madagascar	SRR3145396
BF103	Burkina Faso	SRR3145394
BF111	Burkina Faso	SRR3145395
Bp1651	California, USA, by way of Australia	CP012041, CP012042
Bp22	Singapore	AFBJ01000000
BPC006	China	NC_018527, NC_018529
K96243	Thailand	NC_006350, NC_006351
MSHR1655	Australia	AAHR02000000
MSHR305	Australia	CP006469, CP006470
MSHR3709	Australia	JRFK01000000
MSHR5107	Malaysia	JZXP01000001
MSHR5613	Australia	JQDK01000000
MSHR5848	Australia	AVAJ01000000
MSHR5858	Australia	AVAK01000000
MSHR668	Australia	CP000570, CP000571
NCTC13179	Australia	NC_022658, NC_022659
Pasteur52237	Vietnam	AAHV02000000
PB08298010	Arizona, USA	ARZO01000000
PHLS14	Philippines	ABBJ01000000
PHLS9	Pakistan	ABBL01000000
S13	Singapore	AAHW02000000
T4	Australia	JPNO01000000
VEL	Malaysia	APLI01000000

origins and provided insights on isolates recovered from cases with no clear sources of exposures.

Isolates from Puerto Rico and the isolate from the patient who resided in Trinidad resulted in a clear association, which is surprising because Trinidad is much closer to Venezuela than to Puerto Rico. Isolates PR2013a and PR2013b were obtained from the same soil sample and do not have common SNPs. The soil sample from which PR2013a and PR2013b was isolated was <250 m from the residence of the patient who was infected with isolate PR2012. Not only do the soil isolates cluster with PR2012, but there were only 9 SNPs differences between them and PR2012, which supports the supposition that the area close to the home of the patient was the source of infection. This supposition is analogous to the observation in a case in Australia where only 3 SNPs separated a clinical isolate from an environmental isolate obtained near the home of the patient (*12*).

The association among isolates from patients with links to Mexico was also clear on the basis of clustering in the phylogenetic tree. However, each isolate is relatively distant from each other (e.g., 3606 SNPs distinguish MX2014 from IL2012), which indicates that more genetic diversity has been captured for isolates with origins in Mexico than for those with origins in Puerto Rico.

The subclade consisting of GA2015, RI2013a, RI2013b, and TX2004 appears to be related to the subclade from Mexico. Also, no common SNPs were detected for RI2013a and RI2013b despite difference in colony morphotypes. This finding is consistent with a recent observation in another strain in which no common SNPs were detected among genomes of a variety of colony morphotypes, which indicated that the differences were not caused by mutations, but possibly by differential gene expression or another mechanism (*33*).

TX2004 is an isolate obtained from a patient who has been reported as having the longest incubation period for melioidosis. This patient was a US military veteran who was captured during World War II by the Japanese and interned in various camps in Southeast Asia as a prisoner of war; he did not have a travel history to other regions to which *B. pseudomallei* is endemic. His case of melioidosis was attributed to exposure to *B. pseudomallei* 62 years before development of symptoms (*29*). However, we found that TX2004 belongs to the Western Hemisphere clade and groups with genomes from isolates from melioidosis

patients who had travel histories to Guatemala, Panama, and Peru. This finding, and the fact that TX2004 is ITS type G, suggests that TX2004 might not have been acquired by the patient in the Pacific theater during World War II.

For the 2 isolates obtained from travelers to Aruba, we found no epidemiologic link between the 2 cases (NY2010 and 724644) other than travel to Aruba for vacation. This finding supports the hypothesis that Aruba was the source of exposure and highlights the usefulness of well documented travel records for epidemiologic associations.

We observed clustering of genomes from isolates obtained from 2 green iguanas. The first case (CA2007) was reported in 2007 in northern California, and the iguana died ≈2.5 years later. The second case (CA2013a) was reported in a 1.6-year-old iguana in southern California. The iguanas were purchased from different pet stores and the cases were separated in time. Thus, there was no clear epidemiologic link between the cases. No background information was available on the origin of the iguanas or the pet store suppliers. Both iguanas were not transported after purchase and were confined to the homes of the owners.

Earlier MLST data indicated that both of these isolates had ST518, which had also been seen in isolate PB1007001, obtained from a tourist from Arizona, USA, in whom melioidosis developed after this person was infected in Costa Rica. This finding suggested that the iguanas were infected in Central America before importation and is consistent with the known range of iguanas (Florida to southern Brazil) and the observation that most iguanas imported into the United States come from Central America (*31*,*34*,*35*). The genomes from the 2 isolates are highly related (only 3 common SNPs) and suggests that the iguanas originated in the same region. The same breeding facility could also be involved, assuming that the iguanas were farm raised.

The association of clades with geographic origin promises to be a useful tool when investigating cases of melioidosis in patients without a clear exposure history, such as travel to multiple countries to which this disease is endemic or for patients with no known travel history. Isolate MD2013 was obtained from a retired person from the United States who resided in Ghana and traveled around Africa. He also had previous military service in Asia, although not in regions with a strong history of melioidosis. He sought treatment in the District of Columbia and Maryland, USA, area after symptoms developed. MD2013 has a genome that groups between isolates from Burkina Faso and Madagascar and supports infection with the bacteria in Africa. The grouping of MX2013 with other genomes from Mexico indicates infection occurred in Mexico instead of Vietnam. An isolate from a case in a zoological warehouse worker without a travel history to areas to which melioidosis is endemic has a genome (CA2010) that does not belong within the clade for isolates from the Western Hemisphere. This isolate belongs within a subclade that contains an isolate (PB08298010) from a resident of Arizona who also did not have a clear travel history (*36*). We previously reported that PB08298010 was ITS type G on the basis of an earlier draft genome sequence, but a more recent analysis of a higher-quality whole-genome sequence indicates it has ITS type CE (A. Tuanyok, 2015, pers. comm.) (*5*). The ITS type CE and the location of this subclade support an origin outside the Western Hemisphere, more likely in Asia, which supports the earlier proposal that PB08298010 might have resulted from exposure to contaminated medical supplies from Southeast Asia (*10*,*36*). A similar situation might also be true for a fatal case in a patient in Ohio, USA, who had no travel history outside the continental United States (*32*). Isolate OH2013 groups with isolates from Thailand and is clearly not associated with those in the Western Hemisphere.

The genome sequences analyzed in this study will assist future epidemiologic investigations of melioidosis, especially in cases potentially associated with exposure in the Western Hemisphere. We believe that, as more genome sequences become available, the robustness and resolution of this analysis will improve and thus enhance the ability to associate isolates of *B. pseudomallei* with geographic origin.

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Nontuberculous Mycobacteria Infections at a Provincial Reference Hospital, Cambodia

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Prevalence of nontuberculous mycobacteria (NTM) disease is poorly documented in countries with high prevalence of tuberculosis (TB). We describe prevalence, risk factors, and TB program implications for NTM isolates and disease in Cambodia. A prospective cohort of 1,183 patients with presumptive TB underwent epidemiologic, clinical, radiologic, and microbiologic evaluation, including ≥12-months of follow-up for patients with NTM isolates. Prevalence of NTM isolates was 10.8% and of disease was 0.9%; 217 (18.3%) patients had TB. Of 197 smear-positive patients, 171 (86.8%) had TB confirmed (167 by culture and 4 by Xpert MTB/RIF assay only) and 11 (5.6%) had NTM isolates. HIV infection and past TB were independently associated with having NTM isolates. Improved detection of NTM isolates in Cambodia might require more systematic use of mycobacterial culture and the use of Xpert MTB/RIF to confirm smear-positive TB cases, especially in patients with HIV infection or a history of TB.

Nontuberculous mycobacteria (NTM) include ≈160 species of environmental mycobacteria found largely in soil and water sources, of which ≈40 can be associated with lung disease, the most common clinical presentation of NTM infection (*1*,*2*). NTM are usually less pathogenic than *Mycobacterium tuberculosis* complex (MTBc) and can be isolated without the presence of disease (colonization) (*3*). Pulmonary disease attributable to NTM infection frequently occurs in patients with structural lung disease (*1*,*4*). In HIV-infected patients, disseminated NTM disease occurs more frequently once the CD4 cell count is <50 cells/μL (*5*).

NTM infections are not reportable diseases, and most available data come from sentinel surveillance and laboratory-based studies; consequently, the exact prevalence of such infections is not well known (*6*,*7*). However, detection of NTM isolates has increased worldwide, a trend that might be attributed to several factors, including a surge of HIV infections in the past 2 decades, a better understanding of the clinical and pathological relationship between host and pathogen, improved detection methods, increased natural or artificial environmental exposure (i.e., tap water in developing countries), population aging, and improved survival of patients with structural lung diseases (*8*–*15*). An increase of NTM isolates was also observed after the introduction of liquid culture methods, which are more sensitive in detecting NTM isolates than solid methods (*14*). However, because mycobacterial culture laboratories are lacking in limited-resource countries, the burden of NTM infection is often poorly documented in areas with a high tuberculosis (TB) prevalence. This observation is particularly true in Asia, where no population-based studies have been conducted to document the epidemiology of NTM pulmonary isolates and NTM lung disease (*4*).

Some studies indicate that NTM might play an important role in TB-like disease, which can lead to inappropriate or unnecessary anti-TB treatment (*15*–*18*). Furthermore, smear microscopy cannot distinguish MTBc from NTM, and in many countries with high TB prevalence, access to the XpertMTB/RIF assay (Cepheid, Sunnyvale, CA, USA) is still limited (*19*). Therefore, the effect of NTM isolates on TB case management needs to be further explored in countries with high TB prevalence.

Current guidelines for diagnosis and treatment of NTM infection rely mainly on criteria established by the American Thoracic Society (ATS) in 2007 (*1*,*15*,*20*). Despite their usefulness, the ATS criteria were principally designed for diagnosis of lung disease caused by *M. kansasii*, *M. abscessus*, and *M. avium* complex (comprising *M. avium* and *M. intracellulare*), all of which are common in North

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America and possibly less adapted to the ecologic situation of other regions in the world (*1*,*15*,*21*–*24*). These criteria are less appropriate for the diagnosis of disseminated NTM infection occurring in immunocompromised patients and can require the use of computed tomography scan and advanced diagnostic technologies that are not available in many resource-limited countries (*20*).

The primary objective of our study was to estimate the prevalence of NTM isolates and NTM lung disease among consecutive patients with presumptive TB at the chest clinic of the Kampong Cham Provincial Reference Hospital in Cambodia. Secondary objectives were to identify factors associated with isolation of NTM and to compare the epidemiologic, clinical, and radiologic findings between patients diagnosed with TB, NTM lung disease, and NTM colonization. Finally, exploratory objectives aimed to review the use of the ATS criteria for diagnosis of NTM lung disease and to assess the effect of NTM isolates on TB case management in a setting with high TB prevalence but limited diagnostic capacity.

Materials and Methods

Study Design and Population

The study involved a prospective cohort of patients (≥15 years of age) with presumptive pulmonary TB (cough for ≥3 weeks), ability to produce 2 sputum specimens, and no anti-TB treatment for >7 days during the previous month. The reference hospital received support from Médecins Sans Frontières for diagnosis and treatment of TB for a population of ≈300,000 inhabitants in the Kampong Cham province.

Procedures

At enrollment, patients were interviewed about potential risk factors for NTM exposure and NTM lung disease and underwent a physical examination. HIV testing in accordance with national guidelines and postero-anterior chest radiographs were performed. Three sputum specimens collected during 2 consecutive days were examined by using LED-fluorescent microscopy after auramine-O staining. The 2 best specimens based on macroscopic appearance (purulent and mucopurulent specimens) were decontaminated by using the N-acetyl-L-cysteine-sodium hydroxide method (2% final concentration and 20 min digestion) and then centrifuged. The sediment from the first sample was cultured by using the BBL Mycobacteria Growth Indicator Tube (MGIT) manual system (Becton Dickinson, Sparks, MD, USA) and the second by using Lowenstein-Jensen (LJ) media (*25*). LJ cultures were read once per week for 8 weeks and MGIT cultures once a day for 56 days. Negative culture results were delivered after 8 weeks. The third specimen was stored at –20°C and cultured on LJ and MGIT if NTM was isolated on only 1 of the 2 initial cultures to increase the possibility of isolating the same NTM species in 2 different specimens or in the event 1 of the 2 cultures was contaminated. Identification of MTBc or NTM species used the P-nitrobenzoic acid and Bioline Ag MPT64 Rapid (Standard Diagnostics Inc., Kyonggi-do, Korea) tests. In case of NTM growth on any of the 2 cultures, a subculture on LJ was sent to the Institut Pasteur in Phnom Penh for rapid NTM speciation using 2 DNA strip assays (GenoType Mycobacterium CM and GenoType Mycobacterium AS, Lifescience, Nehren, Germany). An XpertMTB/RIF assay was performed on smear-positive samples according to the manufacturer's guidelines. To reduce risk for specimen contamination with environmental mycobacteria, samples were collected after rinsing the mouth with mineral water and laboratory procedures were carried out with filtered water.

Case Definitions, Treatment, and Follow-up

In the absence of universal case definitions, patients with culture-positive NTM were classified as having NTM lung disease or NTM colonization by a study expert committee based on review of microbiologic, clinical, and radiologic information at baseline and during follow-up (*15*). The definition of NTM lung disease was adapted from the 2007 ATS criteria requiring pulmonary symptoms and abnormal chest radiograph suggestive of TB or NTM disease (i.e., infiltrates, nodular, or cavitary opacities); growth of the same NTM species from ≥2 sputum samples collected at different times; and exclusion of other differential diagnoses, such as TB (*1*). Patients who had NTM isolates but did not meet the eligible criteria for NTM lung disease were classified as having NTM colonization. The decision to initiate treatment was guided by the study expert committee based on the potential risks and benefits of a prolonged course of multiple antibiotics for the patient, taking into consideration age, comorbid conditions, and disease type (*1*,*4*,*11*).

Treatment regimens were based on the 2007 ATS guidelines, and patients were followed until 12 months after completion of treatment with monthly sputum smear microscopy and culture (*1*). Patients classified as having NTM colonization were followed for 12 months. Among these patients, those who remained symptomatic at the first follow-up visit for culture results (2–8 weeks after enrollment) were classified having as symptomatic NTM colonization and were followed every 3 months. Asymptomatic patients or patients with substantial clinical improvement were classified as having asymptomatic NTM colonization and were followed at 6 and 12 months only. Sputum smear microscopy and culture and chest radiograph during follow-up visits were only performed in patients who were still symptomatic. Patients with both MTBc and NTM isolates were classified as having TB. Diagnosis of NTM lung disease could be reconsidered if patients did not respond to TB treatment.

Sample Size and Statistics

By using an NTM disease prevalence estimation of 7% among patients with presumptive TB, a precision of 1.5%, a risk α of 5%, and 10% increase for drop-outs, we determined that a sample size of 1,222 patients was needed (*26*). Data were double entered by using Epi-Data 3.0 software (EpiData Association, Odense Denmark) and analyzed in Stata 10 software (StataCorp LP, College Station, TX, USA). We calculated 95% CIs for prevalences of NTM isolates and NTM lung disease. Patients' characteristics at enrollment were displayed according to the final patients' classification (i.e., as having TB, NTM lung disease, or NTM colonization, or being culture-negative). We performed univariate and multivariate regression analysis using logistic regression model to assess the association between baseline patient characteristics and the isolation of NTM. We included covariates associated with a p value <0.4 in univariate analysis in the initial multivariate model and used a manual backward stepwise approach to obtain the final multivariate model. Statistical significance ($p<0.05$) was assessed with the likelihood-ratio test. The proportion of MTBc isolates detected using the XpertMTB/RIF assay was calculated for smear-positive patients. Criteria used for the diagnosis of NTM lung disease were compared with the 2007 ATS criteria for patients with ≥1 NTM isolate.

Ethics

The study protocol was approved by the National Ethics Committee for Health Research, Phnom Penh, Cambodia, and the Comité de Protection des Personnes, Saint Germain en Laye, France. Patients' informed consent was obtained.

Results

A total of 1,183 patients with laboratory culture results were enrolled during October 1, 2012–April 21, 2014 (Figure). The prevalence of NTM isolates (including those detected in 4 patients with NTM and MTBc isolates) was 10.8% (95% CI 9.1%–12.7%); prevalence of NTM lung disease was 0.9% (95% CI 0.5%–1.7%). Of the 113 patients with NTM colonization, 61 (54.0%) were still symptomatic at the time of culture results. Of the 124 patients classified as having NTM lung disease or NTM colonization, 24 (19.3%) were lost to follow-up, 9 (7.3%) withdrew voluntarily, and 9 (7.3%) died.

The male:female ratio among enrollees was 0.94, and median age was 54 years. Almost half of patients were farmers, and 26.0% had a TB history (median time since the last event 3 years, interquartile range 2–8 years). More than one third of patients had chronic bronchitis, and 12.1% had been hospitalized in the past for respiratory disease (Table 1). TB history (odds ratio [OR] 29.75, 95% CI 7.64–115.85), HIV infection (OR 50.57, 95% CI 7.89–324.16), and previous hospitalizations for respiratory disease (OR 17.4, 95% CI 4.70–64.14) were associated with a diagnosis of NTM lung disease compared with TB. Similarly, among patients with NTM isolates, those with TB history (OR 3.59, 95% CI 0.99–13.05), HIV infection (OR 23.43, 95% CI 3.63–151.19), and previous hospitalizations for respiratory disease (OR 7.84, 95% CI 2.12–28.92) were more likely to be diagnosed with NTM lung disease.

A low body mass index (i.e., <18.5 kg/m^2) was the only clinical parameter that was significantly different between patients with NTM lung disease and TB patients (90.1% vs.

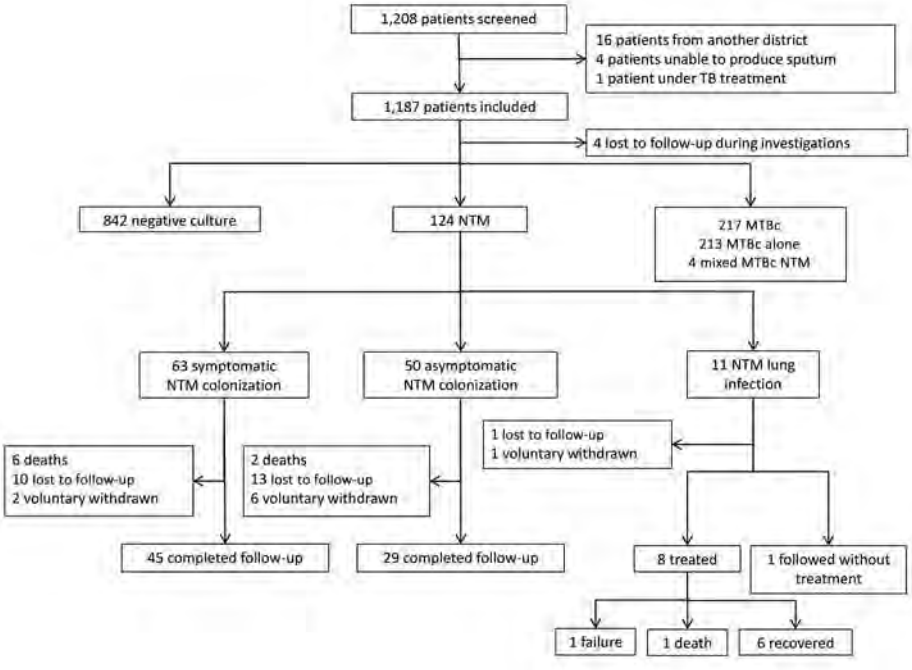

Figure. Schematic summary of results from study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014. MTBc, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacteria; TB, tuberculosis.

56.7%; p = 0.025) (Table 2). On chest radiograph, fibrosis or volume loss (p = 0.031) and bronchiectasis (p = 0.001) were more common in patients with NTM lung disease than in TB patients. Cavitation (p = 0.029), pleural thickening (p = 0.039), and bronchiectasis (p = 0.005) were more frequent in patients with NTM lung disease than in patients with NTM colonization (Table 3).

Only 2 factors were associated with isolation of NTM: history of TB (adjusted OR 1.56, 95% CI 1.04–2.33) and HIV infection (adjusted OR 4.63, 95% CI 1.68–12.77) (Table 4). The most common NTM species were *M. fortuitum* and *M. abscessus* (rapid-growing mycobacteria) and *M. intracellulare*, *M. gordonae*, and *M. scrofulaceum* (slow-growing mycobacteria) (Table 5). Of 124 patients with NTM isolates (excluding 4 patients with both MTBc and NTM isolates), 14 (11.3%) had the same NTM species isolated in 2 separate sputum specimens. The same NTM species was isolated more than once during follow-up in 8/61 (13.1%) patients with symptomatic NTM colonization (3 *M. fortuitum*, 2 *M. intracellulare*, 2 *M. abscessus*, and 1 *M. simiae*) versus 2/49 (4.1%) in patients with asymptomatic NTM colonization (2 *M. fortuitum*) (p = 0.009).

Overall, 217/1183 (18.3%) patients were culture-positive for MTBc, including 142/942 (15.1%) by LJ and 204/1134 (18.0%) by MGIT. The NTM growth was 2.7% (32/1170) on LJ medium compared with 8.0% (95/1,186) in MGIT (p<0.001). Overall NTM growth was 10.0% (117/1,187) when methods were combined.

Of 1,183 patients, 197 were smear-positive (16.6%). Of these, 171 (86.8%) were confirmed to have MTBc (167 by culture and 4 by XpertMTB/RIF only), 11 (5.6%) were positive for NTM, and 15 (7.6%) were both culture and XpertMTB/RIF negative. XpertMTB/RIF detected MTBc in 166/197 (84.2%) smear-positive patients. After exclusion of contaminated results, LJ and MGIT were positive for MTBc in 122/157 (77.7%) and 162/195 (83.1%) of smear-positive patients and in 20/785 (2.5%) and 42/942 (4.4%) of smear-negative patients, respectively.

Table 1. Demographic and epidemiologic characteristics of patients in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

Characteristic	Total, N = 1,187	TB, n = 217	NTM disease, n = 11	NTM colonization, n = 113	Culture-negative, n = 842
Median age, y (IQR)	54 (40–65)	49 (35–62)	54 (46–63)	57 (44–65)	54 (40–65)
Sex					
F	611 (51.5)	87 (40.1)	6 (54.5)	66 (59.5)	451 (53.6)
M	567 (48.5)	130 (60.0)	5 (45.5)	47 (41.6)	391 (46.4)
Occupation					
Unemployed	339 (28.6)	61 (28.1)	4 (36.4)	35 (31.0)	238 (28.3)
Student	35 (2.9)	7 (3.2)	0	5 (4.4)	23 (2.7)
Pensioner	25 (2.1)	1 (0.5)	0	4 (3.5)	20 (2.4)
Farmer	568 (47.9)	109 (50.2)	7 (63.6)	45 (39.8)	406 (48.2)
Trader/seller	57 (4.8)	7 (3.2)	0	8 (7.1)	42 (5.0)
Building worker	18 (1.5)	5 (2.3)	0	1 (0.9)	12 (1.4)
Police/army	14 (1.2)	5 (2.3)	0	0	8 (0.9)
Fisherman	19 (1.6)	2 (0.9)	0	4 (3.5)	13 (1.5)
Administration	33 (2.8)	3 (1.4)	0	4 (3.5)	26 (3.1)
Factory worker	45 (3.8)	13 (6.0)	0	2 (1.8)	30 (3.6)
Other	34 (2.9)	4 (1.8)	0	5 (4.4)	24 (2.8)
Drinking water					
Water well	709 (59.7)	127 (58.5)	7 (63,6)	63 (55.7)	510 (60.6)
Running water	210 (17.7)	36 (16.6)	2 (18.2)	27 (23.9)	144 (17.1)
Rain	64 (5.4)	15 (6.9)	0	7 (6.2)	42 (5.0)
River/lake/pond	131 (11.0)	24 (11.1)	1 (9.1)	11 (9.7)	95 (11.3)
Mineral water	74 (6.1)	15 (6.9)	1 (9.1)	5 (4.4)	51 (6.1)
Water disinfection					
Boiled	701 (59.1)	119 (54.8)	5 (45.5)	68 (60.2)	506 (60.2)
Filtered	121 (10.2)	15 (6.9)	2 (18.2)	13 (11.5)	91 (10.8)
None	364 (30.7)	83 (38.2)	4 (36.4)	32 (28.3)	244 (29.0)
Use of hot tub	13/345 (3.8)	1/63 (1.6)	0	1/37 (2.7)	11/242 (4.5)
Pneumoconiosis risk	27 (2.3)	11 (5.1)	0	1 (0.9)	15 (1.8)
Surgery of the lung	2 (0.2)	0	0	0	2 (0.2)
Past TB	309 (26.0)	12 (5.5)	7 (63.4)	37 (32.7)	252 (30.0)
Chronic bronchitis	447/1,168 (38.3)	99/213 (46.5)	4 (36.4)	34/110 (30.6)	310/830 (33.0)
Hospitalization†	144 (12.1)	14 (6.4)	6 (54.5)	15 (13.3)	109 (13.0)
Smoking					
Current smoker	175 (14.7)	39 (17.9)	2 (18.2)	10 (8.8)	123 (14.6)
Former smoker	266 (22.4)	57 (26.3)	3 (27.3)	23 (20.3)	58 (6.9)
Esophageal motility disorders	1/1,178 (<0.1)	0	0	0	1/836 (<0.1)
HIV positive	19/917 (2.1)	2/179 (1.1)	4 (36.4)	2/84 (2.4)	11/639 (1.7)

*Values are no. patients/no. with available data (%) except as indicated. IQR, interquartile range; NTM, nontuberculous mycobacteria; TB, tuberculosis.

Table 2. Clinical presentation of patients in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

Characteristic	Total, N = 1,187	TB, n = 217	NTM disease, n = 11	NTM colonization, n = 113	Culture-negative, n = 842
Temperature ≥37.5°C	115 (9.7)	32 (14.7)	2 (18.2)	9 (8.0)	72 (8.5)
Median BMI, kg/m^2 (IQR)	19.0 (17.0–21.5)	18.1 (16.2–19.8)	14.8 (13.9–16.2)	18.8 (16.9–20.9)	19.4 (17.2–22.1)
BMI <18.5	524 (44.1)	123 (56.7)	10 (90.9)	52 (46.0)	337 (40.0)
Karnofsky, % (IQR)	90 (90–100)	90 (80–100)	80 (80–90)	90 (90–100)	90 (90–100)
SaO_2, % (IQR)	98 (97–99)	98 (97–99)	99 (91–100)	98 (97–99)	98 (97–99)
Cough	1,185 (99.8)	217 (100)	11 (100)	113 (100)	840 (99.8)
Duration, wks (IQR)	4 (3–12)	8 (4–12)	8 (2–20)	4 (3–10)	4 (3–12)
Chest pain	501 (42.2)	106 (48.8)	7 (63.6)	45 (39.8)	343 (40.6)
Hemoptysis	151 (12.7)	27 (12.4)	0	16 (14.2)	108 (12.8)
Dyspnea	563 (47.4)	118 (54.4)	7 (63.6)	46 (40.7)	392 (46.6)
Loss of appetite	300 (25.3)	63 (29.0)	5 (45.4)	27 (23.9)	205 (24.3)
Loss of weight	753 (63.4)	182 (83.9)	9 (81.8)	62 (54.9)	499 (59.3)
Fatigue	151 (12.7)	44 (20.3)	4 (36.7)	10 (8.8)	93 (11.0)
Pleural effusion	18 (1.5)	3 (1.4)	0	1 (0.9)	14 (1.7)
Adenopathy	5 (0.4)	1 (0.5)	0	0	4 (0.5)

*Values are no. (%) patients except as indicated. IQR, interquartile range; NTM, nontuberculous mycobacteria; TB, tuberculosis.

Out of 128 patients with NTM isolates (including 4 patients with both MTBc and NTM isolates), 12 met the ATS criteria for having NTM lung disease but 4 (33.3%) were finally not confirmed by the study expert committee because of the good clinical response to an antimicrobial drug treatment targeting bacterial respiratory infection (1 for *Klebsiella pneumonia*, 1 for *Pseudomonas aeruginosa*, and 2 empirical treatments). No patient had clinical exacerbation or NTM recurrence during the 12 months of follow-up. Of the 11 patients diagnosed with NTM lung disease by the expert committee, 2 (18.2%) did not meet all the ATS criteria. Both were advanced HIV-infected patients; 1 was symptomatic with normal chest x-ray with only 1 *M. simiae* isolate, and 1 had a nonsuggestive chest radiograph results and several *M. intracellulare* isolates from different specimens (Table 6).

Discussion

We report a high prevalence (10.8%) of NTM isolates in sputum specimens of patients with presumptive TB. NTM growth occurred in one third of patients with positive mycobacterial cultures. However, only 10% of them were diagnosed with NTM disease. The prevalence of NTM isolates was higher than what has been previously reported in Cambodia among HIV infected patients (1%) (*27*). However, at that time, only LJ culture was used, which is likely to explain the difference with our findings. In our study, the NTM growth in MGIT was more than twice as high (8.0%) than on LJ media (2.7%), and LJ missed 2 out the 11 cases finally diagnosed as NTM disease; these findings support the ATS recommendation to use both solid and liquid culture methods for diagnosis of NTM disease (*1*). Also, in the

Table 3. Radiologic presentation of patients in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

	No. (%) patients				
Characteristic	Total, N = 1,187	TB, n = 217	NTM disease, n = 11	NTM colonization, n = 113	Culture-negative, n = 842
Normal	260 (21.9)	2 (<0.1)	1 (9.1)	44 (38.9)	212 (25.2)
Abnormal	927 (78.1)	215 (99.1)	10 (91.0)	69 (61.1)	630 (74.8)
Cavity	189 (20.4)	113 (52.6)	4 (40.0)	7 (10.1)	64 (10.2)
Fibrosis/volume loss	335 (36.2)	76 (35.5)	7 (70.0)	27 (39.1)	224 (35.6)
Infiltrates	866 (93.4)	214 (99.5)	10 (100)	63 (91.3)	577 (91.6)
Miliary	8 (0.9)	8 (3.7)	0	0	0
Adenopathy	103 (11.1)	24 (11.2)	2 (20)	14 (20.3)	62 (9.8)
Nodule/mass	127 (13.7)	36 (16.7)	2 (20)	10 (14.5)	78 (12.4)
Pleural tick	239 (25.8)	68 (31.6)	6 (60)	18 (26.1)	147 (23.3)
Pleural effusion	68 (7.3)	16 (7.4)	0	4 (5.8)	48 (7.6)
Bronchiectasis	148 (16.0)	29 (13.5)	6 (60)	13 (18.8)	99 (15.7)
Extent†					
Minimal	584 (63.1)	83 (38.6)	4 (40.0)	44 (64.7)	451 (71.6)
Moderate	231 (24.9)	84 (39.1)	4 (40.0)	16 (23.5)	126 (20.0)
Advanced	111 (12.0)	48 (22.3)	2 (20.0)	8 (11.8)	53 (8.4)

*NTM, nontuberculous mycobacteria; TB, tuberculosis.
†Minimal: lesions that are slight to moderate density or bronchiectasis without cavitation. Lesions may be present in a small portion of 1 or both lungs, but the total extent of the lesions should not exceed the volume of lung on 1 side, which is present above the second chondrosternal junction (or apex of 1 lung). Moderate: lesions may be present in 1 or both lungs, but the total extent must not be more than the following: 1) scattered lesions of slight to moderate density or bronchiectasis that may extend throughout the total volume of 1 lung or the equivalent volume in both lungs; 2) dense, confluent lesions that are limited in extend to one third of the volume of 1 lung; 3) cavitation with a diameter <4 cm. Advanced: lesions that are more extensive than moderately advanced.

same survey among HIV-infected patients, the prevalence of NTM isolates was 30% and 7%, respectively, in Thailand and Vietnam sites where MGIT was also used (*27*). Our findings are similar to the results from another study in Zambia, where 11% of patients with chronic cough had NTM isolates in their sputum and 0.6% had NTM disease (*16*).

The proportion of NTM disease cases among patients with NTM isolates in our study (10%) is at the lower bound of the range of 9%–71% reported in a systematic review of hospital-based studies in Southeast Asia and is lower than has been reported (30%) in the survey among HIV-infected outpatients in Thailand and Vietnam (*27*,*28*). The prospective follow-up of patients with NTM isolates in our study could partially explain the low proportion of disease. Indeed, retrospective hospital-based surveys tend to overestimate the prevalence because of recruitment bias and small numbers. By using a cross-sectional design only, we might have classified 4 additional cases as NTM disease because

Table 4. Characteristics associated with the detection of NTM isolates in specimens of 1,179 presumptive pulmonary TB patients in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

Characteristic	NTM isolate detected, no. (%)	Univariate analysis		Multivariate analysis	
		OR	95% CI	aOR	95% CI
Age group, y					
<40	24/295 (8.1)	1		1	
40–65	67/575 (11.6)	1.49	0.91–2.43	1.40	0.985–2.31
>65	33/309 (10.7)	1.35	0.78–2.34	1.33	0.76–2.35
Sex					
M	52/571 (9.1)	1		1	
F	72/608 (11.8)	1.34	0.92–1.95	1.36	0.93–2.00
Occupation					
Farmer	52/564 (9.2)	1			
Student, administration	17/138 (12.3)	1.38	0.77–2.48		
Factory, construction	3/63 (4.8)	0.49	0.15–1.62		
Fisherman	4/19 (21.0)	2.62	0.84–8.20		
Pensioner, unemployed	48/395 (12.1)	1.36	0.90–2.06		
Pneumoconiosis risk					
No	123/1,151 (10.7)	1			
Yes	1/27 (3.7)	0.31	0.04–2.39		
Drinking water					
Water well	70/704 (9.9)	1			
Running water	29/209 (13.9)	1.46	0.92–2.32		
Rain	7/63 (11.1)	1.13	0.50–2.58		
River/lake/pond	11/131 (9.2)	0.91	0.48–1.74		
Mineral water	6/72 (8.3)	0.82	0.34–1.97		
Water disinfection					
Boiled	73/696 (10.5)	1			
Filtered	15/121 (12.4)	1.21	0.67–2.18		
None	36/361 (10.0)	0.94	0.62–1.44		
Use of hot tub	38/331 (11.5)	1			
No	1/13 (7.7)	1			
Yes	85/835 (10.2)	0.64	0.08–5.01		
Unknown		0.87	0.58–1.31		
Smoke					
No	86/741 (11.6)	1			
Current smoker	12/173 (6.9)	0.57	0.30–1.06		
Former smoker	26/265 (9.8)	0.83	0.52–1.31		
TB history					
No	80/870 (9.2)	1		1	
Yes	44/307 (10.3)	1.65	1.11–2.45	1.56	1.04–2.33
HIV					
Negative	89/894 (10.0)	1		1	
Positive	6/19 (31.6)	4.17	1.55–11.25	4.63	1.68–12.77
Unknown	29/266 (10.9)	1.11	0.71–1.72		
Hospitalization for respiratory disease					
No	103/1,033 (10.0)	1			
Yes	21/144 (14.6)	1.54	0.93–2.55		
Health seeking behaviors					
No consultation	36/335 (10.7)	1			
Health center	21/234 (9.0)	0.81	0.46–1.44		
Private clinic	19/170 (11.2)	1.04	0.58–1.88		
Self-medication	5/36 (13.9)	1.34	0.49–3.66		
Unknown	43/404 (10.5)	0.99	0.62–1.58		

*aOR, adjusted OR; NTM, nontuberculous mycobacteria; OR, odds ratio; TB, tuberculosis.

Table 5. NTM species detected in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

NTM species	No. (%) patients			
	Overall, N = 123	NTM disease, n = 11	NTM colonization, symptomatic, n = 61	NTM colonization, asymptomatic, n = 50
Slow-growing	56			
M. avium	2 (1.6)	1 (9.1)		1 (2.0)
M. kansaii	1 (0.8)			1 (2.0)
M. scrofulaceum	13 (10.5)	1 (9.1)	5 (8.2)	7 (14.3)
M. intracellulare†	23 (18.7)	5 (45.4)	12 (119.7)	6 (12.0)
M. gordonae	13 (10.5)		8 (13.1)	5 (10.2)
M. interjectum	1 (0.8)		1 (1.6)	
M. simiae	3 (2.4)	1 (9.1)	2 (3.3)	
Rapid-growing	45			
M. fortuitum	28 (22.3)	1 (9.1)	16 (26.2)	11 (22.0)
M. abscessus	14 (11.3)	2 (18.2)	6 (9.8)	6 (12.2)
M. asiaticum	2 (1.6)		1 (1.6)	
M abscessus and *M. asiaticum*	1 (0.8)		1 (1.6)	
Not identified	22 (17.7)		9 (14.7)	13 (26.5)

*NTM, nontuberculous mycobacteria.
†In 3 cases, *M. intracellulare* was recovered with another NTM species: *M. scrofulaceum* (n = 1), *M. interjectum* (n = 1), and *M. fortuitum* (n = 1).

the patients met the ATS criteria at enrollment. However, their clinical assessment during follow-up showed that their cases qualified better as active or transient NTM colonization rather than NTM disease (*4*,*11*).

In our setting, two thirds of patients with NTM lung disease had fibrocavitary disease, with bronchiectasis in 60 and cavititation in 40% (*11*). Post-TB inflammatory bronchiectasis was the most common underlying structural lung disease. However, discussion persists regarding whether NTM invade preexisting bronchiectasis or lead to the initiation of bronchiectasis through postinflammatory bronchiectasis like MTBc does (*29*,*30*). Another possibility is that some NTM disease might previously have been mistakenly attributed to MTBc. One third of patients in our study had advanced HIV infection without underlying lung disease and had disseminated NTM infection with some lung involvement. This finding can explain why only half of them had illness that met the ATS criteria (*3*,*20*).

Among the 113 patients classified as having NTM colonization, the group of symptomatic patients were older and had a history of TB or hospitalization for respiratory disease and bronchiectasis more often than did the group of asymptomatic patients. Also, recurrence of the same NTM species in a sputum specimen during follow-up was more common among symptomatic patients than asymptomatic patients (13.1% vs. 4.1%). Based on these criteria, patients with NTM isolates no longer symptomatic at first follow-up visit were more likely to have NTM contamination or transient colonization without progression, whereas patients who remained symptomatic were more likely to have active colonization or indolent infection, which can progress to active (overt) disease (*4*,*11*,*20*).

Rapid-growing NTM species were very commonly found in our study. The proportion of *M. fortuitum* isolates (28.7%) was much higher than what has been previously reported in surveys conducted in Asia (*31*). However, as expected, *M. fortuitum* was only occasionally responsible for NTM lung disease (*2*). The proportion of *M. abscessus* isolates (14.8%) was close to what has been reported in other studies in Taiwan (18%) and China (14%) and was responsible for 18% (2/11) of the NTM lung disease cases in our study (*28*). This fact is particularly concerning because *M. abscessus* is usually resistant to antimicrobial drugs (*11*). The prevalence of isolation of *M. intracellulare* (23.8%) and *M. scofulaceum* (13.9%) was also very close to what has been reported in previous surveys in Asia (*28*,*32*). Surprisingly, *M. kansasii*, which is frequently isolated in specimens from patients in Asia, was almost absent in our setting (*28*).

If we apply our results to other settings in Cambodia without availability of mycobacterial culture, 10% of patients with presumptive TB (NTM culture-positive) could be wrongly started on TB treatment. Of these patients, those with smear-positive TB would definitely be started on TB treatment. Also, smear-negative patients classified as having NTM lung disease or symptomatic NTM colonization (55/986 [5.6%]) would probably also have been started on TB treatment. Even if NTM isolates represented a small proportion of all smear-positive cases (5%), in settings with high NTM disease burden, confirmation of smear-positive TB using XpertMTB/RIF should be considered. This practice might pose a challenge for national TB programs in countries with low HIV prevalence where XpertMTB/RIF is used as add-on test for smear-negative TB patients (*19*,*31*). Clinicians should also take into consideration the possibility of NTM infection in patients not responding to TB treatment, smear-negative patients with recurrent respiratory infections with bronchiectasis, patients with post-TB lung damage, and HIV-infected patients. Mycobacterial culture should be requested in such cases.

The only factors associated with NTM isolates in patients' specimens were history of previous TB and HIV

Table 6. Comparison of American Thoracic Society classification criteria used to define NTM disease with those used in a study of NTM infections at Kampong Cham Provincial Reference Hospital, Cambodia, October 1, 2012–April 21, 2014*

Radiologic criteria	Clinical criteria, in addition to cough of >3 wks duration: Same NTM species in 2 sputum samples‡	NTM in only 1 sputum sample	NTM and MTBc in 1 sputum sample	Total
Chest radiograph results				
Suggestive†‡	12 (8)‡	24 (1)	4	40
Abnormal but nonsuggestive	2 (1)	44	0	46
Normal	3	39 (1)	0	42
Total	16	108	4	128

*CR, chest radiograph; MTBc, *Mycobacterium tuberculosis* complex; NTM, nontuberculous mycobacterial.
†Presence of nodules, cavities, or bronchiectasis.
‡Criteria corresponding to the NTM lung disease classification according to the American Thoracic Society. Numbers in parentheses correspond to the cases classified as NTM lung disease by the study's expert committee.

infection. Disorders of cell-mediated immunity are known to be associated with an increased risk for disseminated NTM disease (*3*,*15*). However, to our knowledge, HIV has not yet been identified as a risk factor for a positive NTM culture from respiratory specimens (*16*,*33*,*34*).

Our study has limitations. First, even though the prospective design is a strength of the study, 12 months might be too short a duration to assess the progression to NTM lung disease in patients with symptomatic NTM colonization. On the other hand, using findings from patients' follow-up for final classification diverged from the ATS criteria and might have underestimated the proportion of NTM lung disease in our study compared with previous studies. Indeed, 4 out of 12 patients who fit the ATS criteria for lung disease at initial assessment were not confirmed by the study expert review committee. However, this potential underreporting is offset by the fact that 3 patients whose cases did not meet the ATS criteria were classified as having NTM lung disease. Second, assessing the evolution from NTM colonization to disease in patients who dropped out during follow-up (19.3%) was not possible. Third, properly assessing the presence of bronchiectasis in the study population in the absence of computed tomography scan was difficult. Last, the survey included patients with presumptive TB in a limited rural area in Cambodia, so the findings might not be generalizable to other populations.

In conclusion, our study shows that the prevalence of NTM isolates can be high among patients with presumptive TB, which can result in potential misdiagnosis and treatment of TB. In settings without routine use of mycobacterial culture, a sentinel survey of the NTM disease prevalence among patients with clinical suspicion of TB might be recommended. Then, in areas where NTM isolates are frequently detected, such as Southeast Asia, clinicians should consider systematic confirmation of TB in smear-positive patients with XpertMTB/RIF assay and assessment of potential NTM disease in smear-negative patients not responding to TB treatment or in patients with recurrence of respiratory symptoms and structural lung disease. This approach requires specific training to build expertise, good radiography services, and improved access to mycobacterial culture and NTM speciation. Our study highlights the limitations of the 2007 ATS criteria, especially for HIV-infected patients and patients with multiple respiratory infections. Our findings reflect the complex interaction between the NTM exposure–related factors and host-related factors resulting in a spectrum of conditions between colonization and actual disease that can be difficult to differentiate (*4*).

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Risk Factors for *Legionella longbeachae* Legionnaires' Disease, New Zealand

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Learning Objectives

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

- Assess the epidemiology and prognosis of Legionnaires' disease.
- Distinguish the most common chronic illness associated with *Legionella longbeachae* pneumonia.
- Assess risk factors for *L. longbeachae* pneumonia.
- Evaluate potential preventive measures for *L. longbeachae* pneumonia.

CME Editor

Kristina B. Clark, PhD, Copyeditor, Emerging Infectious Diseases. *Disclosure: Kristina B. Clark, PhD, has disclosed the following relevant financial relationships: owns stock, stock options, or bonds from Cooper Companies, Inc.; McKesson Corp; Medtronic; STERIS; Bio-Techne Corp.*

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Legionella longbeachae, found in soil and compost-derived products, is a globally underdiagnosed cause of Legionnaires' disease. We conducted a case–control study of *L. longbeachae* Legionnaires' disease in Canterbury, New Zealand. Case-patients were persons hospitalized with *L. longbeachae* pneumonia, and controls were persons randomly sampled from the electoral roll for the area served by the participating hospital. Among 31 cases and 172 controls, risk factors for Legionnaires' disease were chronic obstructive pulmonary disease, history of smoking ≥10 years, and exposure to compost or potting mix. Gardening behaviors associated with *L. longbeachae* disease included having unwashed hands near the face after exposure

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to or tipping and troweling compost or potting mix. Mask or glove use was not protective among persons exposed to compost-derived products. Precautions against inhaling compost and attention to hand hygiene might effectively prevent *L. longbeachae* disease. Long-term smokers and those with chronic obstructive pulmonary disease should be particularly careful.

Legionnaires' disease is a community-acquired severe pneumonia that causes substantial illness and death (*1*). Of the patients that are hospitalized with the disease, 30% require intensive care unit admission and ≈10% die (*1*,*2*). In most parts of the world, the majority of reported cases are caused by *Legionella pneumophila* (*1*), which sometimes contaminates water (e.g., water from cooling towers) and causes outbreaks. However, Legionnaires' disease is often underdiagnosed because the tests required are not usually performed unless specifically requested. In addition, a substantial diagnostic bias exists in reported cases globally because the most commonly used test, the urinary antigen test, detects only *L. pneumophila* serogroup 1 (*2*).

In 2010, in Christchurch, New Zealand, implementation of an enhanced testing strategy that included PCR testing of respiratory specimens led to a >4-fold increase in the number of detected cases of Legionnaires' disease (*2*). New Zealand has the highest reported incidence of Legionnaires' disease in the world (*1*), and in part because of the more rigorous testing in this country, the epidemiology of detected Legionnaires' disease in New Zealand differs from that of most other countries; a high proportion of cases are caused by *L. longbeachae* (*3*–*5*). *L. longbeachae* can be found in soil and compost-derived products (*6*–*9*). In 2013, a total of 51% of reported Legionnaires' disease cases were caused by *L. longbeachae*, 28% by *L. pneumophila*, and 21% by other *Legionella* species (*3*).

Two small case–control studies on risk factors for *L. longbeachae* pneumonia conducted in South Australia have been published (*10*,*11*). These studies found that preexisting cardiac or respiratory disease, long-term smoking, gardening, exposure to hanging baskets, using potting mix, and eating or drinking after gardening without washing hands were risk factors for this disease. O'Connor et al. suggested that both inhalation and ingestion were potential modes of transmission and advised that long-term smokers and those with respiratory and cardiac conditions should take particular care of their hygiene during and after gardening (*11*).

We conducted a population-based, case–control study to assess the importance of various risk factors for *L. longbeachae* pneumonia hospitalization in a setting with high case ascertainment to build on the South Australia studies and inform public health efforts to prevent Legionnaires' disease. Our main aim was to identify gardening behaviors or types of exposure to potting mix and compost that are associated with the increased risk for Legionnaires' disease. In addition, we assessed several other possible risk factors that could facilitate the introduction of organisms from compost into the lungs.

Methods

Setting

This study was conducted in the region of Canterbury, New Zealand, which has a population of ≈530,000 (*12*). Canterbury contains the city of Christchurch, also known as The Garden City, as well as horticultural and agricultural areas. The Canterbury District Health Board is the publicly funded healthcare provider for the region and includes the local public health unit (Community and Public Health) to which all cases of Legionnaires' disease must be notified. The study was conducted over the course of 2 summers during the peak of Legionnaires' disease activity (October 1, 2013–March 31, 2014 and October 1, 2014–March 31, 2015). Because no cases of Legionnaires' disease had been previously reported in this district in persons <30 years of age, the study population was persons ≥30 years of age who were listed on the electoral roll for the electorates with boundaries within the region served by the Canterbury District Health Board. This project was approved by the University of Otago Human Ethics Committee (H13/065).

Cases

Cases of pneumonia in which the patient was hospitalized and disease onset occurred during peak Legionnaires' season were considered for inclusion into this study. Only confirmed cases that were positive by culture or by PCR for *L. longbeachae* or had a ≥4-fold increase in reciprocal *L. longbeachae* antibody titers were eligible for inclusion (*2*).

Controls

To obtain controls, before each summer, a random sample that was frequency-matched to the expected age distribution of case-patients was taken from the electoral roll. The list of potential controls was randomly sorted and broken into 3 groups; each group was sent an invitation to participate in the study at approximately monthly intervals starting in mid-September in 2013 and at the end of October in 2014. This strategy was used in an effort to interview controls at a rate similar to that of the case-patients. We aimed to recruit 3 controls per case-patient. If no response was received to the first letter, a follow-up letter was sent. If no response was received and the person appeared in the telephone book at the address on the electoral roll, we followed up by phone call.

Data Collection

All notified cases of Legionnaires' disease are followed up by a Health Protection Officer (HPO) from the Public Health Unit. Follow-up usually includes a face-to-face

interview. During the enrollment period, case-patients were invited to take part in our study at these interviews. If they were willing to participate and were on the electoral roll, they were given the usual questionnaire augmented by a structured questionnaire designed specifically for the study that included more detailed questions about possible relevant exposures. For the period of the study, Legionnaires' disease cases were followed up by 1 of the authors (D.S.) or an HPO who had been trained (for consistency purposes) to administer the study questionnaire.

Controls who agreed to take part were telephoned by 1 of the authors (E.K., V.C., or P.S.) or a trained research assistant (an HPO). Those who reported diarrhea, fever, chest pain, or cough lasting >24 hours in the previous 3 weeks were excluded.

The study questionnaire contained questions on demographics, smoking status, and preexisting health conditions. Questions were also asked about garden environment; garden activities; pets; and exposure to soil, compost, and potting mix.

Analysis

We analyzed data using Stata 13 (*13*). All calculations of odds ratios (ORs) used a logistic mixed model that adjusted for age (a continuous variable) and included period (October 1, 2013–March 31, 2014 or October 1, 2014–March 31, 2015) as a random effect. We interpreted ORs as measures of relative risk.

For exposures to purchased compost and potting mix, the products were combined (and referred to as purchased compost products or simply as compost) because their manufacturing processes are similar and because we grouped them together to help maximize precision. We assessed the effects of behaviors that have been encouraged as protective (*14*) (e.g., wearing a mask, wearing gloves, and wetting compost before use) in compost users only. We stratified estimation of the OR of exposure to compost by smoking status to assess whether smoking modifies the effect of exposure to compost.

We identified 2 groups of behaviors that could potentially explain the increased risk for Legionnaires' disease through using compost: behaviors that increase the risk for aerosolizing compost organisms (moving compost around, opening compost bags, and using compost indoors) and behaviors that increase the risk of getting compost organisms near or in the mouth (eating or drinking, smoking, or touching the face after using compost without washing hands). We assumed that both of these types of activities could result in inhalation of *Legionella*, and we conducted a multivariable analysis to assess the relative importance of causal paths that included aerosolization and hand-to-face transfer in explaining the increased risk associated with the use of compost.

We used a causal diagram (*15*) as a guide for multivariable analyses (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/7/16-1429-Techapp1.pdf). Aerosolization of compost and hand-to-face transfer of compost are intermediate factors between the use of compost, which is known to be a risk factor for Legionnaires' disease, and diagnosis of Legionnaires' disease. Our study had insufficient data to precisely estimate these intermediate effects or to definitively determine whether a direct effect of compost remained after adjusting for both aerosolization and hand-to-face activities (which would suggest that other causal pathways in addition to aerosolization or hand-to-face activities exist). However, our assumption was that if the OR associated with compost use reduced to close to 1 in a multiple regression model that included aerosolization and hand-to-face variables, this finding would indicate that we had identified the main causal pathways (*16*).

We did not have enough data to put all the separate relevant variables into a single multivariable model. Our initial intention was to create 1 composite variable indicating experience of any of the aerosolizing activities and 1 variable indicating any of the hand-to-face activities and to include those composite variables in a multivariable model along with compost use. However, all of the case-patients who had used compost in the 3 weeks previous to getting sick had also engaged in ≥1 of the aerosolizing activities, so that composite variable could not be included in the multivariable model. Therefore, we selected 1 of the aerosolization variables as a representative aerosolizing activity as follows. First, we constructed separate models that each included compost use and one of the aerosolizing activities. We then chose the model, and therefore the activity, with the lowest Akaike information criterion (a measure of the relative quality of statistical models) (*17*). This activity was then included in a model with compost use and the composite hand-to-face variable to determine whether there was any direct effect of compost use after adjustment for these 2 mechanisms of exposure. Population attributable fractions, which can be interpreted as the percentage by which disease incidence would be reduced if the risk factor was eliminated, were calculated for these variables from this model (*18*).

Results

Thirty-seven cases of *L. longbeachae* Legionnaires' disease were initially notified to Community and Public Health during the study period (24 in the first summer and 13 in the second). One case was excluded because the patient was not on a Canterbury electoral roll, and 2 patients were interviewed but later excluded from the analysis when *L. longbeachae* infection was not confirmed. Three case-patients declined to participate in the study, yielding an overall response rate of 31 out of 34 (91%) for eligible cases.

Each summer, letters were sent to 301 potential controls (total 602 potential controls) identified from the electoral roll. In total, 214 potential controls (36% of all potential controls and 46% of those who could be contacted) agreed to take part in the study (Figure). Of these, 10 were ineligible, 172 were interviewed, 6 were unable to be interviewed, and 26 were not interviewed for administrative reasons. In the first study period, interviewing stopped after 69 controls had been interviewed (when 3 controls/case was reached), but afterward 2 case-patients who did not have *L. longbeachae* disease were excluded. For the second study period, all controls who agreed to take part were interviewed, except for the 6 whose consent was confirmed too late for the interview to take place.

Demographic Characteristics, Health Conditions, and Smoking

Case-patients were slightly older and more likely than controls to be men (Table 1). The health condition most strongly associated with Legionnaires' disease was chronic obstructive pulmonary disease (COPD; OR 4.2, 95% CI 1.2–14.7) (Table 2). Smoking was associated with an increased risk for Legionnaires' disease, and a dose-dependent response was evident.

Gardening Environment and Pets

All case-patients and all but 4 controls had an outdoor garden on their property, but case-patients were 3 times more likely than controls to have an indoor garden (defined as a glass or tunnel house, hydroponic system, or conservatory) (Table 3). Almost all case-patients had gardened during the 3 weeks before becoming ill, and almost all controls had gardened during the 3 weeks before being interviewed. Using purchased compost products was strongly associated with Legionnaires' disease (OR 6.2, 95% CI 2.2–17.3) and using homemade compost was not. Case-patients were also 3 times as likely as controls to own ≥1 cat.

Behaviors such as moving the hands to the face before handwashing (OR 4.8) and aerosolizing compost (OR 9.9)

Table 1. Demographic characteristics of Legionnaires' disease case-patients and controls, New Zealand, October 1–March 31, 2013–2014 and 2014–2015

Characteristic	Case-patients, no. (%)	Controls, no. (%)
Age, y		
30–39	1 (3)	3 (2)
40–49	4 (13)	8 (5)
50–59	4 (13)	36 (21)
60–69	7 (23)	56 (33)
70–79	10 (32)	49 (28)
≥80	5 (16)	20 (12)
Median age	69	66
Sex		
M	18 (58)	74 (43)
F	13 (42)	98 (57)
Ethnicity*		
European	30 (97)	156 (91)
Māori	1 (3)	6 (3)
Pacific	0	1 (1)
Other	0	8 (5)
Not specified	0	1 (1)
Income		
≤$70,000†	19 (61)	83 (48)
>$70,000	6 (19)	53 (31)
Missing	6 (19)	36 (21)
Possible occupational exposure‡		
Yes	2 (6)	7 (4)
No	28 (90)	165 (96)
Missing	1 (3)	

*If Māori and other ethnicities were ticked, the person's ethnicity was recorded as Māori; if Pacific but not Māori was ticked, the person's ethnicity was recorded as Pacific.
†$70,000 was approximately the reported New Zealand median household income in 2013 (*19*).
‡For case-patients: 1 garden center worker and 1 farm manager. For controls: 4 farmers, 1 soil scientist, 1 gardener, and 1 landscaper.

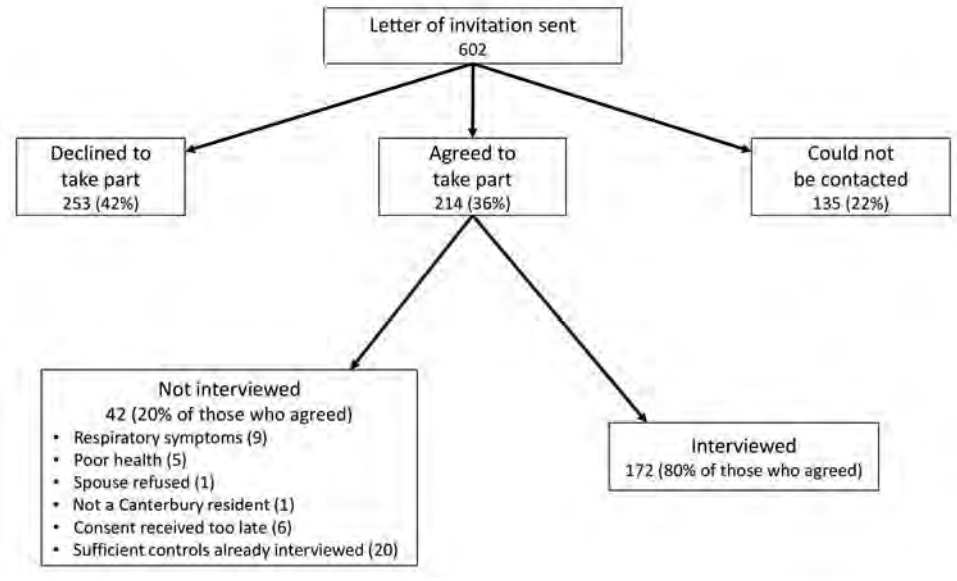

Figure. Flowchart of solicitation and participation of controls for study of *Legionella longbeachae* Legionnaires' disease, New Zealand, October 1–March 31, 2013–2014 and 2014–2015.

Table 2. Univariate analyses of the associations between health conditions or smoking and *Legionella longbeachae* Legionnaires' disease, New Zealand, October 1–March 31, 2013–2014 and 2014–2015*

Health conditions	Case-patients, no. (%)	Controls, no. (%)	OR (95% CI)
Preexisting health conditions			
Cardiac disease	8 (26)	35 (20)	1.2 (0.47–3.0)
Respiratory disease	10 (32)	35 (20)	2.1 (0.87–4.9)
Asthma	8 (26)	28 (16)	1.9 (0.76–4.9)
COPD	5 (16)	9 (5)	4.2 (1.2–14.7)
Diabetes	5 (16)	12 (7)	2.8 (0.88–9.0)
Immunosuppression	6 (19)	16 (9)	2.7 (0.91–7.9)
Smoking			
Current smoker	4 (13)	9 (5)	2.4 (0.66–8.5)
Ever smoked	19 (61)	63 (37)	2.6 (1.2–5.7)
Smoking period†			
Never smoked	12 (39)	109 (65)	1.0
Smoked <20 y	5 (16)	26 (16)	1.6 (0.5–5.1)
Smoked 20 y to <40 y	4 (13)	17 (10)	2.3 (0.6–8.0)
Smoked ≥40 y	10 (32)	15 (9)	5.6 (2.0–16.0)

*COPD, chronic obstructive pulmonary disease; OR, odds ratio.
†Length of time smoking could not be determined for 5 control respondents. Percentages are given for those in which length of time smoking was known.

were associated with the risk for disease (Table 4). None of the behaviors that have been considered possibly protective against Legionnaires' disease were associated with a reduction in disease risk.

Among participants who had smoked for >10 years, those who had used compost had an OR for Legionnaires' disease of 7.9 (95% CI 1.5–43.2); among those who had not smoked or smoked for <10 years but used compost, the OR was 4.6 (95% CI 1.2–17.2). Although these CIs overlap, these results suggest an increased effect of compost use on the risk for Legionnaires' disease in persons who have smoked long-term. The OR for compost users who had smoked for ≥10 years compared with those with neither risk factor was 14.7 (95% CI 3.7–58.4).

In multivariable regression, including compost use and each of the aerosolization variables separately, the model with tipping and troweling compost had the lowest Akaike information criterion (158), and so tipping and troweling was included in a multivariable model with the hand-to-face variable (Table 5). With these 2 variables in the model, the OR for compost use (the direct effect) was 0.97, and the ORs for the indirect effects of compost use through tipping and troweling (6.1) and hand-to-face contact (2.3) were lower than their univariate ORs (8.3 and 4.8, respectively; Table 4) but still raised. CIs were wide, as expected given the small sample size. The population attributable fractions calculated from this multivariable model were 65% for tipping and troweling compost and 30% for making hand-to-face contact before handwashing.

Discussion

Forty years since the first identification of Legionnaires' disease, we still do not have a thorough understanding of how to prevent disease caused by the species *L. longbeachae*. Risk factors for Legionnaires' disease include host characteristics and modifiable behavioral or environmental factors. Most published data have focused on the risk factors for *L. pneumophila* Legionnaires' disease: smoking, older age, chronic

Table 3. Univariate analyses of the associations between garden type, garden exposures, or pets and *Legionella longbeachae* Legionnaires' disease, New Zealand, October 1–March 31, 2013–2014 and 2014–2015*

Environmental factors	Case-patients, no. (%)	Controls, no. (%)	OR (95% CI)
Garden type			
Outdoor garden on property	31 (100)	168 (98)	
Enclosed garden on property†	11 (36)	29 (17)	3.0 (1.3–7.1)
Hanging pots or baskets on property	10 (32)	53 (31)	0.95 (0.41–2.2)
Garden exposures			
Near dripping, hanging pots or baskets	4 (13)	37 (22)	0.55 (0.18–1.7)
Gardened in the past 3 weeks	29 (94)	151 (88)	1.9 (0.42–8.7)
Spent any time gardening outdoors	28 (90)	150 (87)	1.3 (0.36–4.7)
Spent any time gardening indoors‡	10 (32)	54 (31)	1.0 (0.45–2.4)
Used purchased compost	26 (84)	84 (49)	6.2 (2.2–17.3)
Used homemade compost§	7 (23)	40 (22)	1.0 (0.40–2.6)
Pets			
Own dog(s)	7 (23)	40 (23)	0.97 (0.36–2.6)
Own cat(s)	19 (61)	63 (37)	3.0 (1.3–6.8)
Own bird(s)	4 (13)	12 (7)	1.9 (0.55–6.5)

*OR, odds ratio.
†Glasshouse, tunnel house, hydroponics, or conservatory.
‡Including tending to potted plants.
§Irrespective of use of purchased compost.

Table 4. Univariate analyses of the associations between gardening behaviors and *Legionella longbeachae* Legionnaires' disease, New Zealand, October 1–March 31, 2013–2014 and 2014–2015*

Activities performed around the time of gardening	Case-patients, no. (%)	Controls, no. (%)	OR (95% CI)
Hand to face after using compost and before washing hands			
Ate or drank	7 (23)	13 (8)	4.1 (1.4–11.8)
Touched face	12 (39)	31 (18)	3.6 (1.5–8.6)
Smoked	2 (7)	5 (3)	2.2 (0.39–12.0)
Any opportunity for getting hands near face (smoking, eating or drinking, or touching face) before washing hands	16 (52)	36 (21)	4.8 (2.1–11.1)
Possible compost aerosolization			
Opened compost	21 (68)	55 (32)	5.2 (2.2–12.1)
Used compost indoors	7 (23)	9 (5)	6.6 (2.1–20.7)
Tipped or troweled compost	24 (77)	60 (35)	8.3 (3.2–21.5)
Moved compost with hands	15 (48)	52 (30)	2.6 (1.1–5.8)
Purchased compost or moved potting mix around (with hands or by tipping/troweling)	26 (84)	70 (41)	9.9 (3.4–28.3)
Possible protective factors†			
Wore a mask while using compost	5 (19)	12 (14)	1.5 (0.46–4.8)
Wore gloves while handling compost	17 (65)	50 (60)	1.2 (0.49–3.1)
Wet compost down before use	4 (13)	18 (11)	1.6 (0.48–5.4)

*OR, odds ratio.
†Among only those who had used compost in previous 3 weeks.

cardiovascular or respiratory disease, immunosuppression, and exposure to water aerosols (*1*). Data on the risk factors for *L. longbeachae* disease are lacking, beyond the presumed association with compost exposure. Our study highlights some similarities and differences with risk factors for Legionnaires' disease caused by *L. pneumophila.* We found that COPD and smoking are host risk factors for *L. longbeachae* disease. Those who had ever smoked were twice as likely to get *L. longbeachae* disease as those who had never smoked, and the risk was higher for those who had smoked longer. Having COPD, although uncommon (16% of case-patients and 5% of controls), increased disease risk 4-fold.

Use of purchased compost products during the reference period was the strongest exposure risk factor for *L. longbeachae* disease in univariate analysis, and those who had smoked for ≥10 years were more likely than nonsmokers to get Legionnaires' disease after using purchased compost products. The mechanisms by which compost use increases the risk for disease appear to include the activities that increase the likelihood of its aerosolization and activities that could lead to compost being transferred to the face or mouth, presumably leading to inhalation of particles contaminated with *Legionella* bacteria. Our findings suggest that the biggest reduction in *L. longbeachae* disease could be made by eliminating activities that aerosolize compost (population attributable fraction 65%). We also found that hand hygiene might be a useful measure for preventing *L. longbeachae* disease, with the potential to reduce disease by 35%. However, we did not demonstrate a benefit from wearing gloves or masks while gardening.

Our findings are similar to those from the previous studies conducted in South Australia (*10*,*11*) in identifying that having ever smoked, having a history of long-term smoking, recently using compost, and eating or drinking after using compost products before washing hands are risk factors for *L. longbeachae* disease. However, our study did not confirm that preexisting cardiac illness or being near dripping hanging pots or baskets were associated with *L. longbeachae* disease.

We found a previously unidentified association between cat ownership and *L. longbeachae* infection. The association was unaffected by including smoking or use of compost products in the multivariable model (data not shown). However, given the relatively small sample size and potential for a false-positive finding, further investigation of this association is required before any definitive conclusions can be made.

The small sample size was a limitation of this study. Having low numbers of cases resulted in wide CIs for ORs and the limited ability to perform more detailed multivariable analyses. Assessing the effect of different compost handling methods and different compost product exposures on disease risk was not possible. Another limitation was the poor response rate among controls. It is possible that respondents differed from the general population (e.g., a selection for gardeners probably occurred). Because information about the nature of the study provided to participants noted that *L. longbeachae* is found in soil, nongardeners might have perceived that the study investigators were only interested in interviewing gardeners and declined to participate. The lack of nongardeners in the study could cause an underestimation of the effect of gardening-related behaviors on disease risk. On the other hand, recall bias, in which

Table 5. Multivariable analyses of the direct and intermediate effects of types of compost use on *Legionella longbeachae* Legionnaires' disease, New Zealand, October 1–March 31, 2013–2014 and 2014–2015*

Compose use risk factor	OR (95% CI)
Use of compost in previous 3 weeks	0.97 (0.16–5.9)
Tip or trowel compost	6.1 (1.3–29.4)
Hand to face before handwashing	2.3 (0.88–6.1)

*OR, odds ratio.

case-patients overestimate their exposure to risks, could cause overestimation of the effect. The precision of this study did not warrant formal quantitative bias analysis (*20*).

Although we demonstrated no protective effect with use of gloves or masks while gardening, we are reluctant to advise against these practices on the basis of this study alone, especially given the increased disease risk associated with activities that lead to the aerosolization of compost and activities that can lead to the transfer of compost from the hand to the face. Those using face masks and gloves while handling purchased compost products should use single-use disposable masks and wash hands thoroughly after removing gloves but before removing and disposing of the mask; not washing hands before mask removal might be a potential exposure to the face. Smokers (both current smokers and those with a long-term history of smoking) should be made aware that they are at increased risk for Legionnaires' disease and advised to be particularly careful when using purchased compost products.

Transmission of *L. longbeachae* bacteria to humans is still not fully understood, and it is not possible to use compost without moving it around. However, public health messages should encourage persons handling purchased compost products and potting mix to minimize aerosolization (for example, opening bags away from the face and keeping the compost close to the ground when moving it) and to keep hands away from the face until they have been thoroughly washed. Larger studies to assess the risk associated with particular compost-associated behaviors more fully could better inform prevention strategies.

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Environmental Factors as Key Determinants for Visceral Leishmaniasis in Solid Organ Transplant Recipients, Madrid, Spain

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During a visceral leishmaniasis outbreak in an area of Madrid, Spain, the incidence of disease among solid organ transplant recipients was 10.3% (7/68). Being a black person from sub-Saharan Africa, undergoing transplantation during the outbreak, and residing <1,000 m from the epidemic focus were risk factors for posttransplant visceral leishmaniasis.

Visceral leishmaniasis (VL) is an uncommon but potentially fatal complication for solid organ transplant (SOT) recipients (*1*,*2*). Beginning in July 2009, an outbreak of leishmaniasis affected the southwest area of Madrid (*3*). The outbreak was primarily located in Fuenlabrada, which has an annual VL incidence of 21.1 cases/100,000 population (*4*), notably higher than that estimated for the general population in Spain (0.5 cases/100,000 population) (*5*).

Spatial analysis revealed that the highest concentration of cases was in the residential area bordering the park (Bosque Sur) (*6*). A large population of *Lepus granatensis* hares, which serve as a reservoir for *Leishmania infantum*, was present in the area (*7*,*8*), and the *Phlebotomus perniciosus* sand fly in Spain can act as a vector and take blood meals from these hares (*6*,*8*,*9*). Thus, the parkland facilitated the transmission of the leishmaniasis pathogen, which led to the outbreak. This large, urban outbreak provided us the opportunity to analyze the incidence and specific risk factors of VL among SOT recipients.

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The Study

The University Hospital 12 de Octubre in Madrid, Spain, acts as the reference hospital for SOT in South Madrid. We performed a retrospective study of all consecutive adult patients who underwent kidney, liver, or heart transplantation during January 1, 2005–January 1, 2013, and lived in the outbreak area. Patients who underwent SOT before January 1, 2005, were excluded because of the difficulty of ensuing long-term follow-up and the potential of heterogeneity in posttransplant practices. Patients who died or had moved to a different place of residence before outbreak onset were excluded (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/7/15-1251-Techapp1.pdf).

The primary study outcome was the occurrence of VL, the diagnosis of which required confirmation of parasitemia (online Technical Appendix) (*10*). We recorded pretransplant, peritransplant and posttransplant variables and collected various environmental factors prospectively by unblinded, direct interview with the patients. Patients were considered to have frequent contact with dogs if patients reported having dogs at home or taking care of dogs and to have the habit of visiting the park if they reported visiting once a year. The distance between the place of residence and the park was obtained by locating the patient's home address and measuring the shortest linear distance to the nearest border of the parkland by means of an online mapping tool (Google Maps; Google Inc., Mountain View, CA, USA).

The beginning of the exposure period was set as July 2009 (outbreak onset) for patients who underwent SOT before the outbreak and as the transplant date for those who underwent SOT after outbreak onset. In both cases, the exposure period extended to the date of diagnosis of VL, death, or December 2013. We chose to end the study in December 2013 because the incidence of leishmaniasis decreased thereafter because of the implementation of control measures. The clinical research ethics committee of the University Hospital 12 de Octubre approved the study, and participants provided informed consent.

We analyzed 68 patients (Table 1) for a median follow-up of 4.4 (interquartile range 2.39–6.95) years. VL was diagnosed in 7 patients, yielding a cumulative incidence of 10.3% (95% CI 3.1%–17.5%) and an annual incidence of 2,997 (95% CI 1,213–6,161) cases per 100,000 population. Details on disease pathology and therapy were recorded

Table 1. Baseline and clinical characteristics of solid organ transplant recipients in study of risk factors for VL, Madrid, Spain, January 1, 2005–January 1, 2013*

Characteristics	Overall cohort, n = 68	VL, n = 7	No VL, n = 61	p value†
Recipient age, y, mean ± SD	51.1 ± 14.2	53.0 ± 15.5	51.0 ± 13.5	0.721
Male sex, no. (%)	48 (70.6)	6 (85.7)	42 (68.9)	0.664
Race, no. (%)				
White	62 (91.2)	5 (71.4)	57 (93.4)	0.112
Black, sub-Saharan African	4 (5.9)	2 (28.6)‡	2 (3.3)	**0.049**
Other	2 (2.9)	0 (0)	2 (3.3)	1.000
Type of SOT, no. (%)				
Kidney	57 (83.8)	6 (85.7)	51 (83.6)	1.000
Liver	8 (11.8)	0 (0)	8 (13.1)	0.587
Heart	3 (4.4)	1 (14.3)	2 (3.3)	0.282
Donor age, y, mean ± SD	46.1 ± 16.2	49.4 ± 17.4	46.3 ± 16.3	0.596
Cold ischemia time, min, median (IQR)	1,005 (630–1,354)	795 (371–1,365)	1,020 (660–1,360)	0.370
No. HLA mismatches, mean ± SD	4.0 ± 1.2	5.0 ± 1.0	4.0 ± 1.2	0.265
DCD donor, no. (%)	18 (26.5)	3 (42.8)	15 (24.6)	0.370
Transplant during the outbreak, no. (%)	41 (60.3)	7 (100.0)	34 (55.7)	**0.037**
Induction therapy, no. (%)				
Basiliximab	22 (32.4)	0 (0)	22 (36.1)	0.087
Antithymocyte globulin	24 (35.3)	4 (57.1)	20 (32.8)	0.233
None	22 (32.4)	3 (42.8)	19 (31.1)	0.673
Maintenance immunosuppression, no. (%)				
Steroids	56 (82.4)	7 (100.0)	49 (80.3)	0.338
Calcineurin inhibitors	60 (88.2)	6 (85.7)	54 (88.5)	1.000
Mycophenolate mofetil/mycophenolic acid	47 (61.8)	4 (57.1)	43 (70.5)	0.668
mTOR inhibitors	10 (14.7)	1 (14.3)	9 (14.8)	1.000
Complications in the first year after SOT, no. (%)				
Acute graft rejection	19 (27.9)	2 (28.6)	17 (27.9)	1.000
CMV infection	21 (30.9)	4 (57.1)	17 (27.9)	0.189
Bacterial infection	60 (88.2)	6 (85.7)	54 (88.5)	0.190
Environmental factors				
Frequent contact with dogs, no. (%)	26 (38.2)	3 (42.8)	23 (37.7)	1.000
Habit of visiting the park, no. (%)§	19 (31.7)	3 (50.0)	16 (29.6)	0.369
Distance from patient's residence to park, m, median (IQR)	1,220 (849–1,865)	399 (261–985)	1,370 (974–1,880)	**0.001**

*CMV, cytomegalovirus; DCD, donation after circulatory death; HLA, human leukocyte antigen; IQR, interquartile range; mTOR, mammalian target of rapamycin; SOT, solid organ transplant; VL, visceral leishmaniasis.
†The p values refer to the comparison between patients with and without visceral leishmaniasis. Significant values are in bold.
‡The country of birth of both patients with posttransplant VL was Equatorial Guinea.
§Data available for 60 patients.

(Table 2). The mean interval between transplant and diagnosis was 1.34 ± 0.89 years. No patients had visited highly VL-endemic countries.

Black sub-Saharan African SOT recipients were more likely than other recipients to become affected by VL (relative risk 6.40, 95% CI 1.76–23.29, p = 0.049) (Table 1). All 7 episodes of VL occurred in patients who underwent transplantation during the outbreak period (Figure 1).

The median distance between the place of residence and the park was significantly shorter for recipients with VL (399 m) than for those without (1,370 m; p = 0.001) (Figure 2; online Technical Appendix Figure 2). We explored the predictive accuracy of this variable by establishing the optimal cutoff value with the area under the receiving operating characteristic curve analysis. Recipients living <1,000 m from the park (26.1%, 6/23) had a higher incidence of VL than recipients living ≥1,000 m away (2.2%, 1/45; relative risk, 11.74, 95% CI 1.50–91.78; p = 0.005). At 4 years, a lower percentage of the SOT recipients living <1,000 m from the park were free from VL than those living ≥1,000 m away (61.0% vs 98%; p = 0.001 by log-rank test) (online Technical Appendix Figure 3).

Our study suggests that the incidence of VL in SOT recipients is notably higher than that in the general population (*11*). Acquisition of the parasite most likely occurred posttransplant because all but 1 recipient affected with VL (from whom serum samples could be recovered) were seronegative for *Leishmania* spp. before transplantation.

Our findings suggest that environmental factors might be crucial in modulating the incidence of VL in immunocompromised hosts, such as SOT recipients; the distance from the patient's residence to the focus of the outbreak (*6,7*) emerged as a key risk factor. The median distance between the park and the homes of recipients with posttransplant VL was <500 m; the maximum flight distance of female sand flies is 600 m (*12,13*). Therefore, persons living within this radius had a higher chance of being bitten by the VL vector. A similar association was described for the general population during this outbreak (*6*).

Table 2. Disease characteristics, demographics, clinical characteristics, therapy, and outcomes of 7 solid organ transplant recipients with VL, Madrid, Spain, January 1, 2005–January 1, 2013*

Characteristics	Patient no.						
	1	2	3	4	5	6	7
Sex	M	M	M	M	M	F	M
Race	Black	Black	White	White	White	White	White
Linear distance from patient's residence to park, m	794	399	261	1,240	985	233	358
Age at transplant, y	35	34	76	55	68	49	52
Type of SOT	Kidney	Kidney	Kidney	Kidney	Kidney	Heart	Kidney
Donor *Leishmania* spp. serostatus	Negative	NP	Negative	NP	Negative	Negative	Negative
Pretransplant recipient *Leishmania* spp. serostatus	NP	NP	Negative	Positive	Negative	Negative	Negative
Date of transplant	2011 Feb 11	2010 Jan 22	2010 Mar 10	2010 Jul 7	2009 Dec 29	2010 Sep 5	2010 Apr 15
Interval from transplant to VL diagnosis, y	1.17	2.44	0.25	1.4	0.17	1.81	2.21
Fever at admission	Yes	Yes	No	Yes	Yes	Yes	Yes
Pancytopenia	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Splenomegaly	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Serologic testing results for *Leishmania* spp.	Positive	Negative	Negative	Positive	Negative	Positive	Negative
Presence of amastigote forms in bone marrow sample	Positive	Positive	Positive	Positive	Positive	Positive	Positive
PCR assay results of bone marrow sample	NP	NP	NP	Negative	NP	NP	NP
NNN culture results of bone marrow sample	Positive	NP	Positive	Negative	Negative	Positive	Negative
Initial therapy	L-AmB	L-AmB	L-AmB	L-AmB	L-AmB	L-AmB	L-AmB
Relapse	Yes	No	Yes	Yes	No	No	No
Outcome	Renal failure	Graft loss	Cured	Graft loss	Cured	Cured	Cured

*L-AmB, liposomal amphotericin B; NNN, Novy-McNeal-Nicolle; NP, not performed; SOT, solid organ transplant; VL, visceral leishmaniasis.

Undergoing transplantation during the outbreak period was another risk factor for VL. This finding suggests that, in the case of an outbreak in a country with low baseline incidence, pretransplant screening of patients listed for SOT for VL-specific antibodies should be considered and repeated during the posttransplant period for the prompt detection of de novo infection. Recipients should also receive specific counseling to reduce the risk of being bitten by sand flies. In addition, treating physicians must maintain a low threshold of suspicion for VL for persons on immunosuppressive therapy during a VL outbreak.

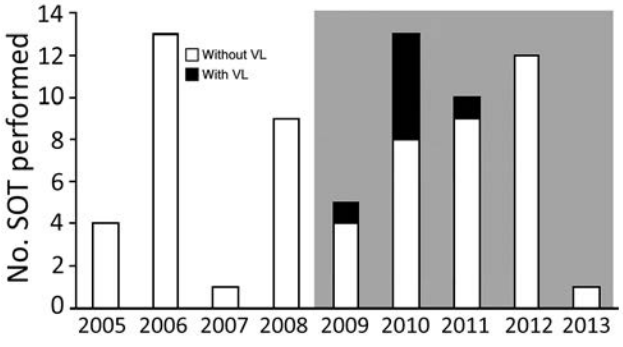

Figure 1. Distribution of VL among solid organ transplant recipients, Madrid, Spain, January 1, 2005–January 1, 2013. Columns represent the number of solid organ transplant procedures performed each year at the University Hospital 12 de Octubre among patients permanently residing in Fuenlabrada, the nearby city affected by the outbreak. Gray shading indicates outbreak period. SOT, solid organ transplant; VL, visceral leishmaniasis.

We found that 28% of posttransplant VL cases occurred in black recipients born in sub-Saharan Africa, even though this subgroup only represented 2.4% of the overall population in the affected area (*14*). An association between sub-Saharan African ethnicity and VL has also been reported in the general population (*4*). No apparent relationship was found between the race of the patient and the frequency of parkland visits. Both black recipients in question came from Equatorial Guinea, a country not considered endemic for leishmaniasis by the World Health Organization (*15*). Therefore, the potential association between genetic susceptibility and posttransplant VL warrants further investigation.

Limitations of this study include the small sample size and that interviewers were not blinded to the diagnosis of VL. However, the objective nature of the questionnaire minimized the potential risk for bias. When assessing degree of exposure to sand flies, we used only indirect variables (i.e., distance between the patient's residence and park, habit of visiting the park) as surrogate measures. Regarding the distance from the park, only linear distances were assessed without considering the potential presence of physical obstacles in the sand fly flight trajectory. Because of these limitations, our findings must be interpreted with caution.

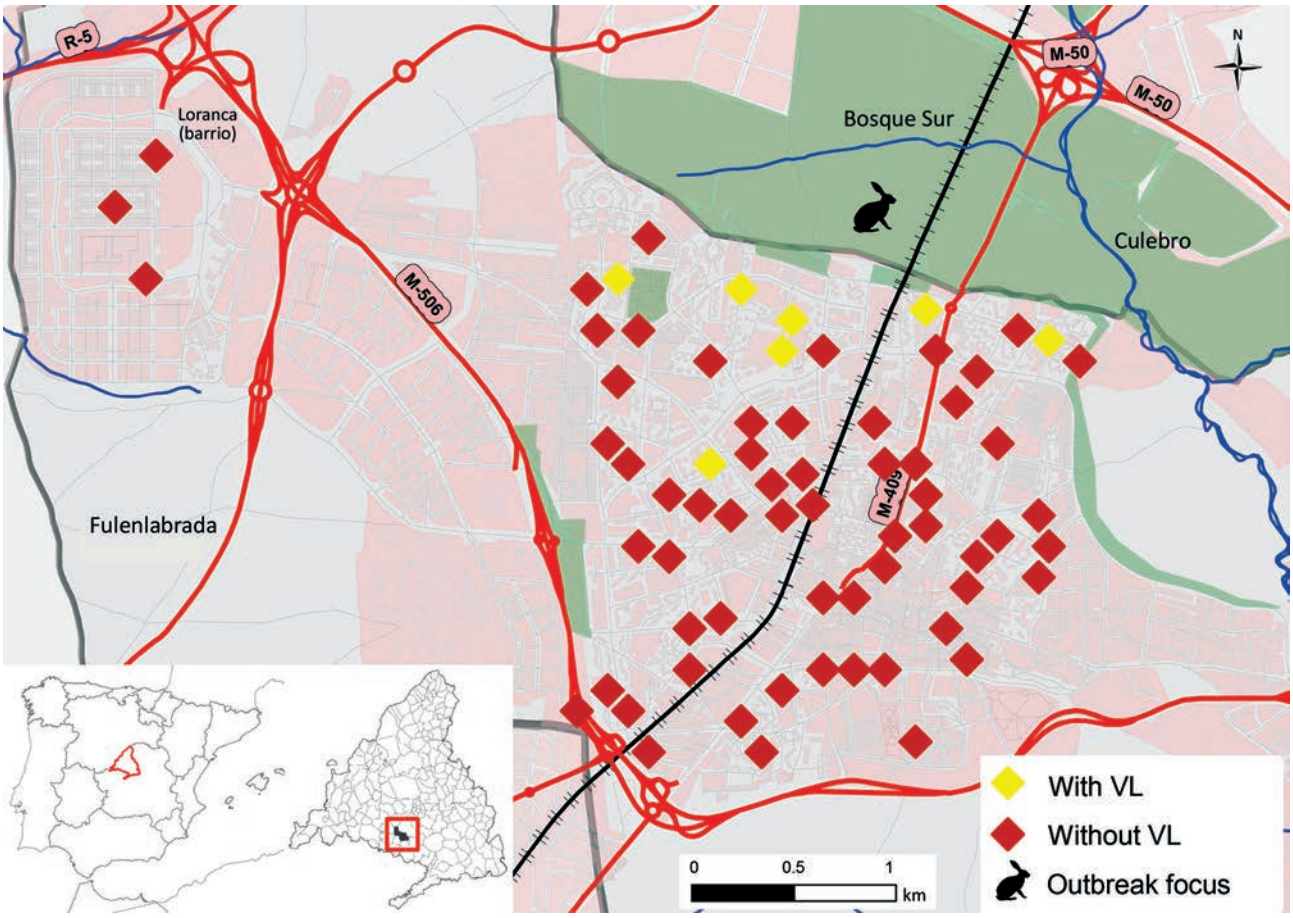

Figure 2. Spatial distribution of solid organ transplant recipients in the southwest area of Madrid, Spain, in relation to park that was focus of visceral leishmaniasis (VL) outbreak, January 1, 2005–January 1, 2013. Map inset shows the location of the outbreak in relation to the rest of Spain. VL, visceral leishmaniasis.

Conclusions

Our study indicates several risk factors (being black and from sub-Saharan Africa, having an SOT during the outbreak, and living <1,000 m from the outbreak focus) useful for helping physicians treat SOT recipients during a VL outbreak. Doctors should select the patients with these risk factors for counseling to minimize their exposure to vectors and active monitoring for prompt diagnosis.

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Locally Acquired *mcr-1* in *Escherichia coli*, Australia, 2011 and 2013

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We identified discrete importation events of the *mcr-1* gene on incompatibility group IncI2 plasmids in *Escherichia coli* isolated from patients in New South Wales, Australia, in 2011 and 2013. *mcr-1* is present in a small minority of colistin-resistant Enterobacteriaceae and appears not to be established locally.

The *mcr-1* (mobile colistin resistance) gene was discovered in *Escherichia coli* isolates collected during 2011–2014 from animals and meat products and from *Klebsiella pneumoniae* from human patients in China (*1*). Since then, *mcr-1* has been identified on plasmids of various incompatibility (Inc) types associated with 0, 1, or 2 copies of the insertion sequence (IS) IS*Apl1* (*2*), and it has been identified in other species (most notably *Salmonella* spp.) and in several other countries (*3*), not including Australia. We analyzed colistin resistance in *Enterobacteriaceae* isolates collected in Sydney, New South Wales, Australia, during 2007–2016.

The Study

We reviewed 4,555 isolates of the family *Enterobacteriaceae* from 2007–2016 that were available for further testing for colistin (polymyxin E) resistance, excluding species that are intrinsically resistant. These isolates were from specimens tested at or referred to the Centre for Infectious Diseases Microbiology Laboratory Services at Westmead Hospital, Sydney, New South Wales, Australia, and were all resistant to third-generation cephalosporins, carbapenems, or both. For antimicrobial drug susceptibility testing, we used the Phoenix Automated Microbiology System (panels NMIC/ID-80, NMIC/ID-101, and NMIC-203; Becton Dickinson, Franklin Lakes, NJ, USA). Of the 4,555 isolates, 96 (2.1%) had a colistin (polymyxin E) minimum inhibitory concentration (MIC) of ≥2 μg/mL, which corresponds to EUCAST (European Committee on Antimicrobial Susceptibility Testing) breakpoints (http://www.eucast.org/clinical_breakpoints/). The 96 colistin-resistant isolates consisted of 44 *K. pneumoniae*, 8 *K. oxytoca*, 18 *E. coli*, 19 *Enterobacter* spp., 5 *Hafnia alvei*, and 2 *Citrobacter freundii* isolates. By using published primers (*1*), we identified *mcr-1* in 2 of the colistin-resistant *E. coli* isolates; these isolates were from patients in different cities in New South Wales.

In September 2011, *E. coli* JIE2288 (colistin MIC of 4 μg/mL) was isolated from the urine of a 70-year-old woman after she had been in the intensive care unit (ICU) at Westmead Hospital, a large metropolitan hospital, for 2 months for management of a subarachnoid hemorrhage. The woman was not administered colistin/polymyxin antimicrobial drugs during hospitalization. The *E. coli* JIE2288 isolate was also resistant to amikacin, gentamicin, tobramycin, amoxicillin/clavulanic acid, cefotaxime, ceftazidime, cefepime, cefoxitin, trimethoprim/sulfamethoxazole, and ciprofloxacin, according to EUCAST breakpoints. The isolate was susceptible to piperacillin/tazobactam (MIC of <4/4 μg/mL) and meropenem (MIC of <1 μg/mL). The patient had no history of overseas travel in the previous 5 years, and while she was in the ICU, no other ICU patients had a recognized colistin-resistant infection and none were receiving colistin treatment. The patient was housed in a single room, and standard infection-control precautions (disposable gown and gloves) were used. The patient was treated with a course of meropenem, and subsequent cultures of her urine did not contain colistin-resistant *E. coli*.

In July 2013, a second colistin-resistant *E. coli* strain (herein designated JIE3685; colistin MIC of >4 μg/mL) was referred to our laboratory after being isolated from the urine of a 71-year-old woman with diabetes mellitus. The woman had sought care from a community physician in Newcastle, New South Wales, for a urinary tract infection. She had had no healthcare contact in the previous 2 years and no history of travel outside Australia in the previous 5 years. JIE3685 was also resistant to cefotaxime, cefepime, ciprofloxacin, gentamicin, tobramycin, and trimethoprim/

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sulfamethoxazole, but it was susceptible to amoxicillin/clavulanic acid, ceftazidime, piperacillin/tazobactam, meropenem, and amikacin.

We obtained colistin-resistant transconjugants from *E. coli* JIE2288 and JIE3685 essentially as previously described (*4*) and, using PCR, confirmed that they carried *mcr-1*. We subjected DNA prepared from the original JIE2288 and JIE3685 isolates and transconjugants to paired-end sequencing (NextSeq 500 platform; Illumina, San Diego, CA, USA) and assembled the sequences by using SPAdes 3.7.1 (http://bioinf.spbau.ru/spades). Using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/), we identified IncI2 replicons in both isolates, and using the MLST tool on the Centre for Genomic Epidemiology website (https://cge.cbs.dtu.dk/services/MLST/), we identified JIE2288 as sequence type (ST) 167 (clonal complex [CC] 10) and JIE3865 as ST93 (CC168). Other ST167 isolates carrying *mcr-1* have been reported; for example, *mcr-1* was carried on a ≈65-kb IncI2 plasmid and the $bla_{\text{NDM-9}}$ gene on a different plasmid in an isolate from chicken meat in China in 2014 (*5*). Furthermore, ST93 (CC168) is a known zoonotic pathogenic strain previously associated with transmission of *mcr-1* from animals to humans in Laos (*6*) and China (*7*).

Most of the IncI2 plasmid from *E. coli* JIE2288 that carries *mcr-1* (designated pJIE2288-1) was assembled as a single contig, missing only the shufflon region that rearranges to change the end of the *pilV* gene (*8*), causing assembly issues that may result in apparent differences between plasmids carrying *mcr-1* (*9*). We obtained the shufflon segments of pJIE2288-1 by mapping raw reads to individual shufflon segments from the archetypal IncI2 plasmid R721 (GenBank accession no. AP002527) and then assembled them in the same order as in R721 to generate a complete plasmid sequence (*8*). An additional plasmid, carrying the $bla_{\text{CTX-M-14a}}$ gene (IncI1, designated pJIE2288-2), was identified in JIE2288. BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) searches with 2 contigs from JIE3685 identified several closely matching plasmids (Table). We used pEC5-1, which had been sequenced by long-read methods, as a reference to resolve a repeated region and as a template for the shufflon, thus enabling complete assembly of a plasmid designated pJIE3685-1. We submitted the sequences of pJIE2288-1 and pJIE3685-1 to GenBank under accession numbers KY795977 and KY795978, respectively.

pJIE2288-1 and pJIE3685-1 each carry *mcr-1* as the only resistance gene (located in the same position), and both lack IS*Apl1*, but they do have differences in the remainder of their IncI2 backbones. pJIE2288-1 (60.733 kb) is closely related to 9 other sequenced *mcr-1* plasmids (differing by only a few single nucleotide changes in backbone regions), some of which carry IS*Apl1* upstream of *mcr-1*, another IS, or both (Table). pJIE3685-1 (60.960 kb) is closely related to 6 other IncI2 *mcr-1* plasmids (again, differing by only a few single nucleotide changes), all of which also lack IS*Apl1* (Table).

We noted differences between pJIE2288-1 and pJIE3685-1 in the *mcr-1* promoter (*11*) and putative ribosome binding site and these or other differences in this region in available *mcr-1* sequences (Figure). We examined the chromosomal *arnBCADTEF*, *pmrAB,* and *phoPQ* genes of each isolate but did not identify any of the changes previously reported in association with colistin resistance in *E. coli* (*12*). This finding suggests that *mcr-1* may be the sole phenotype determinant in these 2 strains.

Table. IncI2 plasmids that carry *mcr-1* and are closely related to pJIE2288-1 or pJIE3685-1 from *Escherichia coli* isolates from separate patients in New South Wales, Australia, 2011 and 2013*

Study plasmid, related plasmids	GenBank accession no.	Genus and species	Source	Country	Year	Insertion sequence
pJIE2288-1	KY795977	*E. coli*	Human urine	Australia	2011	–
pEG430-1	LT174530	*Shigella sonnei*	Human feces	Vietnam	2008	*Apl1*
pHSSH22-MCR1	KX856067	*Salmonella* Typhimurium	Human feces	China	2012	
pHSSH23-MCR1	KX856068	*Salmonella* Enteritidis	Human feces	China	2012	*Apl1*
pHNSHP45	KP347127	*E. coli*	Pig farm	China	2013	*Apl1*, *683*
pHeNE867†	KU934208	*E. coli*	Chicken	China	2013	–
pABC149-MCR-1	KX013538	*E. coli*	Human blood	UAE	2013	*Apl1*
pEc_04HAE12	KX592672	*E. coli*	Human blood	China	2014	*1*
pR150626	KY120366	*Salmonella* Typhimurium	Human	Taiwan		–
pEC006	KY471144	*E. coli*		South Korea‡		*150*
pJIE3685-1	KY795978	*E. coli*	Human urine	Australia	2011	–
pEC5-1	CP016185	*E. coli*	Chicken liver	Malaysia	2013	*1*
pEC13-1	CP016186	*E. coli*	Pond water	Malaysia	2013	–
pS2.14-2	CP016187	*E. coli*	Chicken feed	Malaysia	2013	–
pEc_20COE13§	KY012274	*E. coli*	Human blood	China	2014	–
pBA77-MCR-1	KX013539	*E. coli*	Human urine	Bahrain	2015	*1294*
pP111	KY120365	*Salmonella* Typhimurium	Pig	Taiwan		–

*UAE, United Arab Emirates; –,no known insertion sequences were identified. Blank cells indicate that the information is not available.
†Carries *mcr-1.3* variant (AA111–2GG) encoding MCR-1.3 with a single (Ile38Val) change from MCR-1 (*10*).
‡This information is assumed from a GenBank entry rather than from a published report.
§This plasmid has many missing or extra nucleotides in homopolymeric regions compared with all other related plasmids.

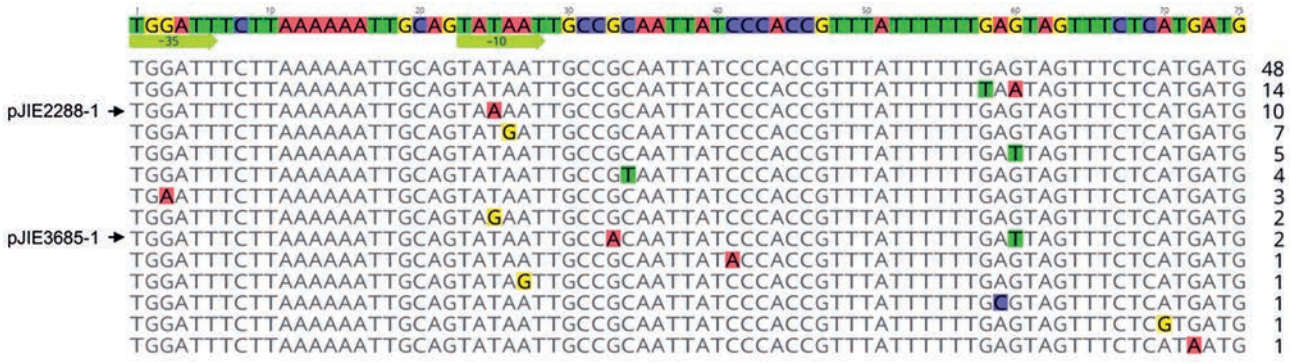

Figure. Differences in promoter and ribosome binding site regions of *mcr-1* in plasmids from *Escherichia coli* in Australia (indicated by arrows) and in other sequences available from GenBank. The sequences end with the ATG start codon of *mcr-1* and a second ATG codon that follows it. The –35 and –10 regions of the proposed promoter (*11*) are indicated by arrows. The numbers to the right indicate how many times each variant has been seen among available sequences.

Conclusions

We describe 2 *E. coli* isolates collected from patients in different cities in Australia ≈2 years apart that belong to STs previously associated with *mcr-1* and which carry this gene on plasmids that are virtually identical to previously described *mcr-1*–bearing plasmids from Asia and the Middle East. Multiple importations of resistant isolates into the community microflora are to be expected, and these 2 case-patients in Australia had no history of travel outside Australia or direct links to each other. Australia's regional neighbors use colistin extensively in agriculture (*13*), but in Australia, its usage in agriculture and healthcare is minimal, and neither colistin nor polymyxin drugs appear in the top 20 antimicrobial drugs prescribed in Australia (*14*). Notwithstanding the limitations of this opportunistic study, our findings indicate that colistin resistance is unusual among Enterobacteriaceae in Australia and that *mcr-1* is neither a key mechanism nor yet widely disseminated in this country despite multiple importation events.

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Postmortem Findings for 7 Neonates with Congenital Zika Virus Infection

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Postmortem examination of 7 neonates with congenital Zika virus infection in Brazil revealed microcephaly, ventriculomegaly, dystrophic calcifications, and severe cortical neuronal depletion in all and arthrogryposis in 6. Other findings were leptomeningeal and brain parenchymal inflammation and pulmonary hypoplasia and lymphocytic infiltration in liver and lungs. Findings confirmed virus neurotropism and multiple organ infection.

From the discovery of Zika virus in Uganda in 1947 through 2007, when an outbreak occurred on Yap Island, Micronesia, only sporadic cases of human infection had been reported (*1*). In early 2015, the virus emerged in Brazil (*2*). Its role in a major public health crisis became apparent when links between Zika virus infection and microcephaly and Guillain-Barré syndrome were established (*1*). Cases of microcephaly associated with Zika virus have been well documented (*3*–*7*). We report a case series of postmortem findings that strengthen this association.

The Study

Postmortem examinations were performed on 7 neonates from the state of Ceará, in northeastern Brazil. Their mothers most likely contracted Zika virus infection during the first trimester of pregnancy in early 2015. Six of the families lived in small towns far from the capital of Fortaleza, suggesting that Zika virus was widespread in Ceará at that time.

During November 2015–February 2016, the 7 autopsies were performed by the Service for Ascertaining Death (Ceará, Brazil). Real-time reverse transcription PCR (RT-PCR) of cerebrospinal fluid and tissue, performed at the Central Public Health Laboratory (Ceará), confirmed congenital Zika virus infection (*8*) (Table). We reviewed medical charts from live patients and their mothers; autopsy reports; and histopathologic reports from 4 pathologists who reviewed the hematoxylin and eosin–stained slides of brain, cerebellum, lung, heart, liver, spleen, kidney, and bladder. Samples were also tested for dengue virus by real-time RT-PCR and for dengue virus nonstructural protein 1 and IgM (*9*,*10*) (Table). After consent was obtained from families, the autopsies were performed as routine cause-of-death investigations.

Of the 7 mothers, all were HIV negative and 5 had had symptoms compatible with viral infection during the first trimester of pregnancy. Neonate survival times ranged from 30 minutes to 6 days after birth; 5 survived <1 hour, 1 died 48 hours after birth, and 1 survived for 6 days. Four neonates were male; for 1 of these in whom the genitalia were ambiguous (neonate 2), the sex was ascertained by identification of undescended testes at autopsy. Gestational ages ranged from 30 to 42 weeks (median 37 weeks) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/16-2019-Techapp1.pdf).

Brain weight was decreased in all neonates; however, body weight was within reference range for gestational age in all except neonate 6 (online Technical Appendix). For 6 neonates, microcephaly was present (Figure 1, panels A, B). One neonate for whom head circumference was within reference limits had morphologic changes typical of microcephaly (Figure 1, panels C, D). Cerebellar hypoplasia was present in neonates 1 and 2 (Figure 1, panel E) and pachygyria in neonate 3 (Figure 1, panel F). Ventriculomegaly was present in all neonates (Figure 1, panels G, H) and arthrogryposis in 6 (Figure 1, panels A, B).

All infants had thinning of the brain parenchyma with severe depletion of neuronal precursors (Figure 2, panel A). In some areas of the brain, the distance from the meningeal membranes to the ependymal epithelium was 3.0 mm (Figure 2, panel A); in 1 neonate, it measured only 0.8 mm (Figure 2, panel B).

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Table. Results of tests for Zika virus in CSF and organs and for dengue virus in brain and CSF for 7 neonates who died of congenital Zika virus infection, Brazil*

Neonate no.	Zika virus rRT-PCR							DENV			
								Brain rRT-PCR	CSF		
	CSF	Brain	Lung	Heart	Liver	Spleen	Kidney		rRT-PCR	IgM†	NS1‡
1	P	P	P	P	N	N	P	ND	N	N	N
2	P	P	N	P	P	P	P	ND	ND	N	N
3	P	P	P	P	P	P	P	ND	ND	N	N
4	P	P	N	N	N	N	N	N	DENV-1	P	P
5	P	P	P	P	P	P	P	N	N	N	N
6	P	ND	ND	ND	ND	ND	ND	N	N	N	N
7	P	ND	ND	ND	ND	ND	ND	ND	N	N	N

*CSF, cerebrospinal fluid; DENV, dengue virus; N, negative; ND, not done; NS1, nonstructural protein 1 (dengue virus antigen test); P, positive.
†By antigen capture assay.
‡By ELISA.

Lymphocytic inflammation was observed in the meninges of 5 neonates (Figure 2, panels B, C) and in the brain parenchyma of 5; for 3 neonates, inflammatory processes were found in both areas. Areas of inflammation varied in intensity and distribution, but periventricular and perivascular cuffing were common (Figure 2, panel D). There was moderate to severe vascular congestion in 6 neonates (Figure 1, panels E, F; Figure 2, panel E). Macroscopic calcification was common (Figure 1, panel G). Three patterns of dystrophic calcification were identified microscopically: individual neuronal mineralization, a fine granular pattern (Figure 2, panel F), and a more coarse dystrophic pattern (Figure 2, panels A, E, G). Coarse calcification in a band-like form (Figure 2, panels A, G) was seen mainly at the junction of gray and white matter and in periventricular areas in association with the inflammatory process. Dystrophic calcification with neuronal mineralization was seen in all neonates. A variable degree of gliosis was present in all neonates, affecting predominantly white matter. Red neurons, neurons with increased cytoplasmic eosinophilia (reflecting acute neuronal injury), were seen in 6 neonates (Figure 2, panel H), and apoptosis was seen in 5 (online

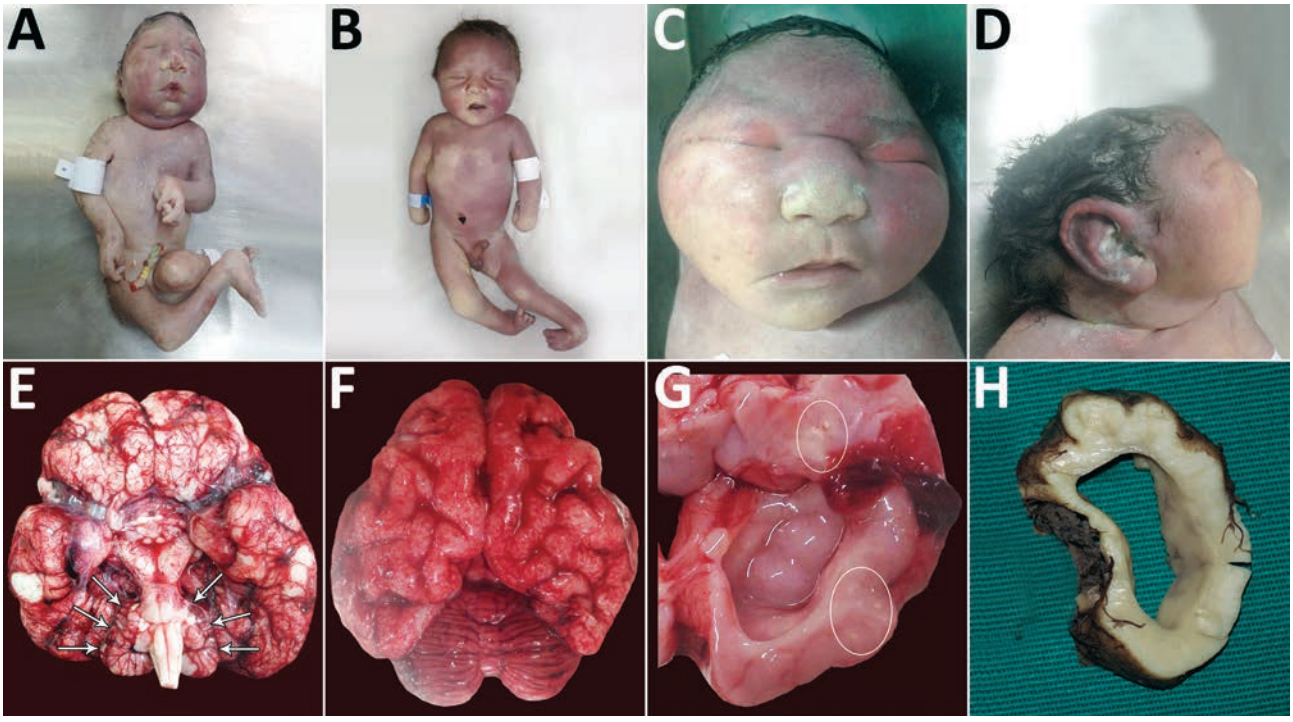

Figure 1. Physical signs in 4 of 7 neonates who died of congenital Zika virus infection, Brazil. A) Neonate 1: typical microcephaly phenotype; arthrogryposis in upper and lower limbs. B) Neonate 7: microcephaly without the typical microcephaly phenotype; arthrogryposis is also present. C) Neonate 3: typical microcephaly phenotype, with head circumference within reference limits, frontal view. D) Neonate 3: typical microcephaly phenotype, with head circumference within reference limits, profile view. E) Brain of neonate 2: symmetric cerebellar hypoplasia (arrows) and vascular congestion. F) Brain of neonate 3: pachygyria and severe vascular congestion. G) Brain of neonate 3: ventriculomegaly and macroscopic calcifications (circles). H) Brain of neonate 7: cross-section showing ventriculomegaly.

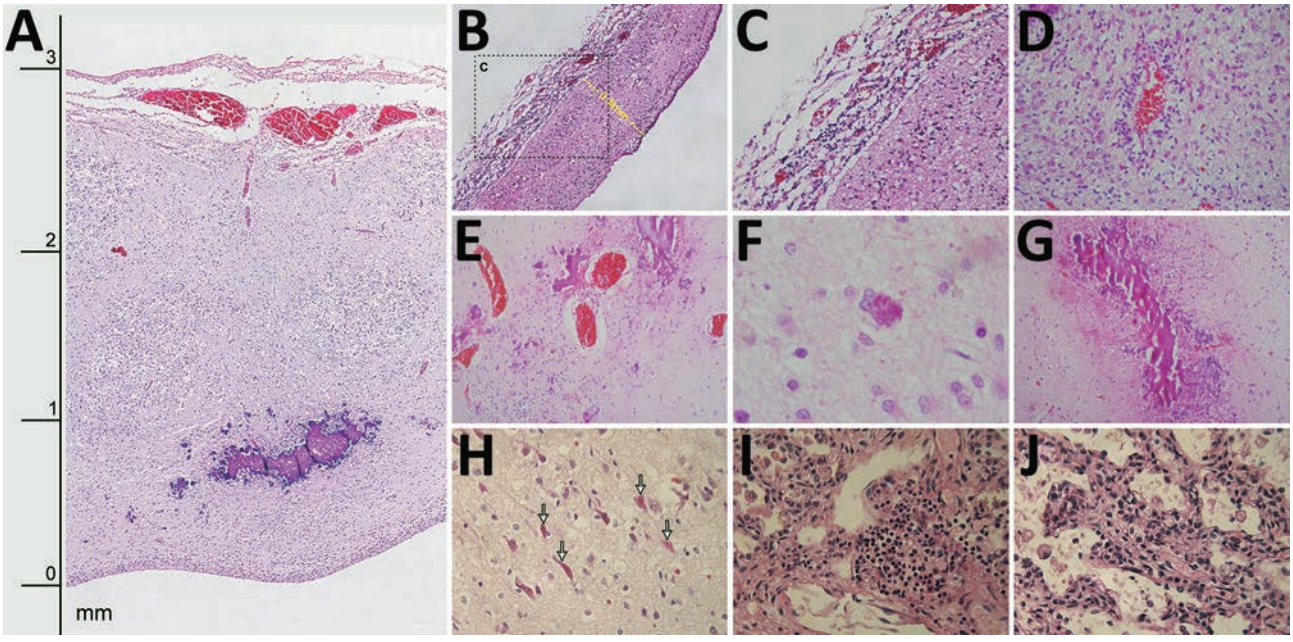

Figure 2. Histologic slides of tissues from 4 of 7 neonates who died of congenital Zika virus infection, Brazil. A) Neonate 1: severe cortical thinning (3 mm) with subventricular dystrophic calcification, reactive gliosis, and marked leptomeningeal congestion as well as marked depletion of neuronal precursors (original magnification ×10). B) Neonate 1: severe thinning of brain parenchyma (0.8 mm) with striking depletion of neuronal precursors and lymphocytic leptomeningitis (original magnification ×10). C) Neonate 1: lymphocytic leptomeningitis (enlargement of box in panel B; original magnification ×20). D) Neonate 6: white matter with lymphocytic perivascular cuffing and severe gliosis (original magnification ×40). E) Neonate 3: marked parenchymal vascular congestion and scattered coarse dystrophic calcification (original magnification ×20). F) Neonate 3: finely granular intracellular calcification (original magnification ×40). G) Neonate 7: band-like pattern of coarse dystrophic calcification at the junction of gray and white matter (original magnification ×10). H) Neonate 6: red neurons (arrows) in brain parenchyma (original magnification ×40). I) Neonate 1: focal interstitial lymphocytic pulmonary infiltrate (original magnification ×40). J) Neonate 1: expansion of alveolar septa with scattered lymphocytic and macrophage infiltrate (original magnification ×40).

Technical Appendix). Additional findings included foci of brain hemorrhage, mainly in periventricular areas.

Pulmonary hypoplasia was present in all neonates (online Technical Appendix); relative lung weight (lung weight/body weight) was 0.004–0.01 g (*11*). Intra-alveolar hemorrhage was seen in 3 neonates; bleeding was severe in neonates 5 and 7. Interstitial lymphocytic pulmonary infiltration (Figure 2, panel I) and expansion of alveolar septa (Figure 2, panel J) was present in neonate 1, who died soon after birth. Fresh frozen lung tissue from that neonate was positive for Zika virus by RT-PCR (Table).

Liver specimens were available from 6 neonates; moderate to severe hydropic degeneration was found in 5 of these specimens. Round eosinophilic cytoplasmic structures suggestive of megamitochondria were observed in 2 neonates, mild to moderate steatosis and Councilman bodies (apoptosis) in 3, hepatocyte necrosis in 1 (neonate 7) (online Technical Appendix), and mild periportal lymphocytic inflammation in 1 (neonate 2).

Kidneys were available from 6 of the neonates. Focal glomerular sclerosis was present in neonates 2 and 6. In neonate 2, tissue was positive for Zika virus by RT-PCR (Table). Moderate lymphocytic cystitis was present in tissue from 1 available bladder. No histologic abnormalities were found on any of the hearts or spleens. Placental tissues were available from 4 neonates; common findings were fibrinoid necrosis, chorangiosis, and amnion hyperplasia. Dengue virus 1 was detected by RT-PCR in cerebrospinal fluid from neonate 4 (Table).

Conclusions

Of the 7 Zika virus–infected neonates examined, 6 did not show intrauterine growth restriction, but all 7 had remarkably decreased brain weight (online Technical Appendix), emphasizing the neurotropism of Zika virus. These findings are similar to those earlier reported for congenital Zika virus infection (*3*–*7*). In contrast, an animal model of congenital Zika virus infection with similar neuropathologic damage was associated with striking global growth retardation (*12*).

The constellation of neuropathologic features (ventriculomegaly, mineralized neurons, and dystrophic calcification with band-like subcortical distribution) differs from features seen in other common infections associated with

congenital abnormalities (e.g., TORCH [toxoplasmosis, other viruses, rubella, cytomegalovirus and herpesvirus infections]) and should raise suspicion for congenital Zika virus infection, warranting further workup. For instance, the pattern of calcifications seen in tissue from patients with congenital cytomegalovirus infection and in toxoplasmosis are predominantly periventricular (*13*).

Pulmonary hypoplasia, defined as lung weight:body weight ratio of ≤0.012 (1.2%), seemed to be a major factor determining death during the perinatal period (*11*). The occurrence of pulmonary hypoplasia and arthrogryposis was most likely a part of fetal akinesia deformation sequence, resulting primarily from central nervous system damage (*14*).

In addition, variable liver damage, a finding commonly seen with infection by other flaviviruses, was found in these neonates (*15*). The detection of Zika virus in tissues that did not show pathologic abnormalities could be the result of viremia or of the tissues sampled; the pathologic abnormalities were mostly focal (samples for PCR could have differed from those used for histopathologic examination). Another possibility could be cross-contamination during the sample collection.

Our report confirms not only the neurotropism of the virus but also the occurrence of pathologic changes consistent with viral infection in multiple organs: liver (Councilman bodies and periportal lymphocytic infiltration), lungs (interstitial lymphocytic pneumonitis), and bladder (lymphocytic cystitis). PCR detection of Zika virus in liver, lung, and kidney tissue also strengthened our hypothesis that Zika virus can infect multiple tissues.

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Porcine Hemagglutinating Encephalomyelitis Virus and Respiratory Disease in Exhibition Swine, Michigan, USA, 2015

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Acute outbreaks of respiratory disease in swine at agricultural fairs in Michigan, USA, in 2015 raised concern for potential human exposure to influenza A virus. Testing ruled out influenza A virus and identified porcine hemagglutinating encephalomyelitis virus as the cause of influenza-like illness in the affected swine.

The commingling of pigs and humans at agricultural fairs has been responsible for most zoonotic influenza A virus (IAV) cases over the past 5 years. During routine IAV surveillance in exhibition swine in the summer of 2015, influenza-like illness (ILI) was noted in swine at 6 of 14 agricultural fairs surveilled in Michigan, USA. Acute outbreaks of ILI in swine at 2 fairs were so severe that animal health and fair officials closed the swine barns to nonessential personnel out of concern for potential human exposure to IAV. Nasal swab specimens were collected from representative swine and tested for IAV (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/17-0019-Techapp1.pdf). IAV was not detected in samples from the pigs at any of the Michigan fairs; however, next-generation sequencing (NGS) identified porcine hemagglutinating encephalomyelitis virus (PHEV) in a specimen from a clinically ill pig.

Following the initial PHEV detection, all samples from the 14 Michigan fairs held in 2015 were screened for the coronavirus; PHEV was detected at 10 (71.4%) of the 14 fairs, with 108 (38.7%) of 279 pigs testing positive. Given the high prevalence of PHEV in Michigan exhibition swine and the uncommon clinical presentation for PHEV (i.e., ILI in market-age pigs), we initiated further epidemiologic investigation.

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The Study

We screened nasal swabs from pigs at 14 Ohio fairs and 14 Indiana fairs for PHEV (detailed methods in online Technical Appendix). Pigs at 4 of the Indiana fairs and 5 of the Ohio fairs exhibited signs of respiratory disease. We detected PHEV in 4 (14.3%) of 28 Ohio and Indiana fairs; 23 (4.1%) of 560 pigs tested positive. The increased risk of PHEV detection in samples from Michigan exhibition swine compared with samples collected from pigs in Ohio and Indiana (risk ratio 9.4, 95% CI 6.2–14.4) indicated epizootic behavior of PHEV in the Michigan fairs.

Although PHEV has been recognized for decades, few PHEV genomes have been publicly deposited. We performed NGS on representative PHEV-positive samples to investigate genetic diversity; 10 complete sequences and 1 partial sequence were obtained. Sequence analysis showed that the 10 complete PHEV strains had 2.1%–2.2% genome difference from a PHEV strain from Belgium (VW572) and 7.2%–7.4% genome difference from human enteric coronavirus (CoV) (HECV) 4408, bovine CoV Kakegawa, and white-tail deer CoV WD470 (Figure 1). Complete genome, nonstructural 2 (NS2) gene, spike gene, and NS4.9 gene phylogenetic analyses indicated 3 distinct clusters, referred to as genotypes 1–3, based on deletions in the NS2 gene (Figure 2; online Technical Appendix). It is likely that the deletions observed in this study contribute to viral evolution and may confer respiratory tropism of PHEV because deletion patterns are common in the genome of porcine respiratory CoV, which has a strong respiratory tropism. In contrast, the 3 other porcine CoVs have a strong enteric tropism (transmissible gastroenteritis virus, porcine epidemic diarrhea virus, and porcine deltacoronavirus).

The presence of ILI in pigs at multiple Michigan fairs, along with the increased risk of PHEV detection at these fairs, supports a causal link between PHEV and respiratory disease. PHEV is a single-stranded positive-sense RNA coronavirus belonging to the family *Coronaviridae*, genus *Betacoronavirus*. The virus is 1 of 5 known porcine CoVs causing disease in swine and is considered endemic worldwide, where it maintains itself by successively infecting groups of animals after replacement or weaning (*2*,*3*). PHEV typically affects pigs <3 weeks of age; clinical syndromes include vomiting and wasting disease and

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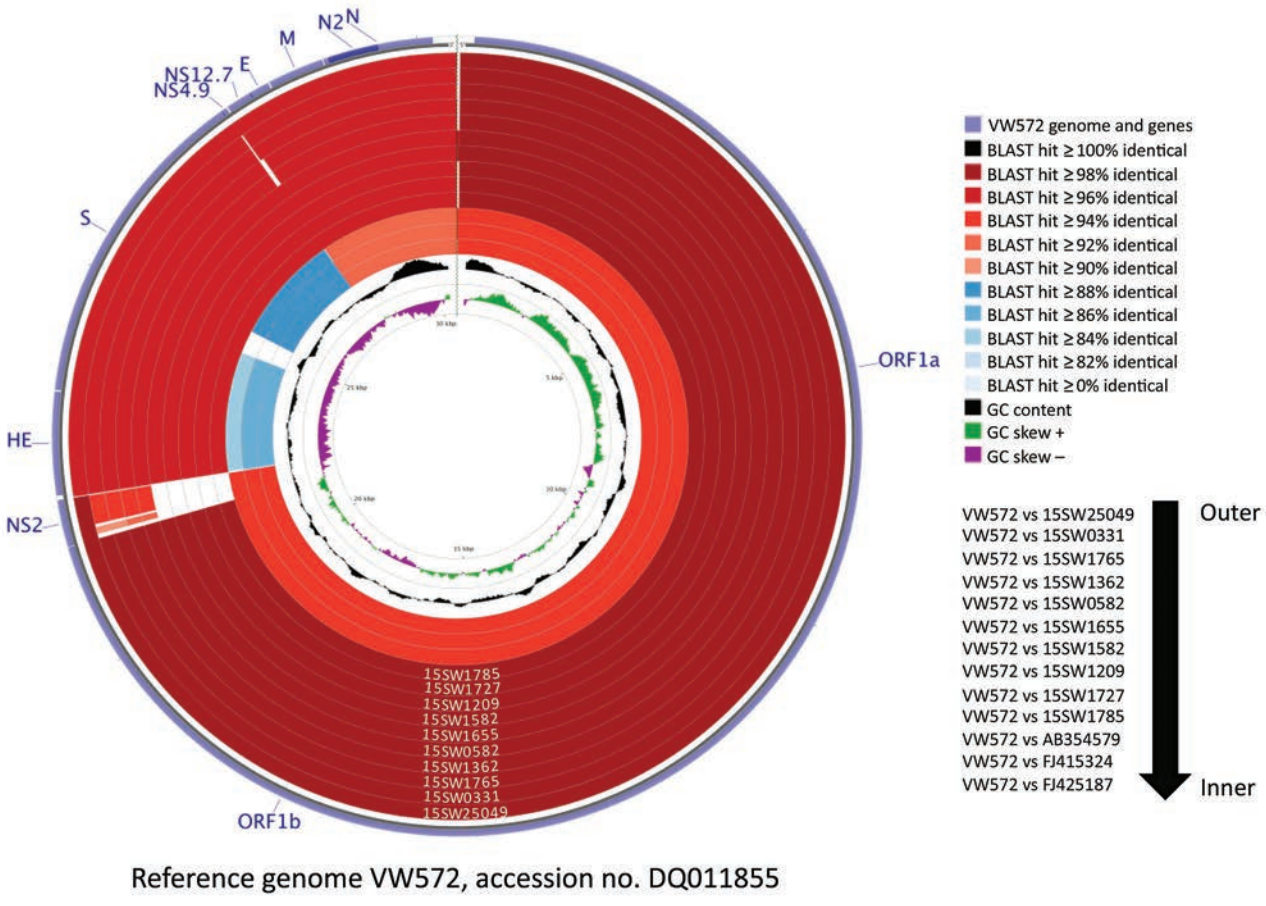

Figure 1. Genomic comparison of 10 porcine hemagglutinating encephalomyelitis virus (PHEV) strains from fairs in Michigan, Indiana, and Ohio, USA, 2015, to 3 non-PHEV coronavirus (CoV) strains from GenBank (bovine CoV Kakegawa, accession no. AB354579; human enteric CoV 4408, accession no. FJ415324;, white-tail deer CoV WD470, accession no. FJ425187) and a reference genome from a PHEV strain from Belgium (VW572, accession no. DQ011855). Analysis was completed by using CGView Comparison Tool software (*1*). The corresponding strain/sample for rings are detailed on the right. The innermost 2 rings display GC content and GC skew. NS2, nonstructural protein 2; ORF, open reading frame.

encephalomyelitis (*4*,*5*). Upper respiratory tract and pulmonary lesions have rarely been described (*6*); however, the primary route of PHEV infection is through upper respiratory tract epithelium. Sneezing and coughing may be the first clinical signs observed in piglets, supporting our premise that PHEV may cause respiratory disease in older swine (*2*). Although there are no data to definitively prove this premise, a previous report suggested an association between PHEV and clinical disease in older animals (*7*). A confounder at breeding facilities is the presence of animals of multiple age groups and bias toward recognizing the classical disease in piglets; our data represent a relatively homogenous group of market-age pigs.

Our findings also appear to highlight a distinct transmission network within Michigan exhibition swine; despite geographic contiguity and no barriers to interstate travel, Michigan samples, compared with those from Ohio and Indiana, yielded different proportions of PHEV detection. This finding is further supported by the observation that PHEV sequenced from Michigan fairs was predominantly genotype 2, which was not detected in Ohio or Indiana (Table). Such a transmission network may be the result of common routes of travel or sites of commingling of swine, including larger swine exhibitions before county fairs. Animal networks have been described in additional species and locations and are not unique to the Michigan fairs (*8*,*9*).

During our interpretation of the data, we considered several limitations. First, no tissues were available to demonstrate pathologic lesions associated with the virus. However, NGS failed to detect the presence of additional pathogens aside from a single sample that contained porcine parainfluenza virus type 1 in addition to PHEV. Second, detection of PHEV in samples could not be directly correlated with respiratory disease in individual animals due to assessment of ILI at the fair level. However, at the fairs where barns were closed because of concerns of IAV,

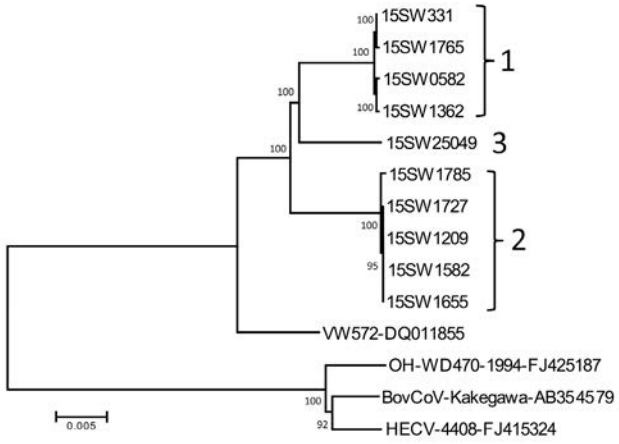

Figure 2. Phylogenetic tree constructed on the basis of the whole-genome sequence of porcine hemagglutinating encephalomyelitis virus (PHEV) strains from fairs in Michigan, Indiana, and Ohio, USA, 2015 (indicated by genotype labels at right), compared with bovine CoV (BovCoV), human enteric CoV (HECV), and white-tail deer CoV and a reference PHEV strain from Belgium (VW572). Reference sequences obtained from GenBank are indicated by strain name and accession number. Numbers along branches indicate bootstrap values. Scale bar indicates nucleotide substitutions per site. CoV, coronavirus.

most swine were affected by ILI, and >50% of pigs were PHEV positive at these locations. Last, it is difficult to conclude whether genotype 2 PHEV was present in Ohio and Indiana, because not all positive samples were sequenced. Although the genotype data may not be statistically significant because of the small sample size, there appears to be a clear difference in genotype distributions.

Conclusions

Our findings provide strong evidence for the role of PHEV as a respiratory pathogen and genomic characterization of clinically relevant strains circulating in US swine herds. The ILI in swine in this study is considered an atypical presentation of PHEV and may reflect unique presentation of PHEV in older but naive swine populations, an atypical form of disease, or increased virulence. Guarded interpretation of our data suggests that, at minimum, PHEV should be considered as a differential diagnosis in clinical outbreaks of ILI in market-age swine. Future surveillance and research are needed to further investigate the association of PHEV with respiratory disease in commercial and exhibition swine.

The rapid government and local authority responses to the outbreaks of respiratory disease in pigs at the fairs involved in our study was justified given the public health threat of IAV. Variant IAV (H3N2v) was responsible for many human cases, including 1 death, during outbreaks in 2011–2016 in which swine-to-human transmission was demonstrated (*10*,*11*). Most recently, in August 2016 there was a regional outbreak of H3N2v virus infection in 18 persons with recent exposure to swine at 7 fairs in Michigan and Ohio (*11*). PHEV is not known to cause any disease in humans, but the PHEV transmission network uncovered in

Table. PHEV obtained from samples of swine at fairs in Michigan, Indiana, and Ohio, USA, 2015*

Fair	Total no. samples	No. PHEV positive	Risk for PHEV positivity	ILI	Strain name	Genotype	GenBank accession no.
Michigan							
A	20	9	0.45	Yes	PHEV-CoV USA-15SW1727	2	KY419111
B	20	20	1.00	Yes	PHEV-CoV USA-15SW1362	1	KY419110
C	20	10	0.50	Yes	PHEV-CoV USA-15SW1582	2	KY419113
D	20	20	1.00	Yes	PHEV-CoV USA-15SW1655	2	KY419109
E	19	7	0.37	Yes	PHEV-CoV USA-15SW25049	3	KY419103
F	20	19	0.95	Yes	PHEV-CoV USA-15SW1209	2	KY419107
G	20	9	0.45	No	NA	NA	NA
H	20	9	0.45	No	NA	NA	NA
I	20	4	0.20	No	PHEV-CoV USA-15SW1785	2	KY419106
J	20	1	0.05	No	PHEV-CoV USA-15SW24992†	2	KY419108
All others, n = 4	80	0	0.00	No	NA	NA	NA
Total, n = 14	279	108	0.39				
Indiana							
K	20	1	0.05	No	NA	NA	NA
L	20	8	0.40	No	PHEV-CoV USA-15SW1765	1	KY419112
M	20	1	0.05	No	PHEV-CoV USA-15SW0331	1	KY419104
All others, n = 11	220	0	0.00	Yes (4)	NA	NA	NA
Ohio							
N	20	13	0.65	No	PHEV-CoV USA-15SW0582	1	KY419105
All others, n = 13	260	0	0.00	Yes (5)	NA	NA	NA
Ohio/Indiana							
Total, n = 28	560	23	0.04				

*Reverse transcription PCR PHEV detection results are shown with calculated risk. Aggregate data for Michigan or Ohio and Indiana are shown. PHEV strain name and corresponding GenBank accession numbers are listed for samples that yielded sequence data. ILI, influenza-like illness; NA, not applicable; PHEV, porcine hemagglutinating encephalomyelitis virus.
†Partial genome (nonstructural protein 2).

Michigan during 2015 may represent a pathway for both intraspecies and interspecies transmission of additional pathogens, including IAV. Although it is inappropriate to make leaps in assuming that this report could predict such a future outbreak, it is worth contemplating potential outcomes had the animals been transmitting a zoonotic agent such as IAV.

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Norovirus GII.P16/GII.2–Associated Gastroenteritis, China, 2016

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During October–December 2016, the number of norovirus outbreaks in China increased sharply from the same period during the previous 4 years. We identified a recombinant norovirus strain, GII.P16-GII.2, as the cause of 44 (79%) of the 56 outbreaks, signaling that this strain could replace the predominant GII.4 viruses.

Noroviruses, the main cause of nonbacterial acute gastroenteritis outbreaks (*1*), are positive-sense, single-stranded RNA viruses within the family *Caliciviridae* (*2*). The genome contains 3 open reading frames (ORFs). ORF1 encodes nonstructural proteins, including an RNA-dependent RNA polymerase (RdRp), ORF2 encodes a capsid protein (viral protein 1 [VP1]), and ORF3 encodes a minor capsid protein (VP2). On the basis of RdRp and VP1 gene sequences, noroviruses are classified into 7 genogroups (GI–GVII) (*3*). GI, GII, and GIV noroviruses can infect humans; GI and GII viruses, the most common, include at least 31 distinct genotypes (*3*).

Since 2002, GII.4 viruses have been the most common norovirus genotype circulating worldwide (*3*,*4*). However, during the winter of 2014–15 in parts of Asia, a new GII.17 strain emerged as the major cause of acute gastroenteritis outbreaks (*5*–*7*), suggesting that non–GII.4 norovirus might become the predominant genotype. We report that, in late 2016 in China, the number of norovirus outbreaks increased significantly over the same period during the previous 4 years (56 in 2016 vs. 6 in 2013, 11 in 2013, 36 in 2014, and 14 in 2015). A GII.P16-GII.2 virus caused 79% of the 56 outbreaks in 2016.

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The Study

Acute gastroenteritis outbreaks, defined as ≥20 patients with vomiting and diarrhea associated with a common source of infection within 1 week, are reported to the National Emergent Public Health Event Information Management System established by the Chinese Center for Disease Control and Prevention (China CDC). Fecal specimens from each outbreak were tested for noroviruses by the local Center for Disease Control and Prevention using commercial real-time reverse transcription PCR (RT-PCR) kits (BioPerfectus Technology Co., Jiangsu, China; Aodong Inspection & Testing Technology Co., Shenzhen, China). At least 3 norovirus-positive samples from each outbreak were transported to the China CDC for study. The China CDC Institutional Review Board for human subject protection approved this study.

During 2012–2016, a total of 313 norovirus outbreaks in 24 provinces and 96 cities in China were reported to the National Emergent Public Health Event Information Management System; 109 (35%) were reported in 2016, mostly in winter. In November and December 2016, norovirus outbreaks increased sharply, accounting for 56 (51%) of the 109 norovirus outbreaks (Figure 1, panel A). These outbreaks occurred mainly in kindergartens (48%) and schools (39%) (online Technical Appendix Table 1, https://wwwnc.cdc.gov/EID/article/23/7/17-0034-Techapp1.pdf).

Partial capsid genes were amplified by conventional RT-PCR for genotyping, as described previously (*8*). Of the outbreaks,25 (78%) of 32 were caused by GII.17 noroviruses during the winter of 2014–15 and 9 (60%) of 15 by GII.3 noroviruses during the winter of 2015–16. In 2016, samples from 94 (86%) of the 109 outbreaks were genotyped. The most common norovirus genotype was GII.2 (49 [52%]), followed by GII.3 and multiple genotypes (8 [9%] each); GII.4Sydney 2012 (7 [7%]); GII.17 and GII.6 (5 [5%] each); and others (12 [13%]). Of the 8 outbreaks involving multiple genotypes, 3 included GII.P16-GII.2 (Figure 1, panel B). Of the 49 GII.2 outbreaks in 2016, 44 (90%) were reported during November and December 2016. Thirty-eight (78%) GII.2 norovirus outbreaks occurred in kindergartens. Samples from all GII.2 norovirus outbreaks were selected for dual genotyping using conventional RT-PCR spanning the ORF1/ORF2 region, as developed by the US Centers for Disease Control and Prevention

[1]These authors contributed equally to this article.

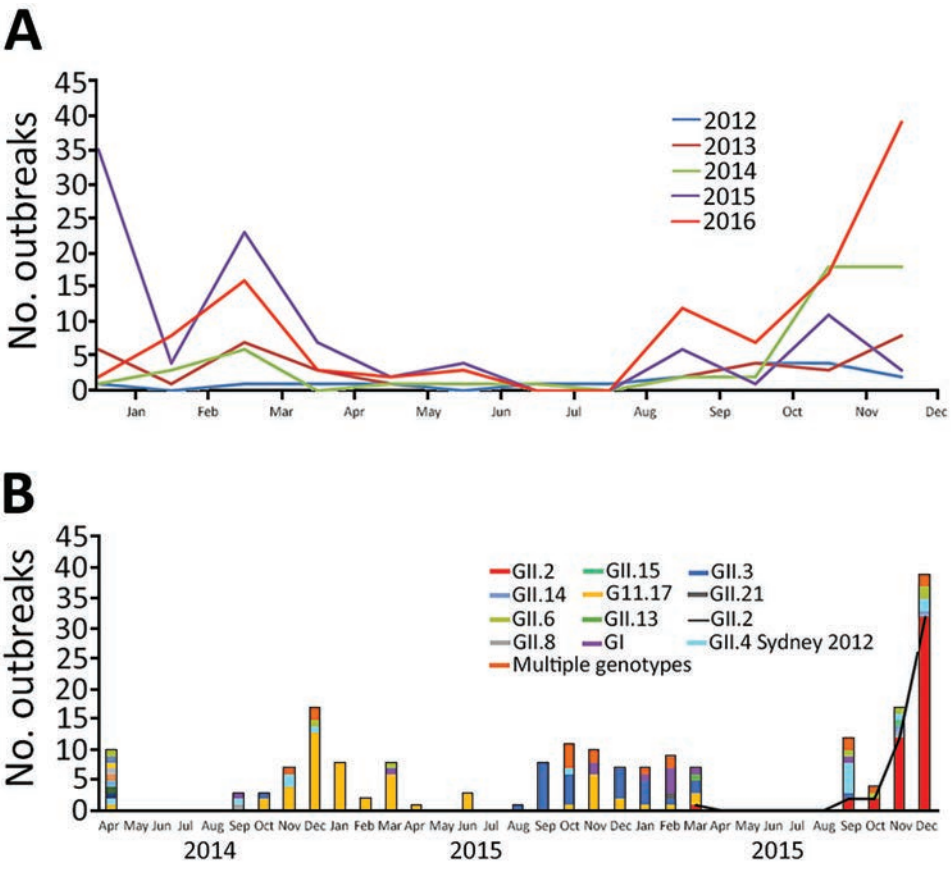

Figure 1. Norovirus outbreaks, China. A) Outbreaks reported to the China Centers for Disease Control and Prevention during 2012–2016. B) Genotype (capsid) distribution of norovirus outbreaks during April 2014—December 2016.

(Atlanta, GA, USA) (J. Vinjé, pers. comm., 2017 Mar 1). Genotyping results showed 48 outbreaks were caused by GII.P16/GII.2 norovirus, and 1 outbreak was caused by the GII.P2/GII.2 norovirus. The first GII.P16/GII.2 norovirus was detected in August 2016.

We obtained complete capsid sequences and nearly complete RdRp sequences from samples from 30 GII.2 outbreaks using nested PCR (online Technical Appendix Table 2). Furthermore, 2 complete genomes (JS1208 and BJSMQ) were determined, showing the highest nucleotide identities (95%) to those of the HS255/2011/USA (GenBank accession no. KJ407074.2) and Miyagi1/2012/JP (GenBank accession no. LC145787.1) strains. Overall, 35 GII.2 VP1 sequences were 99% nt identical to each other, and the 28 RdRp sequences were closely related to the GII.P16 norovirus recently detected in Germany (*9*). All nucleotide sequences obtained were deposited in GenBank (accession nos. KY421121–KY421185).

The predicted 35 GII.2 VP1 amino acid sequences we determined were aligned with those for GII.2 strains from the 1970s to 2016 that are available in GenBank, and <15 aa mutations were found in the VP1 region, suggesting that the GII.2 norovirus is still circulating stably worldwide. This finding is consistent with a recent report showing that the non–GII.4 norovirus remains static (*10*). The x-ray crystal structure of the capsid-protruding domain of GII.2 strain Snow Mountain virus (SMV) (GenBank accession no. KF429769) was recently determined (*11*). Compared with the histo-blood group antigen (HBGA) binding surface of strain SMV, the GII.2 strains in this study had 15 aa mutations in VP1, including 8 aa mutations at residues 335–349 around the HBGA binding site I in the P2 domain, although the conserved set of residues (Asn352, Arg353, Asp382, and Gly445) required for binding the fucose moiety of HBGAs in SMV was unchanged. Sequence analysis of the RdRp region revealed that 28 GII.2 strains in this study differed from GII.P16/GII.4 strain CA3477 by ≈1–5 aa mutations. A sequence analysis of 35 predicted GII.2 VP2 sequences showed mutations (5–10 divergent residues) at the C-terminus of VP2 compared with those of HS255 and Miyagi1. ORF1 of JS1208 and BJSMQ also differed from that of GII.P16/GII.4 strain CA3477 by 11 aa.

Phylogenetic analysis using VP1 showed that 35 strains in this study formed a subcluster independent of the GII.2 subcluster; the strains were most closely related to the norovirus GII.2 subcluster that included HS255 and Miyagi1. However, the RdRp of 28 GII.2 strains formed a single cluster and showed maximum relatedness to those of GII.P16/GII.4 strain CA3477 (Figure 2).

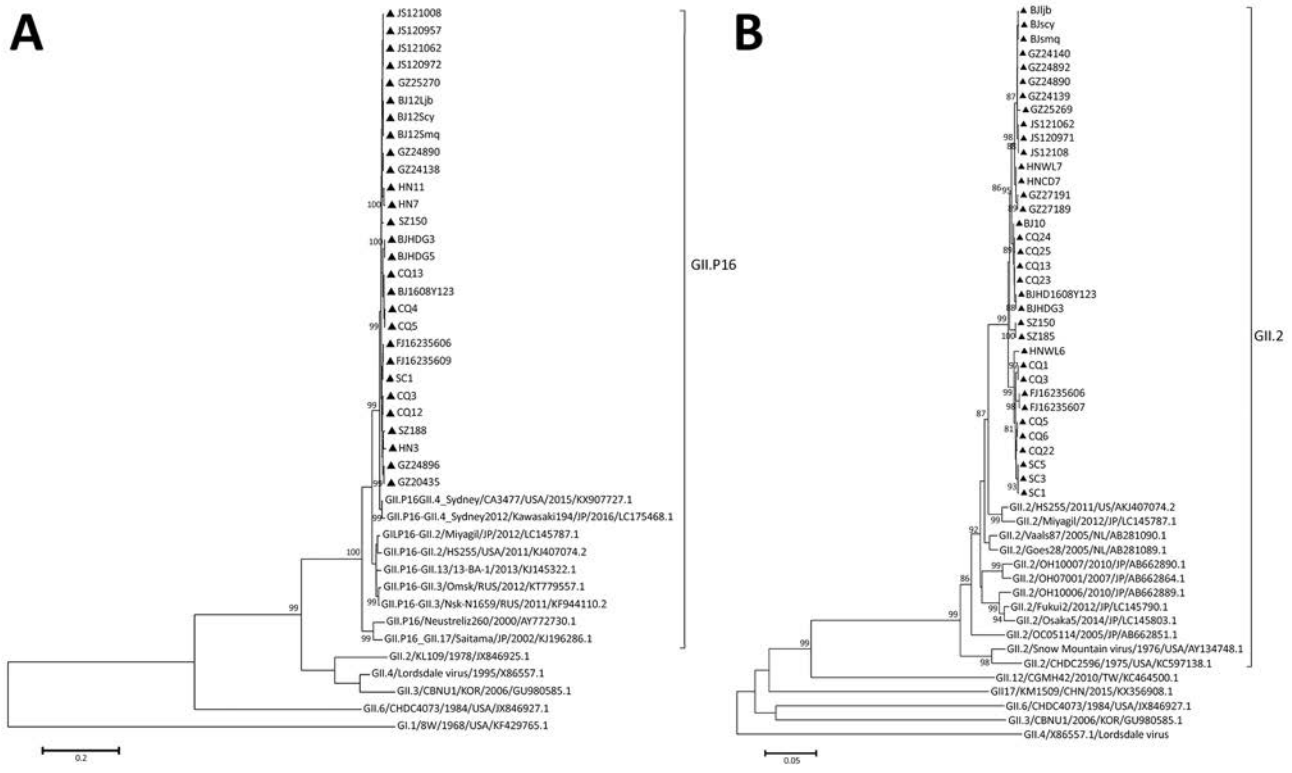

Figure 2. Phylogenetic analyses of the newly identified GII.2 noroviruses in China, reconstructed based on RNA-dependent RNA polymerase (A) and open reading frame 2 (B) with a representative norovirus using the neighbor-joining method with datasets of 1,000 replicates in MEGA 6.0 software (http://www.megasoftware.net). Triangles indicates the positions of the GII.2 norovirus newly identified in 8 cities from 7 provinces. Scale bars indicate nucleotide substitutions per site.

The complete genomes of GII.P2/GII.2 strain Malaysia (GenBank accession no. JX846925.1) and GII.P16/GII.4 strain CA3477 (GenBank accession no. KX907727.1) were used as query sequences to predict possible recombination breakpoints of the JS1208 genome by SimPlot software version 3.5.1 (http://sray.med.som.jhmi.edu/SCRoftware/simplot). The recombination breakpoint was predicted to be at nucleotide position 5088 at the boundary between ORF1 and ORF2.

Conclusions

In China, the number of norovirus outbreaks increased substantially in late 2016, greatly surpassing norovirus activity reported during the same months of the previous 4 years. Most of these outbreaks were associated with a GII.P16-GII.2 strain, similar to a recently reported pattern in Germany (*9*). Seventy-eight percent of the GII.2 outbreaks occurred in kindergartens. The exact reason is unknown but is in line with the model presented by Parra et al. (*10*), in which non-GII.4 genotypes seem to infect infants more frequently because adults have built immunity to different genotypes over time. This finding should be confirmed by serum inhibition/neutralization tests for specific genotypes or strains, such as GII.P16-GII.2, in the population. Continuous surveillance is needed to explore the epidemiologic, clinical, and evolutionary characteristics of this GII.P16-GII.2 strain. Additional studies should explore the mechanisms behind the emergence of this active strain.

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Novel Pestivirus Species in Pigs, Austria, 2015

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A novel pestivirus species was discovered in a piglet-producing farm in Austria during virologic examinations of congenital tremor cases. The emergence of this novel pestivirus species, provisionally termed Linda virus, in domestic pigs may have implications for classical swine fever virus surveillance and porcine health management.

The genus *Pestivirus* consists of 4 approved species within the family *Flaviviridae* (*1*). Besides bovine viral diarrhea virus 1 (BVDV-1), BVDV-2, border disease virus (BDV), and classical swine fever virus (CSFV), several unassigned strains and tentative species are represented by the so-called atypical pestivirus strains. An atypical pestivirus of swine emerged in 2003 in a commercial pig-breeding farm in Australia and was later termed Bungowannah virus (BV). This well-studied virus caused reproductive disorders, stillbirth, and sudden death in piglets, resulting in the loss of ≈50,000 animals in the 2 affected farms. Because of its marked pathogenicity, BV was considered a threat to global pig health, but this virus or relatives were never found at other locations (*2*).

Recently, a novel group of porcine pestiviruses, termed atypical porcine pestiviruses (APPVs), was discovered. These viruses were identified in North America (*3*,*4*) and subsequently detected in Europe (*5*–*8*). There is strong evidence that APPVs are a causative agent behind the type A-II congenital tremor (CT A-II) syndrome of piglets (*4*). CT A-II is characterized by a generalized shaking of the whole body associated with variable degrees of hypomyelination in the brain and spinal cord. However, hypomyelination is also apparent in the brains of fetuses of sheep, cattle, and pigs after late-gestation state infection with BDV, BVDV, or CSFV (*9*). We report results of an investigation of CT in piglets on a farm in southeastern Austria in which a novel pestivirus species was discovered.

The Study

A small-scale piglet-producing farm in Styria, in southeastern Austria, reported major piglet losses from CT in January 2015. Animals from this farm were examined and samples were taken by authorized veterinarians. The CT-affected piglets showed a severe lateral shaking and were often incapable of sucking milk, which led to elevated preweaning death rates (Video, https://wwwnc.cdc.gov/EID/article/23/7/17-0163-V1.htm). The prevalence of CT varied from 20% to 100% within the affected litters. Litters affected by CT were not used for the production of replacement gilts. The outbreak of CT abruptly stopped in July 2015, when all sows had produced 1 CT-affected litter. Only 22.4 piglets per sow were weaned in that year, compared with an average of 25.8 piglets/sow/year before CT symptoms occurred. Pathological examinations were performed as described previously (*8*), confirming the presence of typical CT A-II lesions (Figure 1, panel A).

We tested various samples from the farm using an APPV-specific TaqMan probe-based reverse transcription PCR (RT-PCR) (*8*) but obtained negative results. However, we obtained an amplicon of appropriate length from CT-piglet serum samples by using a novel panpestivirus RT-PCR (PPF 5′-GTKATHCAATACCCTGARGC-3′ and PPR 5′-GGRTTCCAGGARTACATCA-3′), which enables detection of CSFV, BVDV-1, BVDV-2, BDV, BV, and APPV. Surprisingly, sequencing of this RT-PCR product yielded an unknown sequence with a noninterrupted open reading frame. An initial BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi) resulted in "no significant similarity found," but the translated sequence of 270 aa aligned well with different pestiviruses. The viral RNA was detected in all samples (serum, tonsils, lung, liver, spleen, and central nervous system material) of CT-affected piglets and their littermates from the farm.

After inoculating SK-6 cells with serum samples of affected piglets, we detected the amplification of this unknown pestivirus using RT-PCR. We provisionally termed the agent "Linda" (lateral-shaking inducing neurodegenerative agent) to avoid confusion with other pestiviruses. We determined the full genome of Linda virus (LV) using the standard primer walking RT-PCR approach together with rapid amplification of cRNA ends PCR to identify the ultimate 5′- and 3′-termini using the cultured virus. We determined the length of the LV genome (GenBank accession no. KY436034) to be 12,614 nt, with 381 nt 5′-nontranslated region, an open reading frame of 11,772 nt, and 461 nt of 3′-nontranslated region.

We performed a phylogenetic analysis of LV using CLC Workbench 7.6 (CLCBIO, Aarhus, Denmark), which demonstrated LV's divergence from other pestiviruses (Figure 1, panel B). We found the identity between

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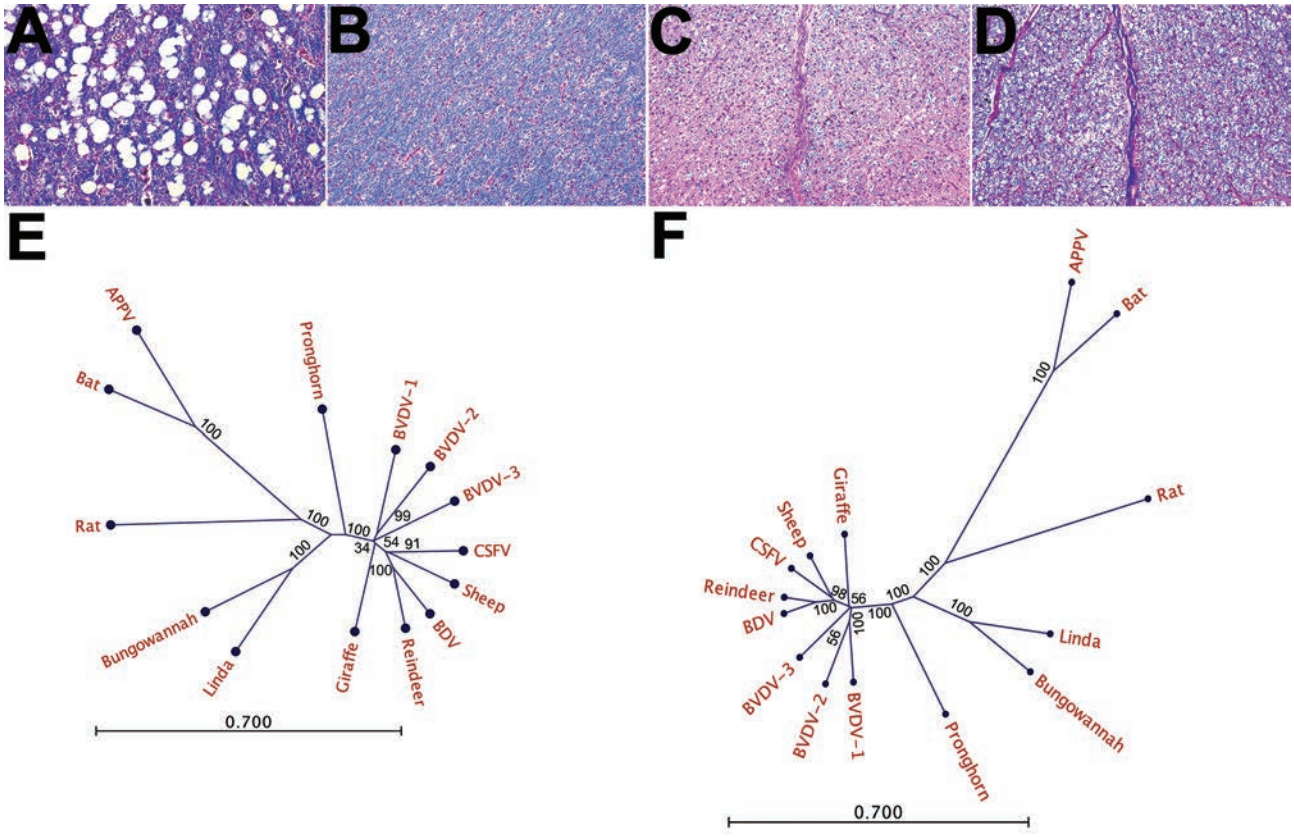

Figure 1. Histologic and phylogenetic examination in investigation of piglets with congenital tremor (CT) on a farm in southeastern Austria, 2015. A) Cerebellar white matter of CT-affected piglet showing multiple sharply bordered vacuoles but normal myelination (stained in blue; luxol fast blue/hematoxylin-eosin staining; original magnification ×10). B) Control piglet with normal cerebellar white matter (original magnification ×10). C) Spinal cord white matter in CT-affected piglet shows a severely reduced amount of myelin (original magnification ×10). D) Control piglet with normal myelination of the spinal cord white matter (original magnification ×10). E, F) Phylogenetic neighbor-joining analysis using the nucleotide (E) and polyprotein (F) sequences of the novel virus isolated from piglets, provisionally termed Linda virus (GenBank accession no. KY436034); approved pestivirus species BVDV-1 NADL (accession no. M31182.1), BVDV-2 890 (accession no. U18059.1), CSFV Alfort_187 (accession no. X87939.1), and BDV X818 (accession no. AF037405.1); and tentative species Bungowannah virus (accession no. EF100713.2), sheep pestivirus Aydin (accession no. NC_018713.1), pronghorn pestivirus (accession no. NC_024018.2), reindeer pestivirus (accession no. AF144618.2), giraffe pestivirus (accession no. NC_003678.1), BVDV-3 D32_00_HoBi (accession no. AB871953.1), APPV NL1 (accession no. KX929062.1), *Rhinolophus affinis* pestivirus (accession no. JQ814854.1), and Norway rat pestivirus (accession no. KJ950914.1). Assumed relationships between the species are shown in a radial branching diagram with numbers indicating the bootstrap values of 1,000 replicates in percentages. Scale bars indicate number of substitutions per site. BDV, border disease virus; BVDV, bovine viral diarrhea virus; CSFV, classical swine fever virus.

LV, approved pestiviruses, and APPV to be only 60%, but we found an identity of 68% between LV and BV. Comparison of the pestiviral polyprotein sequences yielded an amino acid identity of 69% between LV and BV and of <54% with all other pestiviruses (Figure 1, panel C). All known cleavage sites of the NS3 protease were present in LV (*10*). We mapped the NS4A-NS4B cleavage, which takes place at an L/A site in classical pestiviruses and at an L/S site in BV, to the L_{2354}/S_{2355} motive in LV; the intramolecular NS3 cleavage site (L_{1834}/A_{1835}) was conserved in LV (*11*). Each hypothetical mature protein of LV matched best with the mature proteins of BV (Table), even if the identities were relatively low in the structural protein region.

We saw a strong reactivity against LV infected cells when using a BVDV E2-specific antibody termed 6A5 (*12*). This antibody detected the E1-E2 heterodimer of LV (75 kDa) as well as the E2 monomer (50 kDa) in Western blot analysis, although the reactivity against BVDV-1 E2 was much stronger (Figure 2, panel A). With the help of this antibody, we were able to study the in vitro growth of LV. E2 antigen signals of LV were detectable in the cytoplasm of SK-6 and MDBK cells after infection, but the antigen-positive foci were 10-fold larger in SK-6 than in MDBK cells (Figure 2,

Table. BLAST analysis of the 12 putative mature proteins and the polyprotein of novel pestivirus Linda virus from pigs with congenital tremor, Austria, 2015*

Putative mature protein	Amino acid region	Best BLAST hit (GenBank accession no.)	Alignment coverage, %	E-value	Identity, %
N^{pro}	1–182	BV (YP_008992092.1)	96	4×10^{-64}	60
Core	183–283	BV (YP_008992092.1)	95	3×10^{-43}	83
E^{rns}	284–504	BV (YP_008992092.1)	100	5×10^{-117}	74
E1	505–702	BV (YP_008992092.1)	100	3×10^{-82}	67
E2	703–1077	BV (YP_008992092.1)	98	4×10^{-140}	53
P7	1078–1152	BV (YP_008992092.1)	100	1×10^{-25}	60
NS2	1153–1608	BV (YP_008992092.1)	99	0.0	63
NS3	1609–2291	BV (YP_008992092.1)	100	0.0	85
NS4A	2292–2354	BV (YP_008992092.1)	100	2×10^{-28}	81
NS4B	2355–2701	BV (YP_008992092.1)	100	0.0	78
NS5A	2702–3205	BV (YP_008992092.1)	100	0.0	53
NS5B	3206–3923	BV (YP_008992092.1)	99	0.0	73

*BV, Bungowannah virus; E, envelope; NS, nonstructural.

panels B,C). Already in the primary passage on porcine cells, considerably high infectious titers of LV were measured in the supernatant (>10^7 50% tissue culture infectious dose/mL). Using monoclonal antibody 6A5 in immunohistochemical analysis, we detected pestiviral antigen in CT-affected piglets in regions with histological lesions. Specific stains in neurons of the nucleus of the trigeminal nerve (Figure 2, panels D,E), in glial cells in the cerebellar and the cerebral white matter, and in tubular epithelium of the kidneys proved that a pestivirus crossed the blood–brain barrier. As in the case of other pestiviruses, the mechanisms inducing hypomyelination during fetal development remain unclear (*8*,*13*).

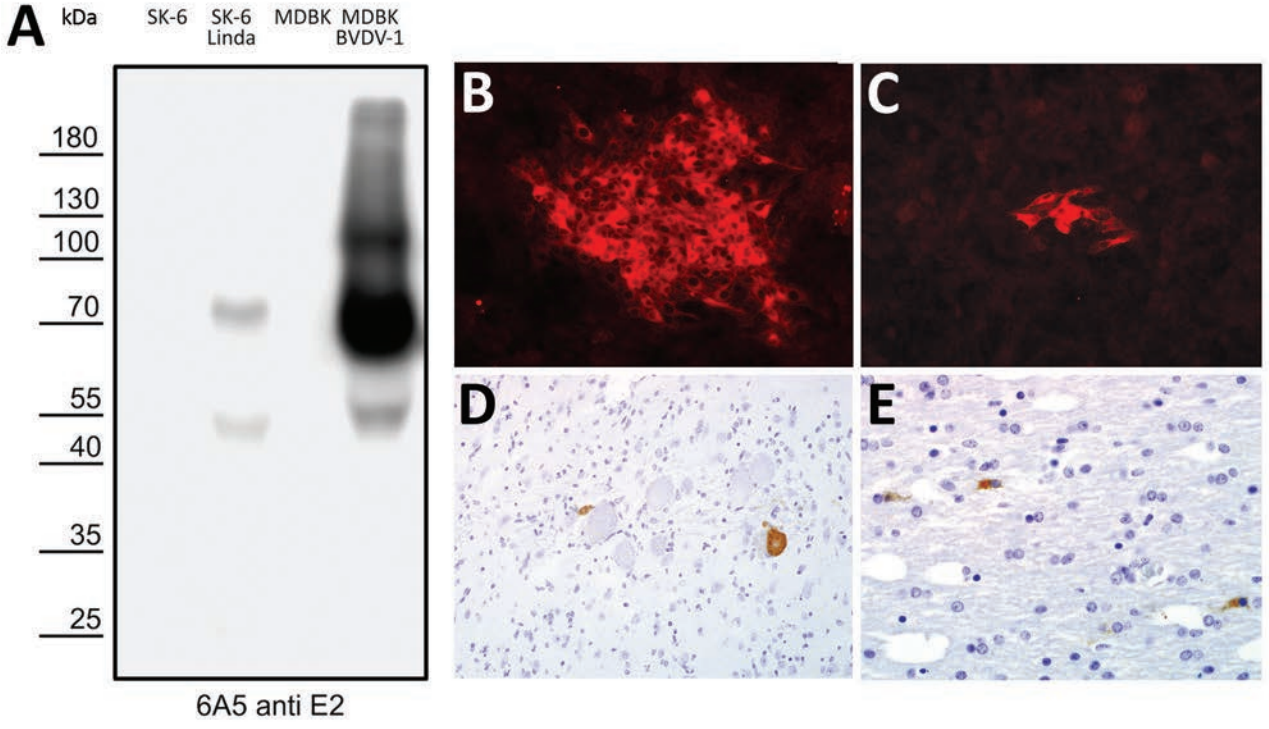

Figure 2. Detection of pestivirus E2 protein with monoclonal antibody 6A5 in investigation of piglets with congenital tremor (CT) on a farm in southeastern Austria, 2015. A) Western blot analysis of cells infected with novel virus provisionally termed Linda virus. Total protein of SK-6 cells infected with Linda virus and MDBK cells infected with BVDV-1 (strain NADL) was probed with the pestivirus E2-specific antibody 6A5. The apparent molecular mass of monomeric E2 (LV 50 kDa and BVDV-1 55 kDa) shows that Linda virus E2 has a lower molecular weight than BVDV-1 E2 as a result of fewer N-linked glycosylation sites. In contrast, the mass of E1-E2 heterodimers is comparable (≈70 kDa in Linda virus and BVDV-1), because Linda virus E1 has an additional N-linked glycosylation site. B, C) Focus size of Linda virus 48 hours after infection of SK-6 cells (B) and MDBK cells (C) (original magnification ×20). D, E) Detection of pestiviral E2 within neuronal tissue of Linda virus–positive, congenital tremor–affected piglets showing positive signals in neurons of the nucleus of the trigeminal nerve (D) (original magnification ×10) and within glial cells in the cerebellar white matter (E) (original magnification ×20). BVDV, bovine viral diarrhea virus.

Conclusions

A previously unknown pestiviral agent was found in CT-affected piglets on a farm in Austria. We observed a severe hypomyelination in the entire white matter of the spinal cord and detected pestivirus antigen in the brain of the CT-affected piglets, suggesting a causal relationship between infection and lesions. Analyses of the assembled genome allowed an unambiguous assignment of LV within the genus *Pestivirus* with regard to the presence of pestivirus-specific genes (N^{pro} and E^{rns}) and sequence homology to other pestiviruses (*14*). In contrast to APPV, which hardly infects cultured cells at all, LV could be easily propagated on porcine cell lines without the need for adaptation, similar to what has been reported for BV. We suggest that LV likely shares a common ancestor with BV.

After its description ≈10 years ago, BV was intensively sought worldwide, but the virus has never been detected outside Australia. The broad cell culture tropism of BV led to the hypothesis that the virus recently jumped from another species to porcine hosts (*15*). The discovery of a related pestivirus with substantial sequence divergence in swine on a different continent suggests that both viruses probably have a porcine origin. The identification of the cross-reacting E2-specific monoclonal antibody 6A5 indicates that a cross-reactivity to related pestiviral proteins exists, which might interfere with the serologic testing for CSFV. Further work is needed to investigate the prevalence and the epidemiology of LV in Europe and to assess its virulence in controlled animal experiments.

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B.L., Lu.S., and T.R. are inventors on a patent application on Linda pestivirus (application no. 62/437, 888). Lu.S. and A.L. examined the animals, provided field samples, and wrote parts of the manuscript. B.L., C.R., Le.S., and T.R. performed the laboratory work and analyzed the data. S.H., B.R.B., and H.W. performed necropsy, histological, immunohistochemical, and ultrastructural examinations on CT animals and wrote parts of the manuscript. B.L. and T.R. interpreted the results and designed the figures. B.L. and T.R. wrote the manuscript. All authors critically analyzed, revised, and approved the manuscript.

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Recombinant GII.P16-GII.2 Norovirus, Taiwan, 2016

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In Taiwan, acute gastroenteritis outbreaks caused by a new norovirus genotype GII.2 increased sharply toward the end of 2016. Unlike previous outbreaks, which often involved restaurants, GII.2 outbreaks mainly occurred in schools. Phylogenetic analysis indicates that these noroviruses are recombinant GII.P16-GII.2 strains.

Norovirus, the leading global cause of epidemic gastroenteritis, is responsible for >90% of all viral gastroenteritis and ≈50% of gastroenteritis outbreaks worldwide (*1*). In Taiwan, variants of the genotype GII.4 had been the most prevalent genotype in norovirus-associated acute gastroenteritis outbreaks until mid-2014 (*2*). Since winter 2014–15, a previously uncommon genotype, GII.17, became the predominant genotype in Taiwan. This GII.17 strain also caused many outbreaks in Japan and was reported sporadically in other parts of the world (*3*,*4*). Since September 2016, we have identified a new GII.P16-GII.2 strain that has replaced GII.17 as the predominant strain in norovirus-associated outbreaks in Taiwan, concurrent with a sharp increase in GII.P16-GII.2 norovirus outbreaks and sporadic cases during the 2016–17 season in Germany (*5*).

The Study

During January 2015–December 2016, a total of 876 acute gastroenteritis outbreaks were reported to the Centers for Disease Control in Taiwan. A total of 576 (65.8%) outbreaks were identified as norovirus by reverse transcription PCR with primer pairs G1SKF/G1SKR and G2SKF/G2SKR for GI and GII genogroup detection, respectively; all positive samples underwent sequence-based genotyping by using the Norovirus Genotyping Tool (Table 1) (*2*,*6*,*7*). Before August 2016, GII.2 strains had been detected only sporadically (Figure 1). By September 2016, GII.2 accounted for 60% of norovirus-associated acute gastroenteritis outbreaks that month and, in December 2016, for 86%. Globally, GII.2 is an uncommon genotype that accounted for ≈1.2% of sporadic norovirus infections in children during 2004–2012; by contrast, GII.4 was responsible for 67.2% of norovirus infections during the same period (*8*). In 2016, the settings of outbreaks involving GII.2 differed from those of non-GII.2; most (72.0%) GII.2 outbreaks occurred in schools, whereas most non-GII.2 outbreaks occurred in restaurants (41.4%) and fewer occurred in schools (31.0%). Within schools, half of GII.2 outbreaks occurred in kindergartens, 33.3% in elementary schools, and 14.9% in junior/senior high schools and colleges/universities (Table 2).

Complete or near full-length open reading frame (ORF) 2 (viral protein [VP] 1) and partial ORF1 (RNA-dependent RNA polymerase [RdRp]) sequences were further obtained from 11 randomly selected Taiwan GII.2 2016 strains by different outbreak and month, as previously described (*2*). However, only 10 and 8 of the RdRp and VP1 sequences, respectively, were of sufficient quality and length for use in analysis. We identified the RdRp region of all GII.2 Taiwan strains as genotype GII.P16 (Figure 2, panel A). Phylogenetic trees were inferred from GII.P16 RdRp and GII.2 VP1 sequences, and in both trees, the GII.2 Taiwan strains form 2 major clusters: 2016 GII.2 Taiwan strains and pre-2016 Taiwan GII.2 strains (Figure 2). Before GII.P16-GII.2 strains appeared, GII.P16 polymerases were found in combination with other strains, such as GII.17 (GenBank accession no. KJ196286), GII.4 Sydney (GenBank accession no. LC175468), GII.10 (GenBank accession no. KC110854), GII.3 (GenBank accession no. KF944110), GII.16 (GenBank accession no. AY682551) in Japan, Korea, and Europe, and GII.13 (GenBank accession no. KM036380) in Taiwan (*9*–*12*). In 2014, GII.P16-GII.2 recombinant strains also were detected sporadically, accounting for only 2 (1.1%) of 170 GII strains in South Korea (*12*). However, these South Korean GII.P16-GII.2 recombinants (GenBank accession nos. KC110856, KC110857) are more similar to the pre-2016 Taiwan GII.P16-GII.2 strains than to the 2016 recombinants in ORF1 phylogenetic tree (Figure 2, panel A). Phylogenetic analysis of VP1 genes of South Korean strains also grouped them with pre-2016 Taiwan GII.P16-GII.2 strains (data not shown). During the 2016–17 norovirus season in Germany, GII.P16-GII.2 strains also were found in nearly half of outbreaks (31/65) and sporadic cases (29/65) (*5*). For the *RdRp* gene, the closest strains to 2016 Taiwan GII.P16-GII.2 strains are the 2016 Germany GII.P16-GII.2 strains (GenBank accession nos. KY357454, KY357459) and a 2016 Japan GII.P16-GII.4 recombinant strain (GenBank accession no. LC175468), which had 98.6%–99.4% identity (Figure 2, panel A) (*10*).

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Table 1. Norovirus detection in acute gastroenteritis outbreaks, Taiwan, 2012–2016

Detection	Outbreaks, no. (%)				
	2012	2013	2014	2015	2016
Total norovirus	175 (57.8)	80 (32.1)	76 (28.8)	262 (60.9)	314 (70.4)
GII.2/GII.2 mixed*	4 (2.3)	1 (1.3)	6 (7.9)	9 (3.4)	75 (23.9)
Non-GII.2	171 (97.7)	79 (98.7)	70 (92.1)	253 (96.6)	239 (76.1)
Total acute gastroenteritis	303	249	264	430	446

*Outbreaks involving either only GII.2 or GII.2 with other GI or GII genotypes.

In Japan, during the 2004 and 2007–2010 norovirus outbreaks, GII.2 strains mostly were associated with GII.P2 polymerase, and few that had GII.P16 polymerase were classified as recombinants. These recombinants are more closely related to pre-2016 Taiwan strains than to 2016 strains (Figure 2, panel A) (*13*). For the VP1 phylogenetic tree, 2016 Taiwan strains were most closely related to the Germany GII.P16-GII.2 strains (GenBank accession nos. KY357459, KY357454) and a Hong Kong GII.2 strain also sampled in 2016 (GenBank accession no. KY421044), which had 97.8%–99.0% identity, and more distantly to a 2011 US strain (GenBank accession no. KJ407074) and a 2012 Japan (GenBank accession no. LC145787) strain (95% identity for each). The pre-2016 Taiwan strains sampled from 2011–2015 outbreaks that were more closely related to Japan chimeric GII.P16-GII.2 strains that were identified during the 2009–10 and 2012–2014 outbreak seasons in Japan (*13*) (Figure 2, panel B). In both trees, all Taiwan GII.P16-GII.2 strains from 2016 form a cluster that shares close genetic distance (>99% similarity), regardless of the type of transmission or setting of the outbreaks, and the 2016 Taiwan and Germany GII.P16-GII.2 strains are too closely related to discern the difference, suggesting that these 2016 GII.P16-GII.2 strains descended from a recent ancestor that might have been imported and spread within a short time. Furthermore, the close relation of pre-2016 Taiwan GII.P16-GII.2 strains to Japan GII.P16-GII.2 strains and 2016 Taiwan GII.P16-GII.2 strains to 2016 Germany and Hong Kong GII.P16-GII.2 strains could indicate that the pre-2016 and 2016 waves of outbreaks each derived independently from GII.2 strains that were circulating elsewhere globally.

Analysis revealed that RdRp and VP1 of 2016 and pre-2016 Taiwan strains share higher degree of amino acid identity (>98%) than nucleotide identity (91%–93%), indicating more synonymous mutations between the 2 groups. Sites in VP1 that can be used to differentiate between pre-2016 and 2016 Taiwan GII.P16-GII.2 strains were identified as $S_{71}A$, $V_{335}I$, $T_{344}A$, $A_{354}G$, and $D_{400}E$, of which 3 of the sites shared the same residue between the 2016 strains and the 1976 Snow Mountain strain (GenBank accession no. AY134748). However, previous studies have shown that for GII.2 strains, antibodies against the 1976 Snow Mountain strain can block and recognize strains as recent as 2010; thus the GII.2 strains have had only limited evolution in antigenic sites (*14*). Recent 2016 GII.P16-GII.2 strains became the predominant strains of norovirus-associated acute gastroenteritis in Taiwan (Figure 1), although whether they were involved in outbreaks before 2004 is unknown because norovirus monitoring in Taiwan did not begin until that year. The recent outbreaks illustrated the potential of GII.2 to turn from a sporadically detected strain into an unprecedented dominating strain.

Conclusions

We report the previously uncommon norovirus genotype GII.P16-GII.2 causing an epidemic in Taiwan in late 2016. Previously, GII.2 strains were infrequently reported among both sporadic cases and outbreaks in Taiwan

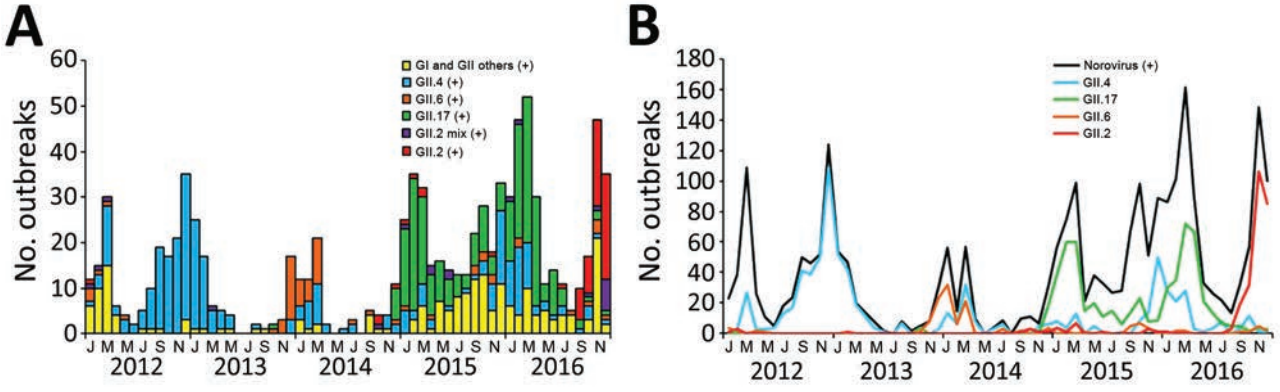

Figure 1. Reported monthly norovirus outbreaks, Taiwan, 2012–2016. A) Outbreaks caused by seasonal predominant strains (GII.2, GII.2 mixed with other genotypes in the same outbreak, GII.17, GII.6, GII.4, and other GI and GII genotypes). B) Monthly trends of all norovirus and predominant norovirus genotypes. +, positive.

Table 2. Settings of norovirus outbreaks, Taiwan, 2016

Setting	GII.2*	Non-GII.2	Total	p value
School	54 (42.2)	74 (57.8)	128	<0.0001†
Kindergarten	28 (66.7)	14 (33.3)	42	<0.0001†
Elementary school, grades 1–6	18 (31.6)	39 (68.4)	57	0.0294†
Junior high, grades 7–9	3 (20)	12 (80)	15	0.064‡
Senior high, grades 10–12, and college/university	5 (35.7)	9 (64.3)	14	0.6033‡
Restaurant§	13 (11.6)	99 (88.4)	112	<0.0001†
Hospital	0	7 (100)	7	0.2035‡
Long-term care facility	1 (3.7)	26 (96.3)	27	0.0101†
Prison/military	0	4 (100)	4	0.5759‡
Other¶	7 (19.4)	29 (80.6)	36	0.5066†
Total	75 (23.9)	239 (76.1)	314	

*Comprises outbreaks with either only GII.2 or GII.2 with other GI or GII genotypes.
†By χ^2 test.
‡By Fisher exact test.
§Includes hotels.
¶Private residences, street food, dormitories, day care centers.

and globally. Before 2014, variants of GII.4, such as New Orleans 2009 and Sydney 2012, followed this paradigm of emergence and rapid replacement every few years, but no non-GII.4 genotypes predominated until the 2014–15 season, when GII.17 emerged in Asia (*4*,*8*). The simultaneous detection and close relation of 2016 Taiwan GII.P16-GII.2 strains with 2016 Germany strains is of importance; given that the previous GII.17 epidemic was limited to Asia, the prevalence of GII.P16-GII.2 should be monitored to assess whether this could become the new predominant strains in other parts of the world. Continued surveillance and unified systems for norovirus typing are critical to monitor the emergence and impact of these and other new norovirus strains.

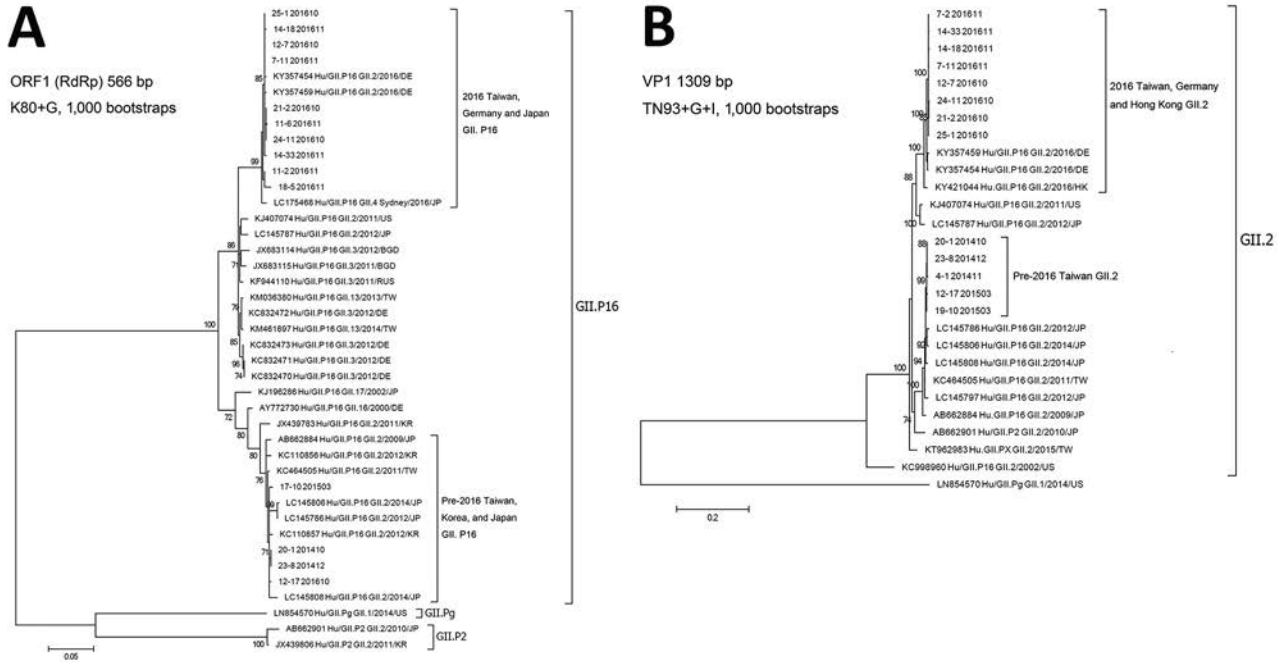

Figure 2. Phylogenetic trees for norovirus GII.2 strains, Taiwan, 2014–2016. A) Partial ORF1 nucleotide sequences in RdRp region (644 nt) of GII.2 strains aligned and the tree generated by using Kimura 80 substitution model with gamma site rates, 1,000 bootstrap replicates, by using MEGA 6.0 software (http://www.megasoftware.net). Bootstrap values of 1,000 replications are shown on the branches. B) Full-length ORF2 nucleotide sequences of GII.2 strains aligned and the tree generated by using Tamura-Nei substitution model with gamma site rates with invariant site using MEGA 6.0 software with 1,000 bootstrap replicates. Bootstrap values of 1,000 replications are shown on the branches. For both trees, norovirus GII.2 reference sequences were downloaded from GenBank; accession number, country, and year are shown. Sequences from Taiwan are indicated by outbreak number and collection year and month. Taiwan GII.2 strains for 2016: 25-1 201610, 14-18 201611, 7-11 201611, 12-7 201610, 24-11 201610, 21-2 201610, 14-33 201611, 11-2 201611, and 18-5 201611; for 2015: 17-10 201503 and 12-17 201503; for 2014: 20-1 20141, 23-8 201412, and 4-1 201411. Corresponding GenBank accession numbers are KY457721–KY457736. BGD, Bangladesh; DE, Germany; HK, Hong Kong; JP, Japan; KR, South Korea; ORF, open reading frame; RdRp, RNA-dependent RNA polymerase; RUS, Russia; TW, Taiwan; US, United States; VP, viral protein. Scale bars indicate nucleotide substitutions per site.

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Emergency Meningococcal ACWY Vaccination Program for Teenagers to Control Group W Meningococcal Disease, England, 2015–2016

Helen Campbell, Michael Edelstein, Nick Andrews, Ray Borrow, Mary Ramsay, Shamez Ladhani

During the first 12 months of an emergency meningococcal ACWY vaccination program for teenagers in England, coverage among persons who left school in 2015, the first cohort to be vaccinated, was 36.6%. There were 69% fewer group W meningococcal cases than predicted by trend analysis and no cases in vaccinated teenagers.

Several countries in Europe, South America, and Australia are experiencing outbreaks of group W meningococcal (MenW) disease, caused by a hypervirulent strain of *Neisseria meningitides* belonging to sequence type 11 (ST11) clonal complex (CC) and associated with severe disease and a high case-fatality rate (*1*). In England, MenW cases caused predominantly by ST11 increased from 19 in epidemiologic year 2008–09 to 176 in 2014–15, which represented 2% and 24%, respectively, of all invasive meningococcal disease (IMD) cases (*2*).

In response to the national increase in MenW cases in England, an emergency immunization program with meningococcal ACWY conjugate vaccine (MenACWY) for adolescents was started in August 2015 (*3*). This program replaced the MenC program for children 13–14 years of age, and there was also a 2-year phased catch-up program for persons 14–18 years of age as of August 31, 2015 (Table 1) (*3*). Vaccine was also offered to new university entrants ≤25 years of age. Compared with persons of the same age who do not attend university, new university entrants have a higher risk for IMD, likely because of social factors that increase meningococcal transmission (*4*). This single-dose vaccination program aimed to directly protect vaccine-eligible cohorts and, in the long term, indirectly protect the wider population by reducing meningococcal carriage (*5*).

Students in the target group who left secondary school in the summer of 2015 and were 18 years of age before September 2015 were the first cohort offered the vaccine through general medical practices, starting in August 2015. Nearly one third of this cohort were accepted into universities in 2015 (*6*). We report impact and vaccine effectiveness data for the first 12 months of the MenACWY program in England.

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The Study

Public Health England conducts enhanced national IMD surveillance in England, where 84% of the UK population resides. National Health Service hospital laboratories routinely submit local invasive meningococcal isolates to the Public Health England Meningococcal Reference Unit for confirmation and characterization, with national PCR testing also offered (*7*). Confirmed case-patients are routinely followed up for additional details, including vaccination history and outcome (*8*).

We assessed whether MenACWY vaccine coverage might be higher among new university entrants. We compared vaccine coverage in June 2016 estimated from data automatically extracted from primary care databases in university-affiliated (n = 79) medical practices on a university campus or recommended by the university on their website and non–university-affiliated (n = 7,543) general medical practices for persons who left school in 2015.

To estimate vaccine impact, we compared confirmed MenW, MenY, and MenB cases in persons who left school in 2015 with projected cases for the first academic year (September 2015–August 2016) after program introduction. To estimate projected case numbers for 2015–16 in the absence of vaccination, we fitted a Poisson regression model with age and time-trend parameters for case-patients 19–24 years of age during 2010–11 and 2015–16 who were not in vaccine-targeted cohorts. We used this model to estimate case projections and incidence rate ratios, which are presented as percentage decrease (1 – incidence rate ratio). MenC cases were excluded because of successful MenC vaccination programs for persons 14–16 years of age and new university entrants available since September 2013.

We assessed vaccine effectiveness among persons who left school during the 2015–16 academic year. Vaccine effectiveness was estimated by using the screening

Table 1. Scheduling of meningococcal ACWY vaccination routines and 3-year catch-up vaccination programs, England*

Birth cohort	School year (age, y, at end of 2014–15 academic year on 2015 Aug 31)	Earliest school year at time of vaccination and type of vaccine received			
		2014–15	2015–16	2016–17	2017–18
2003 Sep 1–2004 Aug 31	6 (11)	NA	NA	NA	Y9 ACWY†
2002 Sep 1–2003 Aug 31	7 (12)	NA	NA	Y9 ACWY†	‡
2001 Sep 1–2002 Aug 31	8 (13)	NA	Y9 ACWY†	‡	‡
2000 Sep 1–2001 Aug 31	9 (14)	Y9 MenC§	NA	Y11 ACWY¶	‡
1999 Sep 1–2000 Aug 31	10 (15)	NA	Y11 ACWY¶	‡	‡
1998 Sep 1–1999 Aug 31	11 (16)	NA	NA	Y13 ACWY#	‡
1997 Sep 1–1998 Aug 31	12 (17)	NA	Y13 ACWY#	‡	‡
1996 Sep 1–1997 Aug 31	13 (18; those who left school in 2015)	Y13 ACWY#	‡	‡	‡

*NA, not applicable; Y, year.
†New routine schedule MenACWY vaccination.
‡Completed MenACWY vaccination of cohort.
§Routine schedule MenC vaccination.
¶School-based MenACWY catch-up cohorts.
#General medical practice–based MenACWY catch-up cohorts.

method (*9*). Vaccine coverage in cases was compared with population vaccination coverage in age-matched peers in England (*10*).

MenW cases in England increased overall by 15%, from 189 in the 2014–15 academic year to 218 in 2015–16. Isolates were available for 178 culture-confirmed cases in 2015–16; a total of 155 (87%) were ST11 CC. Case numbers and incidence increased in every age group except persons 15–19 years of age (26 to 18 cases; 31% reduction) and infants <1 year of age (26 to 17 cases; 35% reduction) (Table 2). Six (33%) of 18 teenage case-patients died, but no infant case-patients died.

By June 2016, vaccine coverage in persons who left school was 36.6%; a total of 79% of these vaccinations were administered during August–September 2015. Vaccine coverage among persons who left school was higher in university-affiliated medical practices than in non–university-affiliated medical practices (56.1% vs. 33.8%; p<0.0001) (Figure 1).

During the first 12 months of the MenACWY vaccination program for teenagers, there were 6 confirmed MenW cases among ≈650,000 persons who left school compared with a projected 19.4 cases (69% decrease, 95% CI 18%–88%), (Figure 2). Five of the 6 cases had ST11 CC (3 were confirmed by PCR only, and ST11 CC war inferred for PorA P1.5.2). None of these 6 eligible case-patients had received MenACWY vaccine, and only 1 (a student from overseas who was not vaccinated) was in a university setting. On the basis of population coverage of 36.6% among persons who left school, early estimated vaccine effectiveness was 100% (95% CI –47% to 100%), but CIs were wide because of small numbers.

One case each of MenY and MenC disease were diagnosed in persons (both not vaccinated) who left school during 2015–16 compared with 3 of each in 2014–15. MenB cases increased from 10 in 2014–15 to 17 in 2015–16. Of the 17 case-patients in 2015–16, six (35%) had received MenACWY vaccine, consistent with national vaccine coverage.

Conclusions

We found a major reduction (69%) in observed MenW cases compared with predicted MenW cases among the first cohort in England to be offered MenACWY conjugate vaccine after the first year of an emergency vaccination program for teenagers, even with a small number of cases. This decrease occurred despite national vaccine coverage of only 36.6% for this cohort. All case-patients who left school in 2015 and had confirmed MenW disease were not vaccinated; the only university case was in an overseas

Table 2. Age distribution of laboratory-confirmed cases of group W meningococcal infection in students, by academic year, England, 2010–11 to 2015–16*

Age group, y	Academic year											
	2010–11		2011–12		2012–13		2013–14		2014–15		2015–16	
	No. cases	Incidence	No. cases	Incidence	No. cases	Incidence	No. cases	Incidence	No. cases	Incidence	No. cases	Incidence
<1	3	0.45	4	0.59	3	0.43	12	1.77	26	3.91	17	2.56
1–4	8	0.31	6	0.23	5	0.19	5	0.18	22	0.80	26	0.94
5–14	1	0.02	1	0.02	3	0.05	1	0.02	4	0.06	7	0.11
15–19	4	0.12	1	0.03	13	0.40	13	0.40	26	0.80	18	0.56
20–24	3	0.09	1	0.03	3	0.08	7	0.19	6	0.17	18	0.50
25–44	1	0.01	3	0.02	6	0.04	6	0.04	7	0.05	10	0.07
45–64	7	0.05	6	0.04	13	0.10	12	0.09	29	0.21	43	0.31
≥65	11	0.13	11	0.13	15	0.17	34	0.37	69	0.72	79	0.81
Total	38	0.07	33	0.06	61	0.11	90	0.17	189	0.35	218	0.40

*Academic years are September 1–August 31. Incidence, cases/100,000 population.

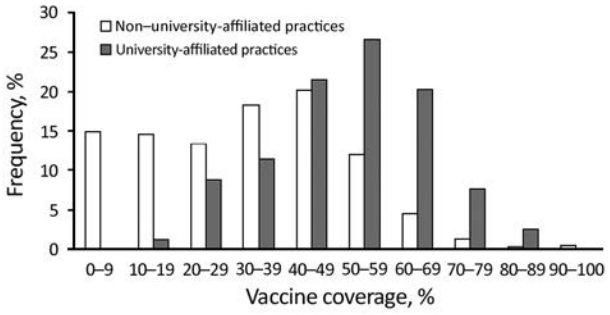

Figure 1. Frequency distribution of meningococcal ACWY conjugate vaccine coverage among teenagers who left school in 2015 in university-affiliated (n = 79) and non–university-affiliated (n = 7,543) general medical practices, England, June 2016. University-affiliated medical practices are either on campus or recommended by universities. The list might not be comprehensive, and non–university-affiliated medical practices will still register students.

student who was not vaccinated. Higher vaccine coverage among university-affiliated general medical practices suggests that persons who left school and were enrolled in universities were more likely to be vaccinated than age-matched peers who did not seek higher education. Some universities have actively vaccinated new entrants and achieved high uptake rates (*11*).

Our initial data on vaccine effectiveness and effect on disease among persons who left school in 2015 are encouraging. However, continued surveillance is vital. Multicomponent MenB vaccine 4CMenB (Bexsero, Basel, Switzerland) was added to the UK national infant vaccination program for infants in September 2015 (*12*). Unlike conjugated polysaccharide meningococcal vaccines, 4CMenB is not capsule specific and has the potential to offer broader protection against all meningococcal strains.

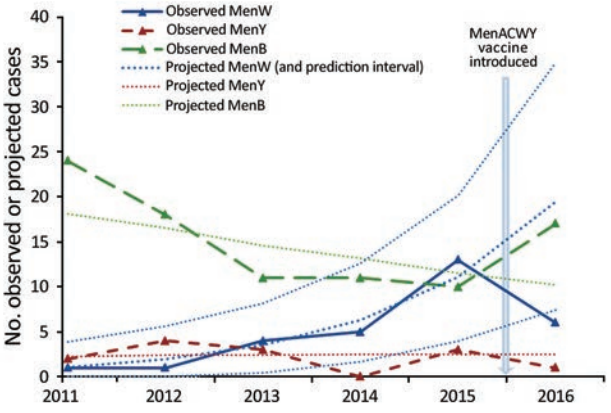

Figure 2. Observed and projected cases of W, Y, and B invasive meningococcal disease in England determined on the basis of trend lines fitted to the prevaccination period (November 2010–11 to 2014–15) and extrapolated to the 2015–16 academic year for the cohort with group W, Y, and B invasive meningococcal disease and who left school. Men, meningococcal.

Antibodies from 4CMenB-vaccinated infants showed potent serum bactericidal antibody activity against the hypervirulent MenW ST11 strain (*13*), which is consistent with the observed decrease in MenW cases among infants.

Public Health England will continue to monitor effects of vaccination programs as more cohorts are vaccinated. Younger cohorts are receiving MenACWY conjugate vaccine through a school-based program; uptake rates are much higher (72%–84%) than for persons who left school and were vaccinated through general medical practices (*14*). Although overall MenW cases increased in 2015–16 compared with 2014–15, the proportionate increase in cases was lower than in previous years, when cases were nearly doubling every year. Whether this finding indicates early signs of an indirect effect is speculative, but 4 months into the 2016–17 academic year, total MenW case numbers are only 8% higher than at the same time in 2015–16.

By the autumn of 2017, MenACWY vaccine will have been offered to all targeted teenagers in the United Kingdom. Because this group also has the highest meningococcal carriage rates (*15*), we hope that preventing carriage through vaccination will reduce cases and deaths in unvaccinated cohorts across all age groups in the coming years.

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etymologia

Meningococcal [*mə-ning"go-kok'al*] Disease

Ronnie Henry

From the Greek *meninx* (“membrane”) + *kokkos* (“berry”), meningococcal disease was first described by Vieusseux during an outbreak in Geneva in 1805. In 1884, Italian pathologists Ettore Marchiafava and Angelo Celli described intracellular micrococci in cerebrospinal fluid, and in 1887, Anton Wiechselbaum identified the meningococcus (designated as *Diplococcus intracellularis meningitidis*) in cerebrospinal fluid and established the connection between the organism and epidemic meningitis. Meningococcus can cause endemic cases, clusters, and epidemics of meningitis and septicemia.

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Association of GII.P16-GII.2 Recombinant Norovirus Strain with Increased Norovirus Outbreaks, Guangdong, China, 2016

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An unusual prevalence of recombinant GII.2 noroviruses (GII.P16-GII.2) in Guangdong, China, at the end of 2016 caused a sharp increase in outbreaks of acute gastroenteritis. This event was another non-GII.4 epidemic that emerged after the GII.17 viruses in 2014 and 2015 and warrants global surveillance.

During the past 20 years, GII.4 genotypes have been responsible for most norovirus outbreaks globally (*1*). New GII.4 variants have emerged every 2 or 3 years, replacing the previous dominant variant. Likewise, in Guangdong, China, as well as in other regions of China, GII.4 noroviruses have caused most outbreaks; several other genotypes, such as GI.3, GI.6, GII.6, and GII.21, were occasionally detected in sporadic cases but rarely caused large outbreaks (*2*).

The Guangdong provincial surveillance network for foodborne disease outbreaks has been active since 2008 and is responsible for the surveillance of norovirus outbreaks in Guangdong. During winter 2014–15, a new GII.17 variant (GII.P17-GII.17 Kawasaki) was first identified in Guangdong and caused a substantial increase in the number of acute gastroenteritis outbreaks (*3*). Epidemics caused by this lineage were detected almost simultaneously in several other provinces of China, and sporadic cases were reported worldwide (*4*). We report an increase in the number of outbreaks associated with a GII.P16-GII.2 recombinant norovirus strain in the last months of 2016 in Guangdong.

The Study

In Guangdong, norovirus outbreaks are highly seasonal; most (>90%) are reported during November–March (*3*). In November 2014, a GII.17 variant was identified in Guangdong and became predominant during the 2014–15 and 2015–16 norovirus seasons (*5*). After June 2016, the number of GII.17 outbreaks decreased, replaced by GII.4 outbreaks (Figure 1). During November and December 2016, a sharp increase in the number of norovirus outbreaks was reported in multiple cities of Guangdong through the provincial surveillance network (Figure 1). Seventeen (81%) of the 21 outbreaks in 10 cities were typed as GII.2; these outbreaks resulted in 760 clinical cases (Figure 1). This genotype was first detected in Guangzhou on November 14, 2016, and spread rapidly thereafter. The sharp increase in the number of outbreaks and the unusual GII.2 genotype prompted us to further characterize these viruses.

We randomly selected 5 fecal samples from each GII.2 outbreak for Sanger sequencing. The full length of *RdRp* and *VP1* genes were sequenced by using specific oligonucleotide primer sets (online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/7/17-0333-Techapp1.pdf) Representative sequences were deposited in GenBank (accession nos. KY485107–KY485126). A primer set (JV12RY and JV13I [*6*]) commonly used for RdRp typing failed to amplify the corresponding fragments of GII.2/Guangdong/2016 strains because of mismatches with these primers. We compared the Guangdong GII.2 viruses with related *VP1* (GII.2) and *RdRp* gene sequences (P16) from GenBank. Molecular clock phylogenetic analysis was performed to analyze the evolution of the *VP1* gene sequences by using the Bayesian Markov chain Monte Carlo framework with a generalized time-reversible nucleotide substitution model (*7*) and an uncorrelated lognormal relaxed clock model (*8*) (Figure 2). Most GII.2 sequences before 2004 uploaded to GenBank were from Japan, the United States, and the Netherlands (online Technical Appendix Figure 1). GII.2 strains detected after 2004 clustered into a major lineage that included the viruses detected in Guangdong in 2016. This lineage, which also included the GII.2 viruses reported in Germany (*9*), was estimated to have emerged during 2011–2014 (95% highest posterior density 2011.7–2014.5) and was divergent from GII.2 strains detected in Japan in 2014. The genetically closest *VP1* gene sequences were GII.2 viruses from the United States in 2011 and from Japan in 2011 and 2012 (Figure 2). Consistent with the tight cluster of the capsid sequences, the

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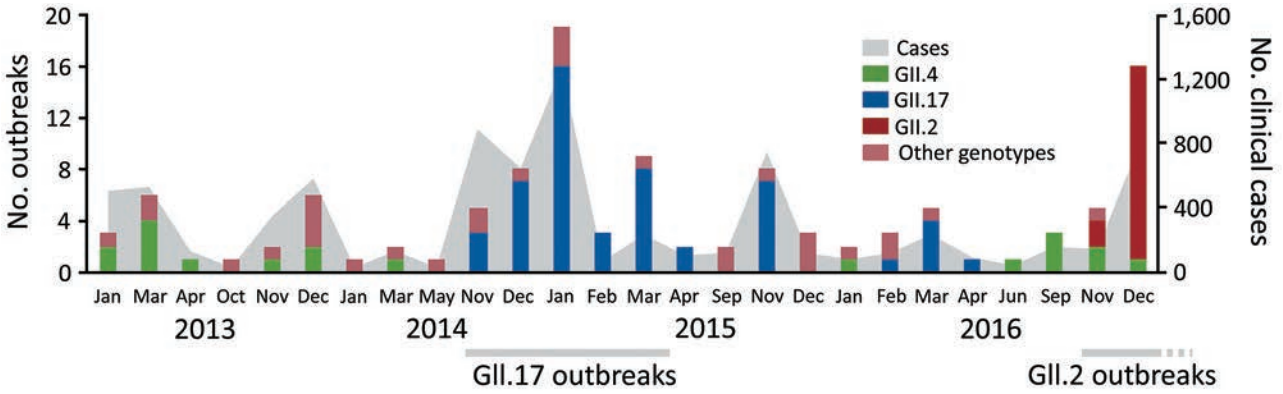

Figure 1. Number of reported GII norovirus outbreaks and confirmed clinical cases, Guangdong, China, January–December 2016.

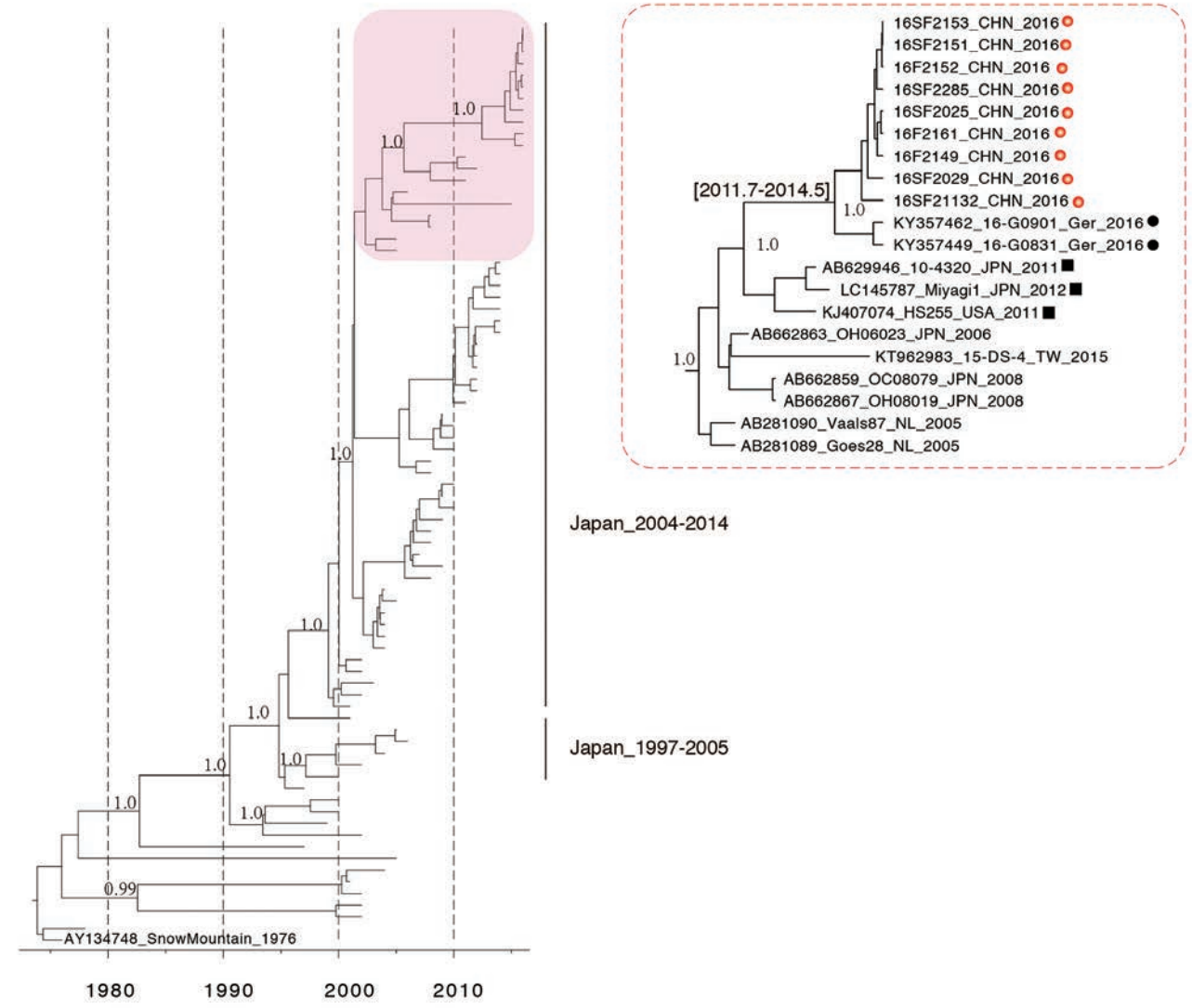

Figure 2. Molecular clock phylogeny of norovirus strain GII.2 VP1 gene sequences. The tree is a maximum clade credibility phylogeny with the GII.2 VP1 sequences, including the Guangdong, China, outbreak strains (red box, enlarged at right). Red dots indicate GII.2/Guangdong/2016 strains; black dots indicate outbreak strains from Germany, 2016; black squares indicate closely related GII.2 strains reported in previous years.

GII.P16 *RdRp* gene sequences of the Guangdong and Germany outbreaks were closely related with GII.P16 sequences from GII.4 Sydney viruses detected in Japan and the United States (online Technical Appendix Figure 2).

Conclusions

We report an increased number of norovirus GII.2 outbreaks during fall 2016 in Guangdong Province, China. The provincial surveillance network reported 17 GII.2 norovirus outbreaks that affected 760 persons during November 14–December 28, 2016. On the basis of complete viral polymerase gene (*RdRp*) and capsid gene (*VP1*) nucleotide sequences, all viruses could be typed as GII.P16-GII.2.

The driving force behind the emergence and spread of GII.2 noroviruses in Guangdong Province is not clear, but the fact that all viruses had a GII.P16 *RdRp* gene might indicate that possession of this polymerase gene makes these viruses more virulent. Similar GII.P16 *RdRp* genes have been reported as part of GII.4 Sydney viruses recently identified in Japan (GenBank accession no. LC153122, GII.P16-GII.4/JP/2016) (*10*) and the United States (GenBank accession no. KX907727, GII.P16-GII.4/U.S./2015) (online Technical Appendix Figure 1), which suggests that recent recombination events of GII.2 and GII.4 Sydney viruses with GII.P16 *RdRp* gene sequences might have been responsible for most of the recent norovirus activity.

The number of GII.P16-GII.2 outbreaks peaked at the end of 2016, similar to the epidemic season caused by the GII.P17-GII.17 variant in 2014. The emergence of GII.2 viruses was almost simultaneously reported in several other regions of China (China National Surveillance Network for Foodborne Disease Outbreaks, February 2017) as well as in Germany (*9*), suggesting the fast spread of this genotype on multiple continents. As a result, the predominant norovirus genotype in Guangdong Province has changed from GII.4 viruses, of which a new variant emerged every 2 or 3 years, to non-GII.4 viruses with GII.17 during the 2014–15 winter season and GII.2 viruses during the 2016–17 season. Since the GII.17 variant has spread to several countries in Asia and has been reported on different continents, the effect of this emerging GII.2 recombinant strain warrants further global surveillance.

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Rabbit Hepatitis E Virus Infections in Humans, France

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Hepatitis E virus (HEV) has been detected in rabbits, but whether rabbit HEV strains can be transmitted to humans is not known. Of 919 HEV-infected patients in France during 2015–2016, five were infected with a rabbit HEV strain. None of the patients had direct contact with rabbits, suggesting foodborne or waterborne infections.

Reports of hepatitis E virus (HEV) infections in humans and animals are becoming more frequent. HEV is a member of the family *Hepeviridae*. HEV strains that infect humans (HEV1, HEV2, HEV3, HEV4, and HEV7) belong to the genus *Orthohepevirus* (*1*). In industrialized countries, HEV transmission is mainly zoonotic, and the most prevalent genotype is HEV3. This genotype is transmitted mainly by direct contact with infected pigs, eating contaminated food products, or the environment (*2*). Genotype 3 includes 3 clades, 2 (3-efg and 3-abchij) of which are found in humans and pigs and 1 (3-ra) of which is found in rabbits (*3*). HEV3-ra has a 93-nt insertion in the X domain of the genome (*4*). This virus has been identified in both farmed and wild rabbits worldwide (*4*) and a pet rabbit (*5*).

HEV3 infections are generally asymptomatic and self-limiting, but symptomatic acute hepatitis develops in some patients, mostly older men. Fulminant hepatitis can occur in patients with underlying liver disease, and HEV3 infections can become chronic in immunocompromised patients, such as recipients of solid-organ transplants, persons with hematologic diseases, and patients infected with HIV (*2*). Although only 1 case of infection with human HEV3-ra has been identified (*6*), the contribution of HEV3-ra to human infection remains uncertain.

The Study

The French National Reference Center for HEV (Paris, France) analyzed 919 HEV strains obtained from patients in France infected during 2015–2016. Strains were obtained in hospitals (90%) or private medical laboratories (10%). A total of 20% of the strains were obtained from immunocompromised patients.

We detected HEV RNA by using a reverse transcription PCR (RT-PCR) 15189 accredited by the International Organization of Standardization (Geneva, Switzerland) (*7*). We used a nested RT-PCR to amplify a 345-nt sequence within HEV open reading frame 2 as described (*8*). To amplify a 365-nt fragment within the X domain, we performed a nested RT-PCR with outer primers 2600-DOM-X-S (5′-TAYCGRGARACYT-GYTCCCG-3′) and 3050-DOM-X-AS (5′-ACATCRA-CATCCCCCTGYTGTATRGA-3′) and inner primers 2685-DOM-X-S (5′-AGYTTTGAYGCCTGGG-3′) and 3050-DOM-X-AS. Phylogenetic analyses were performed by using neighbor-joining methods, a bootstrap of 1,000 replicates, and MEGA version 7.0 software (http://www.megasoftware.net/).

Among the 919 patients, 904 were infected with a genotype 3 strain. The subtype infecting 75 (8.1%) of these HEV3-infected patients could not be determined; 302 (32.9%) patients were infected with clade HEV3-abchij, 522 (56.8%) were infected with clade HEV3-efg, and 5 (0.5%) were infected with clade HEV3-ra (GenBank accession nos. KY611812–KY611816) (Figure). All 5 HEV3-ra strains had an insertion in the X domain of the genome (GenBank accession nos. KY825957–KY825961).

All 5 infected patients were men (median age 52 years, range 38–64 years). None of these men had epidemiologic links to HEV or had traveled abroad; 2 lived in northern France, and 3 lived in southern France. One infected patient was immunocompetent and 4 were immunocompromised. The immunocompetent patient had alcoholic cirrhosis and decompensation of his cirrhosis because of the HEV infection. He cleared the HEV infection spontaneously.

All 4 immunocompromised patients were asymptomatic: 2 were solid-organ transplant recipients, and 2 had hematologic malignancies that were being treated with chemotherapy. Levels of alanine aminotransferase for these 4 patients were persistently high, and plasma HEV RNA was still detected 3 months after the initial evaluation, which indicated a chronic infection (*9*). Three patients were given ribavirin therapy for 3 months. The patients with hematologic malignancies eliminated the virus, but the kidney transplant recipient had a relapse when treatment was stopped.

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The source of infection was unclear because all 5 patients reported they had no direct contact with rabbits. Three patients lived in rural areas, but none recalled any contact with wild or farmed animals. None of them was a hunter. Two patients reported they had eaten rabbit products, but the products were always well-cooked. All patients regularly ate various pork products, and 3 frequently ate raw shellfish (oysters, mussels, or scallops). Two drank tap water, and 3 drank only bottled water. One patient had a vegetable garden (Table).

Conclusions

HEV3-ra can infect humans and its pathogenesis is similar to that of other HEV3 subtypes. There have been frequent reports of autochthonous HEV3 infections in several industrialized countries, particularly France (*10*). The HEV3 subtypes responsible are usually similar to those found in swine. These subtypes belong to clades 3-efg and 3-abchij (*11*,*12*). Their distribution among 919 symptomatic cases (3c, 26.7%; 3e, 2%; and 3f, 47%) in our study was similar to that reported for asymptomatic blood donors in France (*13*). However, HEV3-ra strains were not detected in blood donors, probably because of a small sample size.

The rarity of HEV3-ra infections in humans could be the result of fewer persons eating rabbit than pork products (*14*). In addition, we found that 80% of the HEV3-ra strains were obtained from immunocompromised patients. However, this population represents only 20% of the HEV-infected patients characterized by our laboratory. This HEV3 subtype could be less infectious than other subtypes for humans, but additional studies are needed to verify this hypothesis.

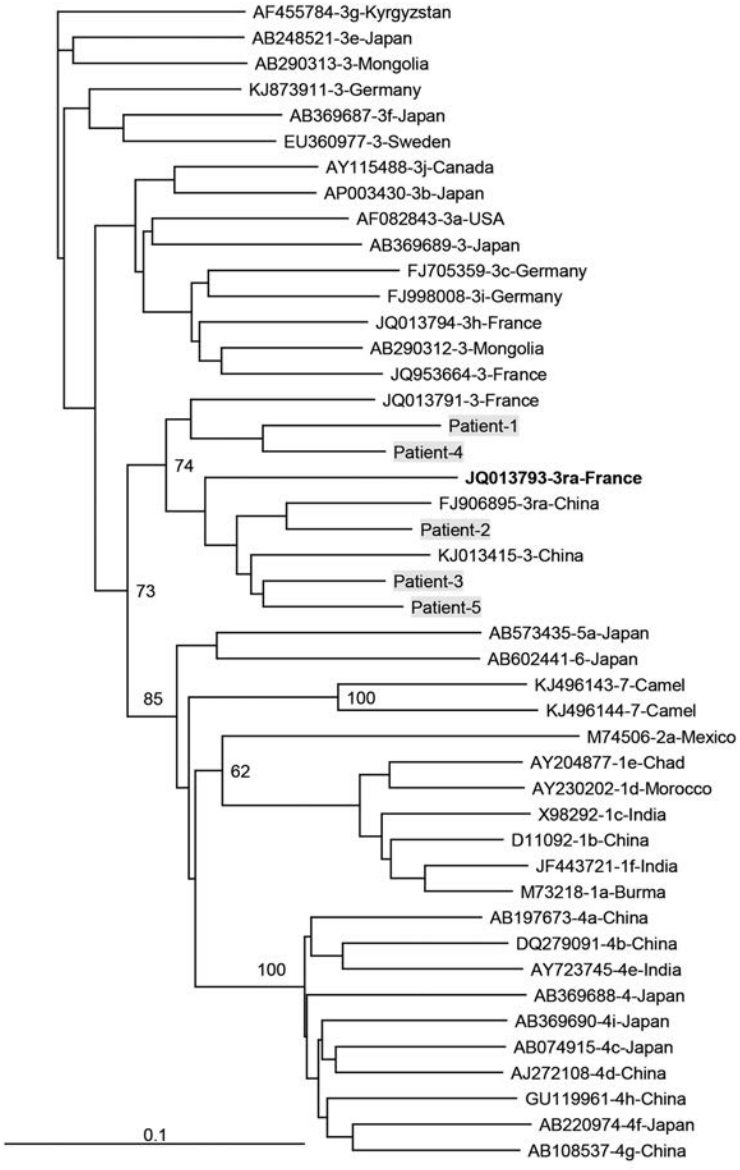

Figure. Phylogenetic tree for a 347-nt sequence within open reading frame 2 of hepatitis E viruses, France. Genetic distances were calculated by using the Kimura 2-parameter method, and the tree was plotted using the by the neighbor-joining method. Values along branches are bootstrap values acquired after 100 replications. Virus sequences obtained from patients in this study (gray shading) were compared with reference sequences of subtypes 3 viruses according to the proposal of Smith et al. (*3*). GenBank accession numbers, genotypes, and countries of origin are listed. Reference sequence in bold is from a hepatitis E virus genotype 3-ra isolated from a human. Scale bar indicates nucleotide substitutions per site.

Table. Characteristics of 5 men infected with rabbit hepatitis E virus, France

Characteristic	Patient 1	2	3	4	5
Age, y	52	38	64	50	62
Location	Southern (urban)	Northern (urban)	Northern (rural)	Southern (rural)	Southern (rural)
Underlying disease or condition	Alcoholic cirrhosis	Kidney transplant	Myeloma	Lymphoma	Heart transplant
Symptomatic acute hepatitis E	Yes	No	No	No	No
Contact with wild animals	No	No	No	No	No
Consumed rabbit	No	Yes	Yes	No	No
Consumed pork	Yes	Yes	Yes	Yes	Yes
Consumed shellfish	No	No	Yes	Yes	Yes
Type of drinking water	Tap	Bottled	Bottled	Bottled	Tap
Vegetable garden	No	No	No	Yes	No

We could not identify the route by which our patients became infected with HEV3-ra. None had any direct contact with rabbits; 2 had eaten well-cooked rabbit. Data from a recent nationwide study in France suggested that waterborne transmission might play a role in HEV epidemiology (*14*). This type of transmission might be the way the patients in our study became infected because we have detected HEV3-ra in environmental samples (F. Abravanel, unpub. data).

To our knowledge, clinical manifestations associated with HEV3-ra have not been reported. We found that HEV3-ra infections caused severe decompensation in a patient with alcoholic cirrhosis. Chronic infections developed in the 4 immunocompromised patients. Patients in industrialized countries infected with other HEV3 subtypes also showed similar clinical findings (*1*). We also found that ribavirin had an antiviral effect on HEV3-ra. Ribavirin eliminated the virus in 2 of the 3 patients given this drug. This finding is consistent with that of a multicenter study, which reported that 78% of persons with chronic HEV3 infections were successfully treated with this drug (*15*).

Our findings emphasize the zoonotic risk for HEV3-ra and expand the spectrum of potential sources of human infection. The route by which HEV3-ra is transmitted to humans needs to be investigated. Longitudinal study of HEV diversity is also needed to assess trends over time.

Dr. Abravanel is an assistant professor at the Virology Department, Toulouse University Hospital, Toulouse, France. Her primary research interest is genetic variability of hepatitis viruses.

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Detection and Genetic Characterization of Adenovirus Type 14 Strain in Students with Influenza-Like Illness, New York, USA, 2014–2015

Daryl M. Lamson, Adriana Kajon, Matthew Shudt, Gabriel Girouard, Kirsten St. George

During the 2014–15 influenza season, 13/168 respiratory samples from students with influenza-like illness (ILI) at a college in New York, USA, were positive for human adenovirus (HAdV); 4/13 samples were positive for HAdV-B14p1. During influenza season, HAdV should be included in the differential diagnostic panel used to determine the etiology of ILI.

Human adenoviruses (HAdVs) contain linear, double-stranded DNA with an average genomic length of 35 kbp. The 51 serotypes identified by seroneutralization and >70 genotypes that have been described by computational analysis of complete genomic sequences are classified within 7 species, designated HAdV-A through HAdV-G (*1*). HAdVs can cause a broad spectrum of disease, including gastroenteritis, conjunctivitis, upper and lower respiratory tract infections, hepatitis, and urinary tract infections (*2*). Most reported illnesses associated with HAdVs are caused by a limited number of types. HAdV type 14 of species HAdV-B (HAdV-B14), which was first identified in the Netherlands in 1955, was rarely reported until the emergence of a new genomic variant, 14p1, was documented in the United States in 2003 by military surveillance (*3*). In 2007 in the United States, sporadic outbreaks of HAdV-B14p1 infection occurred in Oregon (*4*) and at an air force base in Texas (*5*); subsequent outbreaks occurred in other locations in North America (*6*). The most striking genetic difference between variant 14p1 and the prototype strain deWit, which was isolated in the Netherlands in 1957, is a deletion in the fiber gene (*3*). Since its first detection in the United States, the 14p1 variant has been detected in association with acute respiratory diseases of variable severity in several states and countries (*5*,*7*–*10*).

A component of the influenza surveillance conducted by the New York State Department of Health includes the molecular testing and viral culture of respiratory samples submitted by sentinel physicians to the Wadsworth Center Virology Laboratory (Albany, NY, USA). Participating sentinel physicians collect samples from patients with influenza-like illness (ILI), which is defined as fever of >37.8°C plus cough or sore throat. During the 2014–15 influenza season, testing of surveillance specimens detected an increase in samples negative for influenza but positive for HAdV. We report results of sequence analysis for these HAdVs and compare severity of illness for patients infected with different virus types.

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The Study

During the 2014–15 influenza season, staff at the student health clinic of a college in Tomkins County, New York, collected respiratory samples from 168 students seeking care for ILIs and submitted them to the Wadsworth Center Virology Laboratory for testing. We tested all samples by conducting comprehensive respiratory virus culture and influenza virus detection (CDC Human Influenza Virus Real-time RT-PCR Diagnostic Panel; Centers for Disease Control and Prevention, Atlanta, GA, USA). For samples with HAdV-positive results, we performed confirmatory testing by using an adenovirus monoclonal antibody–specific immunofluorescence assay (Light Diagnostics Respiratory Panel Viral Screening and Identification IFA Kit; EMD Millipore, Billerica, MA, USA), and we conducted HAdV molecular typing by partial hexon and fiber gene sequence analysis (*11*,*12*).

Of the 168 samples, 12 (7.8%) were positive for HAdV: HAdV-E4 (8 samples), HAdV-B14 (3 samples), or HAdV-C2 (1 sample), and 1 was suspected to be co-infected with HAdV-E4 and HAdV-B14. For further characterization, we obtained cultured isolates from 2 of the 3 HAdV-B14–positive samples and from the suspected HAdV-E4 and HAdV-B14 co-infected sample. We extracted intracellular genomic HAdV DNA as previously described (*13*) and used it for genome typing by restriction enzyme analysis and for whole-genome sequencing on the MiSeq platform (Illumina, Inc., San Diego, CA, USA). For genomic comparison by whole-genome sequencing, we also processed DNAs from previously characterized HAdV-B14 strains detected in the same geographic region as the study strains; 3 of the strains were isolated in 2011 in New Brunswick, Canada (*10*), and 1 was isolated in

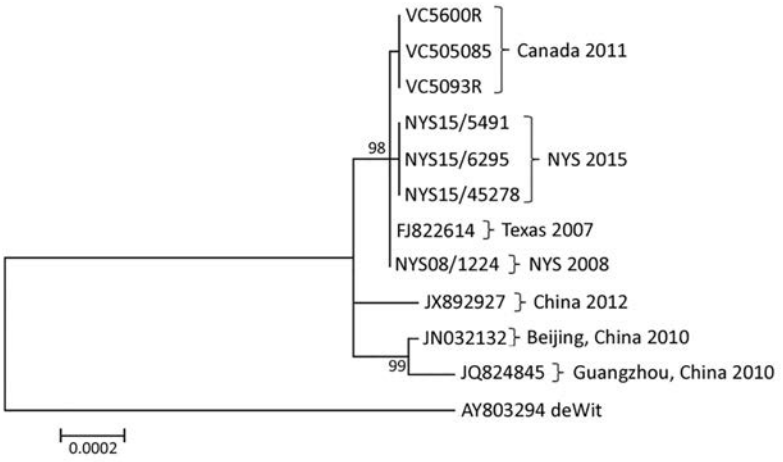

Figure 1. Phylogenetic tree of human adenoviruses constructed using 7 sequences obtained from college students with influenza-like illness, New York, USA, 2014–2015, and reference sequences of isolates from China (GenBank accession nos. JX892927, JN032132, and JQ824845); an isolate from Texas, USA (accession no. FJ822614); and the prototype strain, de Wit, from the Netherlands (accession no. AY803294). The tree was created by using the maximum-likelihood method based on the Kimura 2-parameter model with 500 bootstrap replicates from the whole-genome sequence of the displayed sequences. Pairwise distances were estimated by using the maximum composite–likelihood approach. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA6 (*14*). Scale bar indicates number of substitutions per site. NYS, New York state.

2008 in New York (*3*). We uploaded the fully annotated whole-genome sequences from this study to GenBank (accession nos. KY201426–32).

By in silico restriction enzyme analysis of their genomic sequence, we confirmed that 7 HAdV-B14 strains were genome type 14p1: of them, 3 were among the 4 strains isolated in New York during 2014, 1 was isolated in New York during 2008, and 3 were the strains isolated in Canada during 2011. The fourth 2014 HAdV-B14–positive specimen from New York did not grow in culture, but sequence analysis of the hexon gene and reactivity on a HAdV-B14–specific real-time PCR assay (*4*) identified the virus as HAdV-B14. We aligned and analyzed the 7 HAdV-B14 genomic sequences from this study along with 5 HAdV-B14 sequences available from GenBank (accession nos. AY803294, FJ822614, JN032132, JQ824845, and JX892927). The resulting tree (Figure 1) demonstrated that the HAdV-B14 strains from New York college students with ILI during the 2014–15 influenza season were closely related to strains circulating in North America during previous years, but they were distinguishable from strains isolated from 3 patients in 2011 in New Brunswick. The HAdV-B14 strains isolated from the New York students differed by 1–29 nt from the Canada 2011, Texas 2007, and New York 2008 strains and from strains isolated in China in 2010 and 2012; one aa change was observed in each of the following proteins: E1A, E1B, protein VI, and E3 20.8-kDa.

We did not perform restriction enzyme analysis on the isolate from the patient sample suspected to be co-infected with HAdV-E4 and HAdV-B14. However, virtual digest of the individual de novo–assembled, contiguous, whole-genome sequencing identified HAdV-E4 as genome type 4a1 and HAdV-B14 as 14p1 (data not shown).

Although we could not review patient charts, ILI symptoms and signs were noted on the original test request submission forms, which were available for our evaluation of New York case-patients (Figure 2). All 3 of the HAdV-B14–infected patients had fever, compared with only 5 of 8 HAdV-E4–infected patients (Figure 2). One of the HAdV14–positive patients had fever, nausea, vomiting, arthralgia, malaise, and myalgia; this combination of signs and symptoms was not noted for any other patients. However, the HAdV-C2–infected patient showed similar symptoms, along with bronchitis.

Conclusions

Outbreaks of HAdV-associated febrile respiratory illness are common among nonvaccinated trainees in military recruit training centers (*15*). The military trainee environment and the college student setting share similarities, including population age (18–21 years) and multiple-occupant residences, which result in close human proximity and facilitate virus transmission. Thus, it is not surprising that HAdV infections are also prevalent among college students. Without active surveillance for HAdV and other respiratory pathogens, outbreaks of acute respiratory illness may go undetected, leading to rapid spread and, subsequently, to difficult containment.

Molecular testing is the primary laboratory surveillance tool. However, clinicians and laboratories in New York detected infections with HAdV and other respiratory pathogens by using viral culture on a portion of respiratory samples collected as part of the influenza surveillance program. During the 2014–15 influenza season, several samples submitted from colleges in New York tested positive for HAdV, but cases of HAdV-B14 infection were identified in only 1 college. Those infections may represent isolated cases, but during influenza season, clinicians and laboratories should be aware of HAdV as a possible cause of ILI and of the potential for HAdV-B14 to circulate at low frequency. This HAdV type has demonstrated

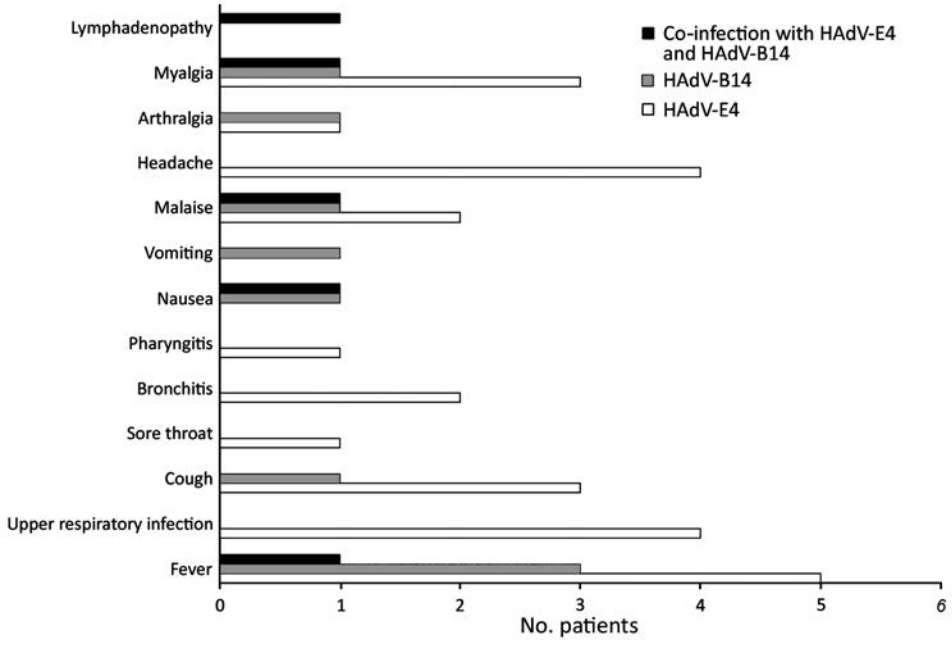

Figure 2. Symptoms experienced by 12 of 13 students with influenza-like illness who were found to be infected with human adenovirus (HAdV)-E4 (n = 8) or HAdV-B14 (n = 3) or co-infected with HAdV-E4 and HAdV-B14 (n = 1), New York, USA, 2014–2015.

potential to cause severe illness and has been responsible for widespread outbreaks (*3–6*,*8*,*9*).

Our findings highlight the benefit of including college student populations in sentinel surveillance efforts. During influenza season, HAdV should be included in the differential diagnostic panel used to determine the etiology of acute respiratory disease to prevent the unnecessary use of influenza antiviral drugs. Furthermore, our findings demonstrate the power of next-generation sequencing for phylogenetic analysis of HAdV strains for investigation of outbreaks.

Acknowledgments

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Next-generation, whole-genome sequencing, and sequence data analyses was performed at the Wadsworth Center, NYSDOH. Genomic characterization by Restriction enzyme analysis was performed at the Lovelace Respiratory Research Institute.

Mr. Lamson is Assistant Director of Special Projects in the Laboratory of Viral Diseases at the Wadsworth Center, New York State Department of Health. His research focuses on molecular epidemiology and sequence analysis of adenoviruses and enteroviruses with next-generation and di-deoxy Sanger sequencing.

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Francisella tularensis subsp. *holarctica* in Ringtail Possums, Australia

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The occurrence of *Francisella tularensis* outside of endemic areas, such as North America and Eurasia, has been enigmatic. We report the metagenomic discovery and isolation of *F. tularensis* ssp. *holarctica* biovar *japonica* from diseased ringtail possums in Sydney, Australia. This finding confirms the presence of *F. tularensis* in the Southern Hemisphere.

Tularemia is a highly infectious zoonotic disease caused by the bacterium *Francisella tularensis* that affects humans and other animals (*1*,*2*). Globally, tularemia has been identified in a wide range of animal hosts; rabbits and rodents are the most important reservoirs (*3*). Tularemia in humans is typically acquired through direct exposure to infected animals during hunting, although biting insect vectors such as ticks and mosquitos, as well as waterborne and environmental sources, have been reported (*1*). Outbreaks are not uncommon in disease-endemic areas, and a positive correlation exists between the population density of the animal reservoir and the number of human tularemia cases (*4*).

Ulceroglandular tularemia is the most common form of disease in humans, accounting for ≈75% of reported cases, and is characterized by fever, ulceration at the site of infection, and enlargement of local lymph nodes (*5*). Untreated infection by *F. tularensis* can be life-threatening. Given its multiple routes of transmission, including inhalation of contaminated dust, the bacterium is considered a category A bioterrorism agent in most jurisdictions.

Tularemia is endemic to parts of the Northern Hemisphere, including North America and Eurasia, and most cases in humans are caused by the *F. tularensis* subspecies *tularensis* (type A) and *holarctica* (type B). Notably, regions in the Southern Hemisphere, including Australia, have been largely considered tularemia-free. In 2003, a divergent *Francisella* spp. was isolated from a human foot wound in the Northern Territory, Australia (*6*), and has since been reclassified as *F. hispanensis* (*7*). However, in 2011, a case of ulceroglandular tularemia was reported in an adult bitten by a wild ringtail possum (*Pseudocheirus peregrinus*) in western Tasmania, Australia (*8*). No isolate was obtained in this case, although infection by *F. tularensis* was suggested by both 16S rRNA sequencing and serology (*8*). Two additional cases of suspected human tularemia were reported in Tasmania in 2011, close to the site of the original infection; 1 of these involved exposure to ringtail possums (*9*). Together, these cases suggest a possible wider distribution of *F. tularensis* in the Southern Hemisphere and a potential reservoir in ringtail possums in Australia.

The Study

As part of a wider study of neglected and undiagnosed disease syndromes observed in Australia wildlife, we investigated the possible infectious cause of several deaths in ringtail possums that were most often associated with acute necrotizing enteritis or hepatitis. In total, 8 possums were included in this study, each representative of a distinct mortality event in Sydney, New South Wales, during 2000–2009. Liver and brain tissue were collected at the time during necropsy and stored at –80°C.

To identify potential pathogens in these possum samples, we used an unbiased, high-throughput RNA sequencing approach (RNA-Seq; Epicentre, Madison, WI, USA). Total RNA was first extracted from the 8 affected liver tissues and pooled before host ribosomal RNA depletion and library preparation by using Ribo-Zero Gold (Illumina; San Diego, CA, USA) with TruSeq Stranded mRNA Library preparation kit (Illumina). The library was sequenced by using the Illumina HiSeq2500 platform, producing 50,740,088 paired-end sequence reads (100 nt lengths). These data were assembled de novo using Trinity (*10*) and screened for viral, bacterial, and fungal pathogens by comparisons to existing databases by using nucleotide and protein Blast searches (*11*).

No viral or fungal pathogens were apparent in the de novo assembled transcripts. Strikingly, however, *F. tularensis* was abundant, representing ≈85% of the bacterial contigs identified. Other, less abundant bacteria were species from the genera *Serratia*, *Streptococcus*, *Escherichia*, and *Bacillus*. Remapping the RNA-Seq data against the complete genome

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[1]These authors contributed equally to this article.

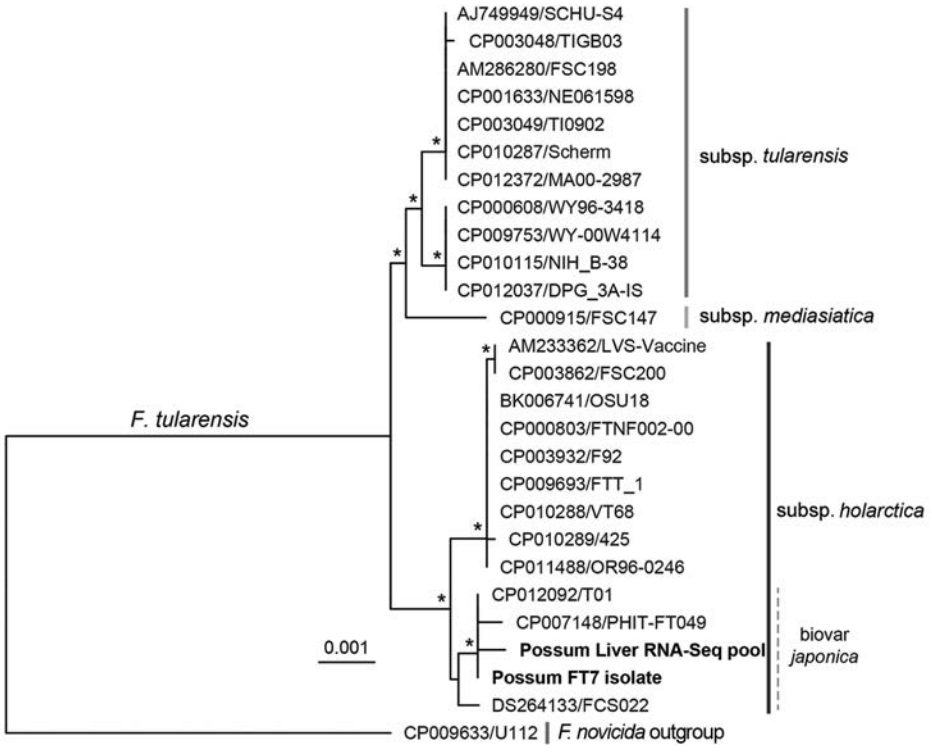

Figure 1. Multilocus phylogenetic analysis of *Francisella tularensis* isolates, including ringtail possum sequences from Australia. The alignment comprised 5 concatenated housekeeping genes (*LepA*, *RecA*, *GyrB*, *AtpD*, and *TrpB*) from the ringtail possum FT7 isolate and RNA-Seq pool (boldface) as well as National Center for Biotechnology Information whole-genome reference sequences (27 sequences, alignment length 7,009 nt). The coverage of each gene from the RNA-Seq data was 71.8%–100%, with mean depths of 1.4×–4.4×. Phylogenetic analysis was performed by using the maximum-likelihood method in PhyML (Hasegawa-Kishino-Yano plus gamma substitution model with 1,000 bootstrap replicates) and rooted by using a *F. novicida* isolate (U112). Isolates are labeled with GenBank accession number and name, and subspecies are indicated. Scale bar indicates number of substitutions per site, and asterisks represent nodes supported by bootstrap values >70%.

of the *F. tularensis* subsp. *holarctica* reference strain T01 (GenBank accession no. NZ_CP012092) produced an assembly of 80,516 reads, providing 46.9% genome coverage (891,561/1,899,676 nt) at a mean depth of 4.2× and pairwise identity of 99.8%. This relatively high abundance suggested that *F. tularensis* subsp. *holarctica* RNA was probably present in the pooled possum liver sample.

To confirm the infection, individual liver samples were independently screened for *F. tularensis* at the Emerging Infections and Biohazard Response Unit (EIBRU) at Pathology West, Westmead Hospital, Sydney. This screening involved culturing on enriched cysteine heart agar blood culture medium with antibiotic supplementation (*12*), and assays provided through the US Centers for Disease Control and Prevention's Laboratory Response Network that included direct fluorescent antigen (DFA) testing and real-time PCR. No serum was available for serologic testing. Of the 8 liver samples present in the RNA-Seq pool, 2 were positive (samples 2 and 7) for *F. tularensis* by both DFA and PCR (all 3 gene targets, FT1, FT2, and FT3, were positive). Both of these *F. tularensis*–positive samples were obtained from ringtail possums found in the Sydney north shore area and were associated with 2 separate mass mortality events in May 2002 (sample 7) and August 2003 (sample 2). An *F. tularensis* isolate was also obtained from sample 7 (denoted FT7) that was identified by morphology, biochemical tests, mass spectrometry, and confirmatory testing by DFA and real-time PCR. Whole-genome sequencing was performed on an Illumina NextSeq500 by using Nextera XT libraries prepared from FT7 genomic DNA in addition to control stocks held by the Emerging Infections and Biohazard Response Unit to eliminate them as possible sources of contamination. Raw sequence data are available from the National Center for Biotechnology Information (BioSample accession no. SAMN06114577).

A multilocus phylogenetic comparison of the FT7 isolate and the original possum liver RNA-Seq data revealed that the sequences clearly clustered together within an Asian subclade of the *holarctica* subspecies that includes biovar *japonica* (Figure 1). The differences between the sequences (3 nt across the multilocus region of 7,009 nt) are probably best explained by sequence quality with poor coverage (only 1×) in the RNA-Seq data at these positions. A subsequent whole-genome single nucleotide polymorphism analysis (Figure 2) and subspecies-specific PCR producing an 839-bp product confirmed FT7 as a member of biovar *japonica* (*13*). Although it is difficult to draw conclusions from such a conserved region, we note that the sequences from the FT7 isolate and the RNA-Seq data both matched, with 100% identity, the 16S rRNA and *recA* gene sequences identified in the human tularemia case in Tasmania (*8*). Testing of additional archived tissues from 8 similarly affected ringtail possums and 3 rabbits did not identify further cases positive for *F. tularensis*. Hence, there are

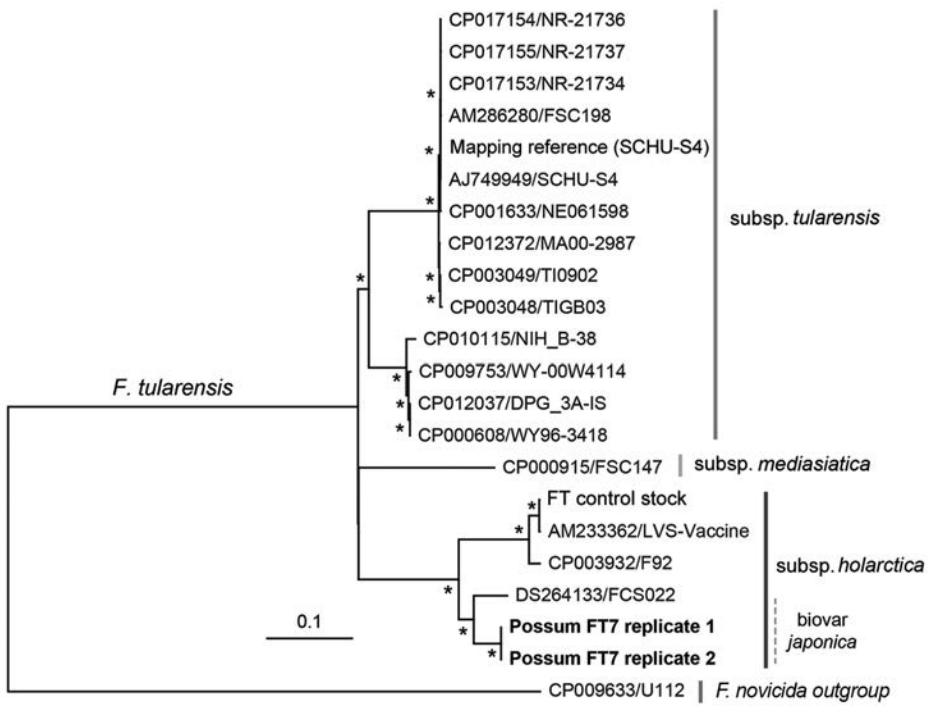

Figure 2. Maximum-likelihood phylogeny of whole-genome single nucleotide polymorphisms of the FT7 *Francisella tularensis* isolate from a ringtail possum in Australia (boldface) with other *Francisella* species, including biovar *japonica*. The single nucleotide polymorphism analysis was performed by mapping reads against a reference genome sequence *F. tularensis* subsp. *tularensis* SCHU-S4 (GenBank accession no. NC_006570.2) in addition to 16 genomes of the *F. tularensis* complex obtained from GenBank. Isolates are labeled with GenBank accession number and name, and subspecies are indicated. Scale bar indicates number of substitutions per site, and asterisks represent nodes supported by bootstrap values >70%.

probably multiple etiologies for the acute necrotizing enteritis or hepatitis observed.

Conclusions

The clinical, gross, and histologic findings, in addition to the genetics and microbiology, confirm the diagnosis of *F. tularensis* infection in ≥2 ringtail possums associated with 2 mortality events in Sydney. Native ringtail possums in Australia might therefore be a natural reservoir or end-stage host of tularemia and serve as useful sentinels of disease activity that could pose a threat to human health through occasional exposure. A better understanding of the ecology of *F. tularensis* subsp. *holarctica* in Australia is necessary to contribute to public health and to wildlife welfare and conservation.

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The Summer of Seventy-Six— *Legionella pneumophila* Monologue

Arvind Valavane, Rama Chaudhry

The scientific community has understood that the microbes around and within us are the driving force of our life (*1*). However, the next generation is being brought up with a fear of encountering microorganisms. This generation has developed a deeply rooted view that all microorganisms are dangerous. Moreover, anthropocentric investigations have created an unrealistic view of the microorganisms in nature (*2*). Only a negligible percentage of the known microorganisms cause disease in humans, and we have created a mess in handling them.

We believe that the perspective of the next generation toward microorganisms should be changed. Activities involving creation of bacterial monologues (to think from microbes' perspective) have been shown to enhance the conceptual understanding of key topics in microbiology and improvement in the attitude of students toward science (*3*). Use of such methods would inculcate the right interest among the next generation and teach them to respect nature.

We present a poem called *The Summer of Seventy-Six*, which deliberates about the pathogen turned environmental bacterium *Legionella pneumophila,* by walking in its shoes.

Learning my native dance, I
Enjoyed the filmy cover, until you
Gave me a chance to
Invade an unclean tower.
On the summer of seventy-six, I got a
Name, only to sing some veterans' fame,
Every corner I took the blame,
Like you never played the vexing game.
Little aerosols made the incident and your
Ailing was a mere accident.

Protozoans are my natural host,
Naegleria makes a happy toast.
Even in hot springs I can thrive,
Under the soil I can jive.
Macrophages are my motel room,
On my way I make them bloom,
Prevention stops the moody gloom,
Hauling me before the boom.
In a way I am a pathogen, popping your
Lungs' oxygen, but you clearly know, I am
Affected by heat, copper, and a halogen.

Human-made modifications of nature are the cause for troubles of humanity, and if we embark on disturbing nature we should be ready to pay for it. One classical example of our carelessness was reflected at the American Legion Convention held in 1976 in Philadelphia, Pennsylvania, USA, which led to the discovery of genus *Legionella*. It was a team of scientists, led by Dr. Joseph McDade from the Centers for Disease Control (Atlanta, GA, USA), that isolated the bacterium from infected lung tissues in the wake of the initial outbreak, which later led to determination of the source (*4*). This team even provided answers for some retrospective unsolved respiratory illness.

Artificial water distribution systems are the main reservoirs of *Legionella* species, apart from natural freshwater sources and soil (*5*). The list of species identified in the genus *Legionella* keeps increasing day by day, and more than half of them have been implicated in human illness (*6*). The infection is considered preventable because person-to-person transmission of *Legionella* spp. has never been reported, except for 1 recent probable case (*7*).

In spite of advances in communication and technology, there is a vast gap among the industrialized and developing nations in terms of public health safety regarding this bacterium. When industrialized nations are considerably monitoring *Legionella* spp., developing nations are completely out of the picture (*8*,*9*). Moreover, considering the increasing tourism industry among developing nations, we should keep in mind that travel-associated Legionnaires' disease is not uncommon (*10*).

Thus, creating awareness among developing nations, collaboration on research projects for evidence-based learning, exchange of resources, and sharing of knowledge should happen among us to coexist with this bacterium. After all, we are breathing in its world and not the other way around!

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Mycobacterium gordonae in Patient with Facial Ulcers, Nosebleeds, and Positive T-SPOT.TB Test Result, China

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Mycobacterium gordonae is often regarded as a weak pathogen that only occasionally causes overt disease. We report a case of *M. gordonae* infection in the facial skin, nasal mucosa, and paranasal sinus in an immunocompetent patient and review previous cases. The T-SPOT.*TB* test might be useful in diagnosing such cases.

Nontuberculous mycobacteria are increasingly more involved in causing human infections. *Mycobacterium gordonae*, a type of slow-growing nontuberculous mycobacterium, is generally regarded as a weak pathogen, although it has caused some disease in humans (*1*–*3*). We describe a case of *M. gordonae* infection in the facial skin, nasal mucosa, and paranasal sinus of an immunocompetent patient in China.

In March 2016, a 60-year-old woman with no history of tuberculosis or immunosuppression sought treatment for a 2-year history of asymptomatic facial ulcers and intermittent nosebleeds. The primary lesion was a small erythema on the left cheek that, without incurring trauma, developed into an ulcer. The condition gradually worsened. One year before visiting our hospital, she received a diagnosis of lupus erythematosus; she had also been referred to otorhinolaryngology, where she was treated unsuccessfully with nasal endoscopic surgery. The skin lesions were erythematous, covered with yellow crusts, and on both sides of the patient's face (Figure, panel A). A nasal examination revealed small scabs in the nasal vestibule. Lymph nodes were not palpable.

Laboratory test results indicated the patient was negative for autoantibodies and HIV. A computed tomography (CT) scan of the paranasal sinuses showed ethmoid and maxillary sinusitis (Figure, panel B). Histologic examination indicated that a large number of lymphocytes, a few histiocytes, and plasma cells had infiltrated the lesion (Figure, panel E). Periodic acid-Schiff and acid-fast stains were negative for bacteria, and tissue culture was negative for fungus. Mycobacterial infection was suspected, but because mycobacteria are slow-growing, another test was needed to inform clinical practice. An alternative diagnostic test, the T-SPOT.*TB* (Oxford Immunotec Ltd., Oxford, England), an interferon-γ release assay, was performed and showed positive results (tuberculosis [TB] antigen – Nil = 20; ≥6 was positive). After 3 weeks of culture at 32°C in Löwenstein–Jensen medium, smooth, creamy, yolk-yellow bacteria were observed (Figure, panel F). Ziehl–Neelsen staining indicated they were acid-fast bacilli. Sequence analysis of 16S rRNA revealed a 99% similarity with *M. gordonae* strain Y27, and the *hsp65* gene showed a 100% similarity with *M. gordonae* strain FJ09081. Further sequence analysis showed 16S rRNA was 99% homologous and *hsp65* 96% homologous with *M. gordonae* ATCC 14470. The diagnosis of *M. gordonae* infection was made.

After diagnosis, the patient was empirically treated with clarithromycin (500 mg/d) and moxifloxacin (400 mg/d). At the 4-month follow-up, no new nosebleeds or skin lesions were reported, and skin examination revealed scattered atrophic scars on the face (Figure, panel C). CT scans showed substantial improvement of the ethmoid and maxillary sinuses (Figure, panel D); T-SPOT.*TB* results were negative (TB antigen – Nil = 0). Treatment continued for 2 more months.

A drug sensitivity test showed that the isolate from the patient was sensitive to clarithromycin, ethambutol, and moxifloxacin but less sensitive to rifampin, rifabutin, and isoniazid (*4*). No adverse drug reactions or recurrences were noted for up to 3 months after treatment was completed.

M. gordonae is found widely throughout the environment. Infections caused by *M. gordonae* usually occur in the lungs (and only occasionally in other organs) of immunocompromised patients. A cutaneous infection with *M. gordonae* is unusual and a paranasal sinus infection even rarer.

Seven cases of cutaneous infection with *M. gordonae* have been reported; all were in women 38–80 years of age. The infection can affect persons who have not experienced trauma or been exposed to immunosuppressants. The most common lesions caused by *M. gordonae* are nodules that slowly enlarge and become ulcerated over several months. Lesions usually are located on the face, at distal extremities, or at sites of previous trauma (*1*,*5*–*8*). In this case, the infection caused lesions throughout the facial skin, nasal mucosa, and paranasal sinus, probably because the delay in accurate diagnosis allowed for wide dissemination of the pathogen.

Common laboratory methods for diagnosis of nontuberculous mycobacterial infection include histopathologic stainings, tissue culture, PCR, and gene sequencing.

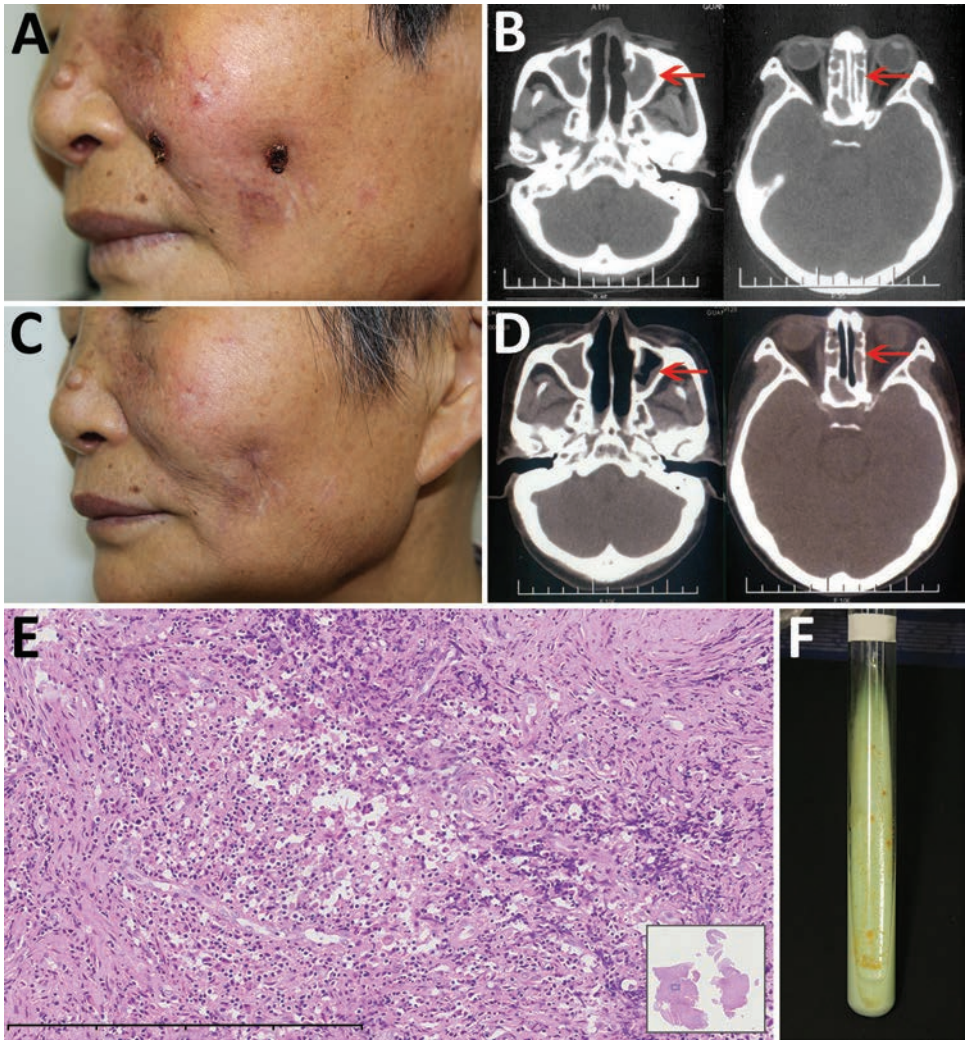

Figure. *Mycobacterium gordonae* infection in a 60-year-old immunocompetent woman, China. A) Facial lesions before treatment. Ulcers were erythematous and covered with yellow crusts. B) Computed tomography images before treatment show heterogeneous hypersignal in the ethmoid and left maxillary sinus (arrows). C) Facial lesions after treatment. Atrophic scars are seen at sites of previous lesions. D) Computed tomography images after treatment show recovery of the ethmoid sinus and left maxillary sinus (arrows). E) Hematoxylin and eosin stain of nasal mucosa showing the infiltration of a large number of lymphocytes, a few histiocytes, and plasma cells. Scale bar corresponds to 400 μm. Inset shows the nasal mucosa sample (original magnification ×20). F) Tissue culture 3 weeks after incubation shows yolk-yellow bacteria growing in Löwenstein–Jensen medium.

Tissue culture and sequencing usually provide the most reliable evidence for diagnosis; however, tissue culture has a low sensitivity and is time-consuming, making early diagnosis difficult.

In this case, the initial patient sample was positive by T-SPOT.*TB*, and after mycobacterium-specific treatment and clinical improvement, the convalescent-phase patient sample was negative by T-SPOT.*TB*. These results were highly suggestive that the patient had a mycobacterial infection and that the infection was adequately treated.

Although it is generally believed that a positive T-SPOT.*TB* result means the patient has an *M. tuberculosis* infection, positive results have been reported for infections caused by nontuberculous mycobacteria (*2*,*9*). The T-SPOT.*TB* assay uses the *M. tuberculosis* antigens ESAT-6 and CFP-10. The *ESAT-6* and *CFP-10* genes are located within the *M. tuberculosis* region of difference 1 (RD1), a DNA sequence that is also present in *M. marinum*, *M. kansasii*, *M. szulgai*, and *M. gordonae* (*10*). Because these genes are present in other mycobacterial genomes, the T-SPOT.*TB* assay might be useful for diagnosing infections with multiple RD1-possessing mycobacteria. However, further studies are needed to confirm its diagnostic value.

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Norovirus GII.17 as Major Epidemic Strain in Italy, Winter 2015–16

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In winter 2015–16, norovirus GII.17 Kawasaki 2014 emerged as a cause of sporadic gastroenteritis in children in Italy. Median patient age was higher for those with GII.17 than GII.4 infection (55 vs. 24 months), suggesting limited cross-protection for older children.

Noroviruses are a major cause of acute gastroenteritis in children and adults. Although >30 norovirus genotypes can infect humans, the genotype GII.4 has been associated with most norovirus-related outbreaks and sporadic cases of gastroenteritis since the mid-1990s. Norovirus strains undergo genetic and antigenic evolution through accumulations of point mutations and intragenotype and intergenotype recombination.

During the winter season 2014–15, a novel NoV GII.P17_GII.17 variant, Kawasaki 2014, emerged in several countries in Asia, replacing the previously dominant variant, GII.4 Sydney 2012 (*1*–*4*). Kawasaki 2014 is phylogenetically distinct (Kawasaki-308 lineage) from earlier GII.17 variants that circulated in 2013 and 2014 and from the original 1978 GII.17 strain, which had a GII.4 open reading frame (ORF)1 gene (*4*). The spread of the variant Kawasaki 2014 in Asia was unexpected because this was the first time a variant other than GII.4 acquired such epidemiologic relevance. Until now, the Kawasaki 2014 variant had been reported in a limited number of cases in countries outside of Asia (*5*,*6*), including some in Europe (*6*–*8*), with most cases occurring sporadically.

The Italian Study Group for Enteric Viruses (http://isgev.net) monitors the epidemiology of enteric viruses in children through hospital-based surveillance in 3 geographically distinct areas: northern Italy, in Parma (University Hospital); southern Italy, in Bari (Pediatric University Hospital "Giovanni XXIII"); and on Sicily island, in Palermo (ARNAS Civic Hospital). The official total pediatric population (0–14 years of age) includes 426,569 newborns, infants, and children (as of January 1, 2015).

From January 2015 through February 2016, the Italian Study Group for Enteric Viruses analyzed 2,603 fecal samples of children (0–14 years of age) with diarrhea.

The collection of fecal samples was part of the process of diagnosis of acute gastroenteritis, and we obtained verbal informed consent for analysis from families or caretakers. Norovirus prevalence was 12.8% (95/740) in Parma, 7.5% (95/1,273) in Bari, and 21.4% (126/590) in Palermo, yielding a calculated norovirus national prevalence of 12.1% (316/2,603).

We performed multitarget analysis in the diagnostic regions A (ORF1, polymerase) and C (ORF2, capsid) of the norovirus genome (*9*). We characterized a subset (57.6%; 182/316) of the 2015–16 norovirus-positive samples either completely (44.0%; 139) or partially (13.6%; 43). Of the latter, 6 were characterized only in diagnostic region A (327 nt, at positions 4538–4865 relative to U07611 reference) and 37 only in diagnostic region C (342 nt, at positions 5307–5649 relative to U07611 reference) (Figure).

In 2015, norovirus GII.P17_GII.17 Kawasaki 2014 was detected in 2 cases in February (*8*) and 1 in September, accounting for only 3.3% of the 90 norovirus-positive samples from 2015 that were fully typed; the recombinant strain GII.P4 New Orleans 2009_GII.4 Sydney 2012 (38.9%; 35/90) and the pandemic variant GII.Pe_GII.4 Sydney 2012 (32.2%; 29/90) were predominant. Conversely, of the 49 strains fully typed for January and February 2016, the variant Kawasaki reached 18.4% (9/49) prevalence and represented the third most common strain in Italy, after GII.P4 New Orleans 2009_GII.4 Sydney 2012 (34.7%; 17/49) and GII.Pe_GII.4 Sydney 2012 (24.5%; 12/49). Occurrence of related cases or outbreaks was ruled out on the basis of the patients' anamnestic data.

We determined the sequence of a large portion of the genome at the 3′ end (3.2-kb, including partial ORF1 [822 nt], full-length ORF2 [1621 nt], and ORF3 [866 nt]) for 3 representative GII.P17_GII.17 strains from this study (GenBank accession nos. BA202/16–16: KX592170; PA31/2016: KX592171; and PA39/2016: KX592172). After phylogenetic analysis, the Kawasaki 2014 variants from Italy segregated into the Kawasaki-308 genetic subclade, together with other GII.P17_GII.17 sequences available in GenBank, including those of noroviruses detected in China and Hong Kong during 2014–15 and in the United States in November 2014 (*8*) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/16-1255-Techapp.pdf).

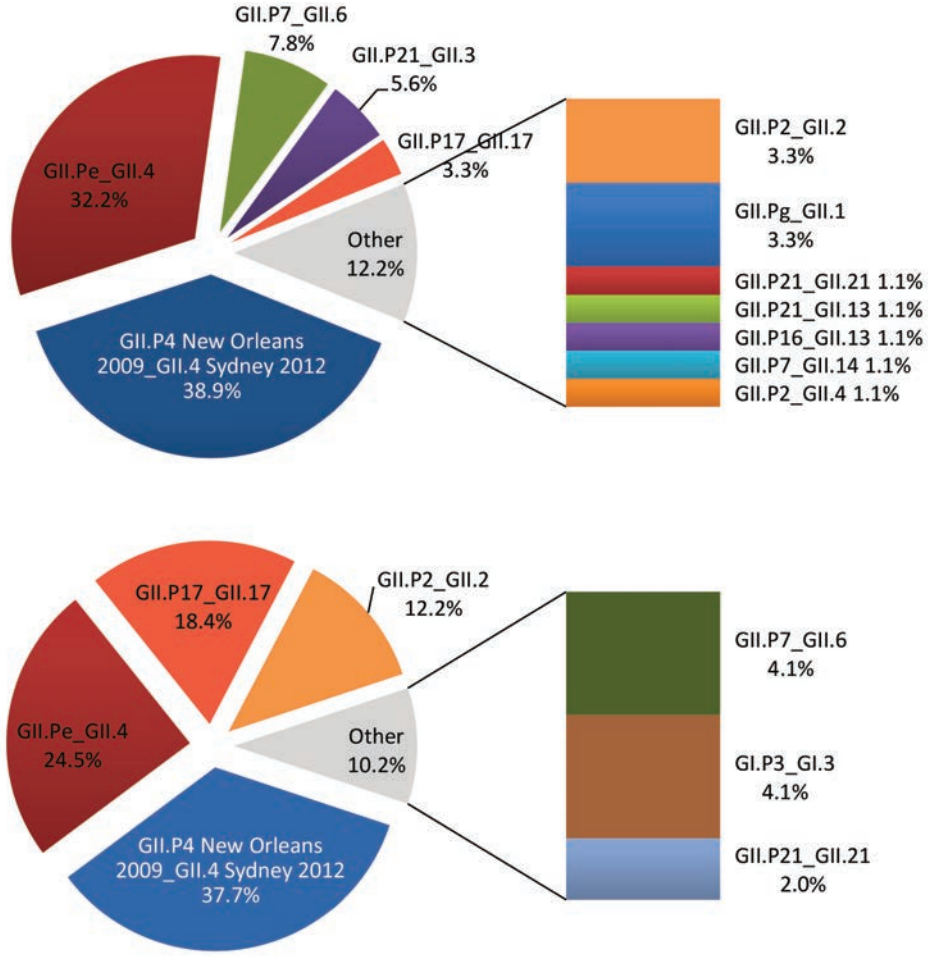

Figure. Norovirus genotypes for fully typed strains detected during January–December 2015 (A) and during January–February 2016 (B) in Italy by the Italian Study Group for Enteric Viruses surveillance system.

Analysis of demographic data revealed a significantly higher median age for patients with GII.P17_GII.17 infections (55 [SD 49.8] months) than for patients with GII.4 infections (24 [SD 13.6] months) ($p<0.005$; 2-tailed Mann U-test, $p = 0.00433$ [95% CI 0.4–6.5]). These observations are consistent with a lack of specific herd immunity in the population, meaning that the GII.17 virus can infect older patients more easily than GII.4 viruses can, as observed in Hong Kong (*4*).

Our analysis indicates that, in Italy in winter 2015–16, the epidemiologic pattern of norovirus GII.17 viruses markedly changed, suggesting increased circulation of the variant Kawasaki 2014 among children, although GII.4 variants (the capsid variant Sydney 2012 with the GII.Pe or GII.P4 polymerase) were still predominant. The mechanisms driving the global spread of norovirus GII.17 could include the broad range of co-receptors used by these viruses (*10*) or the limited cross-antigenic relationships with the predominant GII.4 strains that could trigger mechanisms of antigenic escape. Norovirus GII.17 could present a challenge for the development of norovirus vaccines because it is not clear whether, and to what extent, there is cross-protection between vaccine antigens and GII.17 viruses (*6*).

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Disseminated *Mycobacterium genavense* Infection in Patient with Adult-Onset Immunodeficiency

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We report a case of disseminated *Mycobacterium genavense* infection resulting from neutralizing anti–interferon-γ autoantibodies in the patient. We identified M. genavense targeting the hsp65 gene in an aspiration specimen of the

lymph node. Adult-onset immunodeficiency caused by neutralizing anti–interferon-γ autoantibodies, in addition to HIV infection, can lead to disseminated nontuberculous mycobacterial infection.

Mycobacterium genavense is a ubiquitous nontuberculous mycobacteria (NTM), first described as a human infection in the 1990s as a primary cause of fatal disseminated infection in HIV-infected patients with low CD4 counts (*1*). *M. genavense* also is recognized as an opportunistic pathogen in patients without HIV who have severe immunodeficiency, such as that after solid-organ or hematopoietic stem cell transplantation or immunosuppressive therapy (*2*,*3*). The diagnosis of *M. genavense* infection is clinically challenging because of the difficulties in routinely culturing the organism and the absence of specific symptoms, even in fatal infections. Therefore, diagnosing *M. genavense* infection in patients without known evidence of immunodeficiency is particularly difficult. We report a previously healthy 66-year-old man with multiple lymphadenopathies in whom disseminated *M. genavense* infection resulting from the presence of neutralizing anti–interferon-γ (anti–IFN-γ) autoantibodies was diagnosed.

In November 2015, the patient sought care at Asahi General Hospital (Chiba, Japan) for a 2-week history of right-side neck swelling and abdominal pain. His vital signs were within reference ranges. Except for right cervical lymphadenopathy, findings on physical examination were unremarkable. HIV antibodies were undetectable, and CD4/CD8 lymphocyte counts were within reference ranges. No mediastinal or lung involvement was detected on chest computed tomography (CT) scan. Gallium-67 single-photon emission CT/CT revealed high-intensity accumulation of the right cervical and ileocolic lymph nodes (online Technical Appendix Figure, https://wwwnc.cdc.gov/EID/article/23/7/16-1677-Techapp1.pdf). Acid-fast bacilli (AFB) staining of the lymph-node aspiration specimen yielded positive results; however, findings on solid media culture and PCR for detecting *M. tuberculosis*, *M. avium*, and *M. intracellulare* were negative. After a 6-week outpatient follow-up, the patient returned with newly developed right axillary lymphadenopathy. An aspiration specimen of the lymph node showed positive AFB staining and was submitted for molecular biologic analysis. *M. genavense* was identified on amplification and sequencing analysis targeting the *hsp65* gene (*4*). We strongly suspected neutralizing anti–IFN-γ autoantibodies as the cause because the whole blood IFN-γ level with mitogen stimulation was low, as determined using an IFN-γ–releasing assay (QuantiFERON TB 3G; Cellestis, Carnagie, VIC, Australia). A high serum-neutralizing anti–IFN-γ autoantibody titer and inhibited STAT1 (signal transducer and activator of transcription 1) phosphorylation through IFN-γ stimulation in the leukocytes were confirmed, leading to a diagnosis of disseminated *M. genavense* infection. Clarithromycin, ethambutol, rifampin, and amikacin were administered. Lymphadenopathy improved after 6 weeks, and amikacin was discontinued. No relapse occurred during 16 months of treatment.

Recent studies have described disseminated NTM infection in patients in Asia with adult-onset immunodeficiency resulting from neutralizing anti–IFN-γ autoantibodies (*5*–*7*). Disseminated infection mainly involves the lymph nodes, followed by the osteoarticular system, bone, lungs, and skin (*6*,*7*). The pathogen comprises rapidly and slowly growing mycobacteria; *M. avium* complex and *M. abscessus* are the most frequently detected. Although the long-term outcome is unclear, most patients need long-term antimicrobial therapy, and some relapses occur after treatment discontinuation (*6*,*7*). Adjuvant rituximab therapy has been used for refractory disease (*8*).

Although disseminated *M. genavense* infections formerly only were known to occur in HIV-infected patients, the epidemiologic shift to infections in patients without HIV reflects the introduction of combination antiretroviral therapy and increasing use of immunosuppressive agents (*2*). In 2 previous series comprising 14 HIV-negative patients with *M. genavense* infection, most patients had known evidence of immunodeficiency; of the 12 patients treated with immunosuppressive agents, 5 had sarcoidosis, 5 were solid-organ transplantation recipients, 1 had non-Hodgkin lymphoma, and 1 had rheumatoid arthritis. Only 2 patients were identified with adult-onset innate immunodeficiency (*2*,*3*); 1 patient had innate interleukin-12 receptor deficiency and 1 had idiopathic CD4 lymphocytopenia.

Needle aspirates and tissue biopsy provide higher NTM diagnostic yields than does swab sampling but are insufficiently sensitive. Therefore, less frequently encountered mycobacterial species are identified by gene sequencing, reverse hybridization, and high-performance liquid chromatography (*9*). Moreover, the identification of *M. genavense* infection using standard mycobacterial culture methods is difficult. Acidified solid media testing with blood and charcoal is probably the most suitable method (*10*); however, accurate diagnosis requires additional molecular biologic analysis, such as amplification and sequencing of the 16S ribosomal RNA, *hsp65*, or *rpoB* genes. In this case, we identified *M. genavense* using a direct molecular biologic method for aspiration specimens from the lymph node.

Little is known about death among HIV-negative patients with *M. genavense* infection, although some patients reportedly have died (*2*,*3*). Although their conditions eventually improve, despite a lack of early identification of *M. genavense*, delayed diagnosis might influence death. Direct molecular biologic methods could better identify *M. genavense* infection and improve prognosis.

We report a case of disseminated *M. genavense* infection resulting from neutralizing anti–IFN-γ autoantibodies in the patient. *M. genavense* infection should be considered in the differential diagnosis of mycobacteria detected with AFB staining but not with culture, even in patients without known evidence of immunodeficiency. Adult-onset immunodeficiency acquired by neutralizing anti–IFN-γ autoantibodies, in addition to HIV infection, can lead to disseminated NTM infection.

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Live Cell Therapy as Potential Risk Factor for Q Fever

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During an outbreak of Q fever in Germany, we identified an infected sheep flock from which animals were routinely used as a source for life cell therapy (LCT), the injection of fetal cells or cell extracts from sheep into humans. Q fever developed in 7 LCT recipients from Canada, Germany, and the United States.

Gram-negative intracellular bacteria (*Coxiella burnetii*) cause Q fever, a zoonotic disease usually subclinical in livestock and humans. Typically, human patients show signs and symptoms, such as fever, severe headache, nausea, pneumonia, or hepatitis, 2-3 weeks after infection. Chronic Q fever develops in ≈1%–5% of patients (*1*).

On August 5, 2014, a local health department in the Federal State of the Rhineland Palatinate in southern Germany alerted the Federal State Agency for Consumer and Health Protection (FSACHP) (Landau, Germany) after detecting a cluster of 8 patients with pneumonia in a rural community during a 6-week period. The local health department and FSACHP started a joint outbreak

investigation to identify cases, find the source of the outbreak, and stop disease transmission.

On August 12, five of 8 patients tested by ELISA and immunofluorescence test (IFT) by the local health department had results consistent with acute *C. burnetii* infection. The local health department issued a public health warning in the local media and advised anyone in the affected community with influenza-like symptoms or pneumonia in the past 4 months to be tested for Q fever by their general practitioner. In addition, the department offered free testing to pregnant women and persons with cardiovascular risk factors (e.g., heart valve defects), irrespective of symptoms (*2*).

Case-patients were defined as persons who had phase II IgM or IgG titers for Q fever by ELISA or IFT in 2014. Data for these case-patients were entered into the German Electronic Surveillance System for Infectious Disease Outbreaks (*3*). Thirteen residential case-patients (6 men, 7 women) who lived in the affected county (Bad Duerkheim) were identified; 11 reported symptoms compatible with Q fever, of whom 6 were hospitalized (Figure). Median age for residential case-patients was 50 (range 32–59) years for women and 44 (range 26–56) years for men.

Because all residential case-patients lived within 1.5 km of a flock of 1,000 sheep, the FSACHP tested random samples from these sheep. Of 61 sheep tested, 25 were positive for *C. burnetii* by ELISA of serum samples and 2 by PCR of vaginal swab specimens. During the investigation, the local health department discovered that young rams and pregnant ewes in the flock had been used as donor animals for live cell therapy (LCT) at 2 medical facilities in a district 10 km from the farm. LCT is injection of fetal cells or cell extracts from sheep into humans. The flock was banned for LCT production, and veterinary control measures (e.g., indoor housing and immunization of sheep) were initiated to stop transmission.

Staff members of LCT facilities were offered serologic testing by ELISA or IFT. Sixteen persons with occupational cases (3 men, 13 women) were reported; 10 showed onset of disease, and 2 were hospitalized (Figure). Median age for occupational case-patients was 48 (range 28–62) years for women and 45 (range 35–50) years for men.

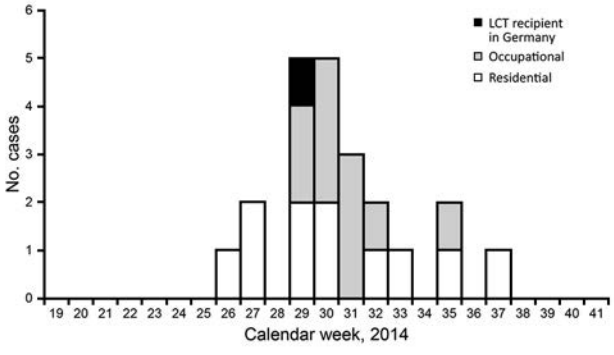

Figure. Residential (n = 11), occupational (n = 10), and recipient (n = 1) cases of Q fever related to live cell therapy (LCT), by week of symptom onset compatible with Q fever, Rhineland-Palatinate, Germany, 2014

LCT is an alternative treatment (without medical evidence of effectiveness) that is marketed worldwide online. It consists of intramuscular injections of cell suspensions from fetal sheep to human recipients for rejuvenation (anti-aging) and other ailments. Apart from national recipients, medical tourists from North America and Asia travel to Germany to receive injections. In August, the FSACHP was notified of a patient from Canada who received LCT injections on May 28, 2014, and became ill in June, before *C. burnetii* was detected in the asymptomatic donor sheep herd (*4*,*5*).

Newspaper coverage in October of the Q fever outbreak and the potential link to the LCT recipient from Canada alerted an LCT recipient in Germany who had recovered from a previously unidentified illness after receiving LCT in Rhineland Palatinate (*4*,*6*). The patient became ill (fever, severe diarrhea, and fatigue) 1 day after receiving LCT injections on July 14, lost 9 kg, and was hospitalized for 10 days. She was positive for Q fever by IFT in October 2014 and had a phase II IgG titer (1:65,536) that was higher than her phase I IgG titer (1:4,096), which indicated a recent infection. She had no other contact with sheep or sheep products during her stay at the LCT facility or thereafter.

A pharmacovigilance report by the Paul Ehrlich Institut (Langen, Germany) indicated that LCT treatment was the probable cause of the Q fever (*7*). The county ordered both LCT facilities to advise all 830 LCT recipients treated since January 2014 to consult their general practitioner about their possible risk for Q fever. This advice prompted 5 US citizens who received injections on May 30 in one of the clinics to be tested for Q fever. An investigation by the US Centers for Disease Control and Prevention (Atlanta, GA, USA) identified recent Q fever in all 5 patients (phase II IgG titers $\leq$1:65,536 at 2–6 months postinjection) (*5*).

Several facilities offer LCT in Germany, although the federal ministry of health recently released an assessment stating that the use of LCT is unsafe (*8*). Therefore, practitioners worldwide should be informed that working at an LCT clinic or receiving LCT injections should be considered potential risk factors for Q fever.

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Novel Avulaviruses in Penguins, Antarctica

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We identified 3 novel and distinct avulaviruses from Gentoo penguins sampled in Antarctica. We isolated these viruses and sequenced their complete genomes; serologic assays demonstrated that the viruses do not have cross-reactivity between them. Our findings suggest that these 3 new viruses represent members of 3 novel avulavirus species.

Avian paramyxovirus (APMV) belongs to the genus *Avulavirus*, family *Paramyxoviridae*. There are 13 recognized *Avulavirus* species, each with 1 member, called avian paramyxovirus 1–13 (APMV-1–APMV-13) (*1*). A putative APMV-14 also has been recently described but not yet formally recognized (*2*).

In the past decade, APMV-10 through APMV-14 have been reported because of the intensification of surveillance of avian influenza A viruses (*3*–*6*). Most of the avulaviruses have been detected in wild birds associated with mild or no clinical disease; only Newcastle disease virus (a strain of APMV-1), APMV-2, and APMV-3 might cause substantial disease in poultry (*7*). Previous studies have described the presence of APMV-1, APMV-3, APMV-7, APMV-8, and other as-yet uncharacterized avulaviruses in Antarctic penguins (*8*). As a part of avian influenza surveillance expeditions in Antarctica during 2014–2016, we identified 3 novel avulaviruses in Gentoo penguins.

Cloacal, fecal, and serum samples were collected from Gentoo penguins (*Pygoscelis papua*) and Adélie penguins (*P. adeliae*), at 7 Antarctic locations (online Technical Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/23/7/17-0054-Techapp1.pdf) during 2014–2016. Diagnostic tests, virus isolation, and serologic assays confirmed the identity of these paramyxoviruses (online Technical Appendix).

We successfully isolated virus from 12 cloacal samples from Gentoo penguins on Kopaitic Island; these viruses showed positive hemagglutination titers ranging from 4 to 128 hemagglutination units. From these 12 isolates, only 5 were further confirmed by reverse transcription PCR and Sanger sequencing (*9*), suggesting the presence of new avulaviruses. All PCR-positive isolates were pooled and submitted for next-generation sequencing by using MiSeq 250 paired cycle run (Illumina, San Diego, CA, USA) (*10*).

By using next-generation sequencing, we obtained the genomic sequences of 3 novel avulaviruses that were

named as follows: Antarctic penguin virus A (APVA), Antarctic penguin virus B (APVB), and Antarctic penguin virus C (APVC) (GenBank accession nos. KY452442–KY452444). Genome lengths of the 3 new avulaviruses ranged from 14,926 to 15,071 nt. The 6 genes for avulaviruses (coding for the nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and RNA-dependent RNA polymerase protein) were identified in these virus genomes (online Technical Appendix Figure 2, panel A). The sequence assembly was validated by coverage mapping (online Technical Appendix Figure 2, panel B). The genomes described here are coding-complete; future experiments are needed to sequence the absolute terminus of the nontranslating region.

The 3 avulaviruses reported in this study showed 57%–60% genome-wide nucleotide identities to all other avulaviruses, as well as 32%–50% protein identities in the hemagglutinin-neuraminidase protein gene and 31%–48% in the fusion protein gene (online Technical Appendix Figure 2, panel C). These new avulaviruses have 64%–67% genome-wide identity among each other. Accordingly, phylogenetic analyses (whether conducted by using genomes or specific genes) revealed that the new viruses form a monophyletic cluster with APMV-1, APMV-9, APMV-12, and APMV-13 (Figure; online Technical Appendix Figure 3). Recently, a cutoff of ≤60% identity of nucleotide distance on whole genome has been proposed to differentiate avulaviruses (*3*); however, APMV-12 and APMV-13 and these 3 newly discovered viruses have higher identity. Thus, we suggest that this criterion requires further validation.

Phylogenetic analysis and pairwise comparison suggests that APVA, APVB, and APVC might each represent novel avulavirus species, which we recommend naming *Avian avulavirus 15*, *16*, and *17*, respectively (pending approval by the International Committee on Taxonomy of Viruses). We performed a hemagglutination inhibition assay by using APMV-1, APMV-2, APMV-3, APVA, and APVC antisera against isolates confirmed. No cross-reactivity was observed between APVA, APVB, and APVC. These viruses also did not show cross-reactivity against APMV-1, APMV2, and APMV-3 antisera. Antigenic results support the idea that novel viruses are 3 distinct species.

We observed cytopathic effects during infection of MDBK cells and Vero cells in all isolates evaluated. These effects were characterized by cell rounding and detachment of the monolayer, but syncytia were not evident (online Technical Appendix Figure 4).

We also performed a hemagglutination inhibition assay by using APVA and APVC viruses. Three serum samples from Adélie penguins from Kopaitic Island reacted against APVC (titers 10–40), and 1 reacted against APVA (titer 40) (online Technical Appendix Table). This result suggests that these novel avulaviruses can also infect Adélie penguins.

We report the successful virus isolation and whole-genome sequencing of avulaviruses in Antarctic penguin populations. Our analyses show that these viruses are genetically and antigenically divergent, indicating that Antarctic

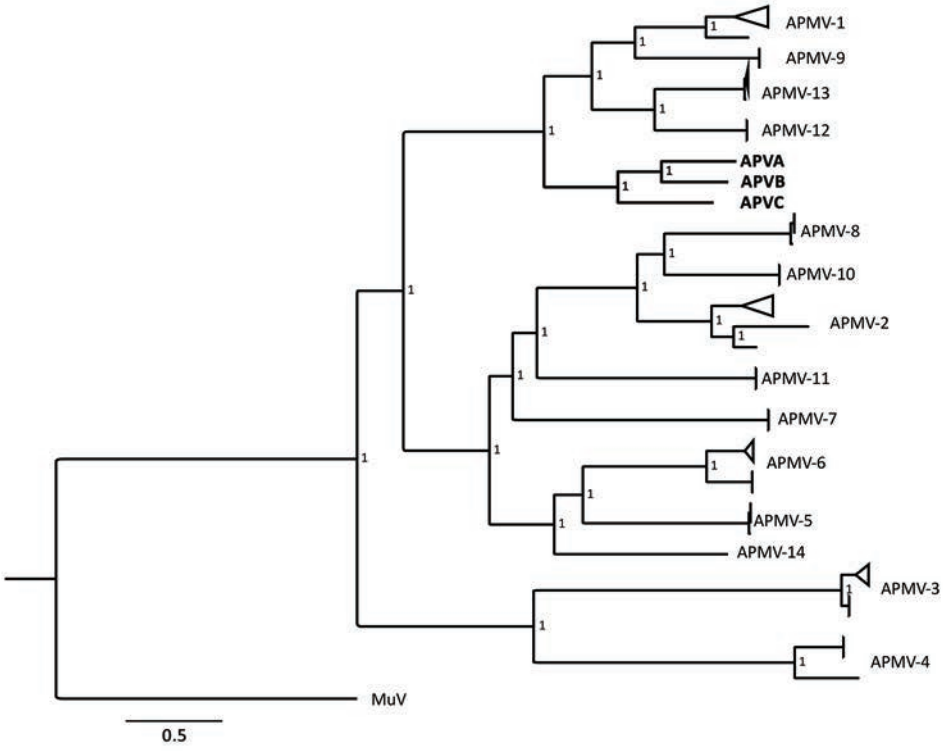

Figure. Bayesian phylogenetic tree based on concatenated nucleoprotein, phosphoprotein, matrix protein, fusion protein, hemagglutinin-neuraminidase protein, and RNA-dependent RNA polymerase protein gene sequences of 80 avulaviruses analyzed in a study of avulaviruses in penguins, Antarctica. Mumps virus was used as outgroup. Bold indicates the 3 novel viruses isolates in this study. The best-fit model of nucleotide substitution was generalized time reversible plus gamma plus invariant sites. The analysis was considered complete if the average SD of the split frequencies was <0.01 and effective sample size was >200. The values represent the posterior probabilities of each node. Scale bar indicates nucleotide substitutions per site. APMV, avian paramyxovirus; APVA, Antarctic penguin virus A; APVB, Antarctic penguin virus B; Antarctic penguin virus C; MuV, mumps virus.

penguins harbor multiple avulaviruses. An important limitation is that the new viruses were not tested serologically against APMV-4 through APMV-13; however, genetic and antigenic differences between the new viruses support the idea that they are new species.

These data suggest that in Antarctica a much greater diversity of avulaviruses exists than previously recognized. Therefore, additional studies to evaluate the presence of these new viruses in other birds in Antarctica are needed to better understand the ecology and transmission of avulaviruses in this pristine environment.

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Rickettsia sibirica mongolitimonae Infection, Turkey, 2016

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In 2016, *Rickettsia sibirica mongolitimonae* was diagnosed for a man in Turkey. He had been bitten by a *Hyalomma marginatum* tick, from which PCR detected rickettsial DNA. Sequence analysis of the DNA identified *R. sibirica mongolitimonae*. Immunofluorescence assay of patient serum indicated *R. conorii*, which cross-reacts. PCR is recommended for rickettsiosis diagnoses.

The first case of human infection with *Rickettsia sibirica mongolitimonae* was reported in France in 1996 (*1*). The infection is called lymphangitis-associated rickettsiosis

because of the lymphadenopathy and lymphangitis that occur with this infection but not with other spotted fever group rickettsioses (*2*). We describe a case of *R. sibirica mongolitimonae* infection with no lymphadenopathy and lymphangitis.

On May 1, 2016, a 53-year-old man was admitted to an emergency department in Adana, Turkey, for fever, headache, and maculopapular rash. He reported that 1 week earlier he had removed a tick from his umbilicus while farming in Adana, in the Mediterranean region of Turkey. He stored the tick in a glass jar and 2 days later sought care for high fever from his family doctor; administration of cefdinir produced no improvement. Four days later, he was hospitalized for fever (39°C), nausea, and malaise. Physical examination detected maculopapular rash and a black necrotic eschar at the center of an erythematous lesion on the patient's umbilicus (online Technical Appendix Figure, panel A, https://wwwnc.cdc.gov/EID/article/23/7/17-0188-Techapp1.pdf). The patient had no sign of lymphadenomegaly or lymphangitis. Initial laboratory examination of serum showed 10.1 $\times$ 10^9 leukocytes/L, 221 $\times$ 10^9 thrombocytes/L, 13 g/dL hemoglobin, and 3.74 mg/dL C-reactive protein (reference range <0.5 mg/dL). A blood sample was sent to the National Microbiology Reference Laboratory in Ankara, Turkey. Doxycycline (100 mg 2×/d) was administered for suspected rickettsial disease. After 48 hours, the patient's fever resolved, and his condition rapidly improved. He was discharged on day 5 of hospitalization, and doxycycline was stopped on day 10 after initiation.

Immunofluorescence assay of serum for typhus group rickettsiae IgM and IgG produced negative results. At the time of hospital admission, *R. conorii* IgM and IgG titers were 1:48 and 1:320, respectively. At a 1-month follow-up visit to the outpatient clinic, the patient's *R. conorii* IgM and IgG titers had increased to 1:384 and 1:640, respectively.

The removed tick, provided by the patient, was stored in 70% ethanol and sent to the Protozoology and Entomology Laboratory of Ankara University Faculty of Veterinary Medicine for identification of the tick species and PCR (online Technical Appendix Figure, panel B). Use of the morphological keys of Apanaskevich and Horak (*3*) led to tick identification as a *Hyalomma marginatum* female. DNA was extracted from the whole tick as described by Orkun et al. (*4*). Rickettsial DNA was detected by PCR with primers Rr. 190.70 and Rr. 190.701, which amplify the outer membrane protein A gene (*ompA*) of *Rickettsia* spp. (*5*). PCR and sequencing were conducted as described by Orkun et al. (*4*). The obtained nucleotide sequence was compared with sequences in the GenBank database, obtained by nucleotide sequence homology searches performed by BLAST analysis (http://www.ncbi.nlmn.nih.gov/BLAST). The gene sequence obtained in this study has been deposited in GenBank (accession no. KY513920).

PCR detected rickettsial DNA in the tick removed from the patient, and after sequence analysis, we determined that the rickettsial DNA belonged to *R. sibirica mongolitimonae*. According to nucleotide BLAST analysis, the obtained isolate is 100% similar to the reference strain *R. sibirica* subsp. *mongolitimonae* HA-91 (GenBank accession no. U43796) and *R. sibirica* subsp. *mongolitimonae* Bpy1 (GenBank accession no. KT345980) obtained from a biopsy sample from a human patient in Spain.

Although the climate and geography of cities like Adana in the Mediterranean region of Turkey are suitable for agents of Mediterranean spotted fever, we are unaware of any confirmed cases of *R. conorii* infection in this region. One reason may be limited access to diagnostic tools for rickettsial diseases. Another may be that doxycycline, the most effective treatment option for all rickettsial diseases (*6*), is easily administered for suspected cases of rickettsiosis with no differential diagnosis.

In Europe, *R. sibirica mongolitimonae* was detected in *Hyalomma excavatum* ticks in Greece and Cyprus; in *Rhipicephalus pusillus* ticks in France, Portugal, and Spain; and in *Rhipicephalus bursa* ticks in Spain (*6*). In 2016, *R. sibirica mongolitimonae* was isolated from 2 *H. marginatum* ticks in the central Anatolian region of Turkey (*7*).

Nearly 35% of patients with *R. sibirica mongolitimonae* infection experience rope-like lymphangitis and other highly specific manifestations (*8*). The eschar on the patient reported here was located below the umbilicus, and he had no sign of inguinal lymphadenopathy or lymphangitis on the abdominal wall.

The best sample to use for detection of spotted fever group rickettsiae is skin biopsied from the inoculation eschar (*9*). We did not perform a biopsy because we had the vector tick removed from the eschar. Also helpful for rickettsiosis investigations are serologic analyses by immunofluorescence assay. In our laboratory, only *R. conorii* serologic tests are performed for spotted fever group rickettsiae; for the patient reported here, these test results were positive for *R. conorii*. However, cross-reactions are common among *Rickettsia* spp. in the spotted fever and typhus groups (*10*), and cross-reactions on serologic tests should be considered. Whenever possible, PCRs should be performed for rickettsiosis diagnoses.

Mr. Kuscu works at the Cukurova University Faculty of Medicine as an infectious diseases and clinical microbiology specialist. His primary research interests are zoonoses and vectorborne diseases such as Crimean-Congo hemorrhagic fever and sandfly fever.

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Contaminated Stream Water as Source for *Escherichia coli* O157 Illness in Children

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In May 2016, an outbreak of Shiga toxin–producing *Escherichia coli* O157 infections occurred among children who had played in a stream flowing through a park. Analysis of *E. coli* isolates from the patients, stream water, and deer and coyote scat showed that feces from deer were the most likely source of contamination.

In the United States, recreational water is a relatively uncommon source of Shiga toxin–producing *Escherichia coli* (STEC) O157 outbreaks (*1*). We describe an outbreak of STEC O157 infections among children exposed to a contaminated stream in northern California, USA, and provide laboratory evidence establishing wildlife as the source of water contamination.

In May 2016, four cases of Shiga toxin (Stx) 1– and 2–producing *E. coli* O157 infection were reported to a local health department in northern California; investigation revealed a common source of exposure. The case-patients, ranging in age from 1 to 3 years, had played in a stream adjacent to a children's playground within a city park. Exposure of the case-patients to the stream occurred on 3 separate days spanning a 2-week period. Two case-patients are known to have ingested water while playing in the stream. Two case-patients were siblings. All case-patients had diarrhea and abdominal cramps; bloody diarrhea was reported for 3. One case-patient was hospitalized with hemolytic uremic syndrome.

The stream is a second-order waterway located in a northern California community of ≈7,500 residents. At the time of exposures, stream flow was <30 ft^3/s. The land upstream is not used for agricultural activities such as livestock production. The community is serviced by a public sewer system; inspection of sewer lines indicated no breach to the system.

Water samples were collected from the exposure site 7 days after the last case-patient was exposed and weekly thereafter for 17 weeks; samples were tested quantitatively for fecal indicator organisms. Throughout the study period, all water samples exceeded recreational water quality limits for *E. coli* and enterococci levels (*2*). Water samples were also cultured for STEC isolation and PCR detection of stx_1 and stx_2 (*3*). Stx1- and Stx2-producing *E. coli* O157 were isolated from stream water each week for the first 4 weeks. Additionally, an Stx2-producing *E. coli* non-O157 strain was isolated from the stream in the first week of sampling. Enrichment broth cultures of water samples were also positive by PCR for *stx*1 and *stx*2 for the first 4 weeks of sampling. Thereafter, both stx_1 and stx_2, or stx_2 only, were intermittently detected in enrichment broth cultures for 9 additional weeks.

In the absence of an obvious source (e.g., upstream agricultural operation or sewer leak), wildlife was considered as a possible contributor to water contamination.

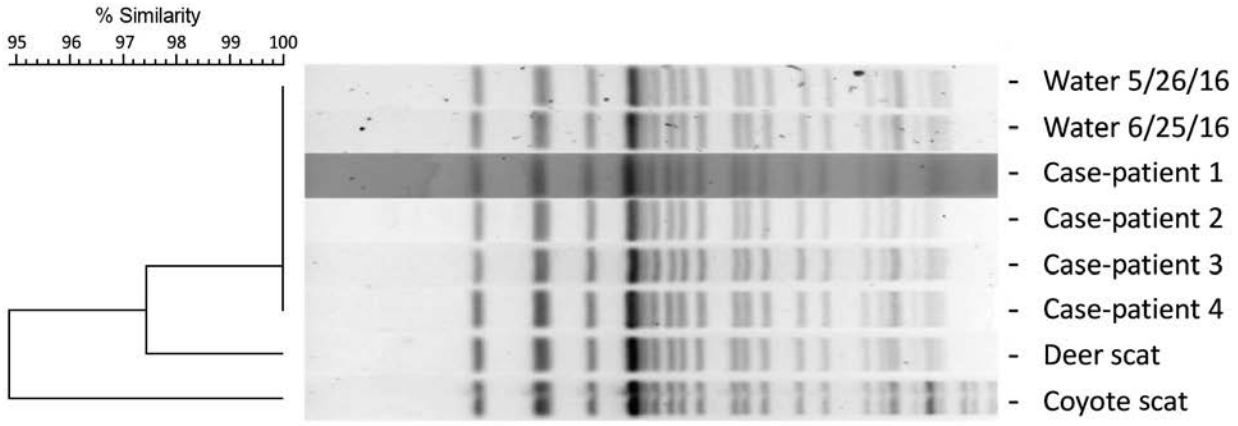

Figure. Pulsed-field gel electrophoresis (PFGE) analysis of Shiga toxin 1– and 2–producing *Escherichia coli* O157 isolates digested with *Xba*I. A dendrogram displaying PFGE pattern similarity is shown at left. The PFGE profiles for the case-patients and water isolates were identical and designated as pattern EXH01.0238 by PulseNet (https://www.cdc.gov/pulsenet/). The PFGE patterns for the deer and coyote scat isolates shared >95% similarity with pattern EXH01.0238. Dates on water samples indicate date of collection.

Thirteen fresh wildlife scat specimens were collected along the stream for STEC culture and PCR. Of the 13 scat specimens, 8 originated from deer, 2 from raccoon, and 1 each from coyote, turkey, and river otter. Six scat specimens (4 deer, 1 coyote, 1 river otter) were positive for stx_1 and stx_2 or for stx_2 by PCR (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/17-0226-Techapp1.pdf). Stx1- and Stx2-producing *E. coli* O157 were isolated from deer scat and coyote scat. An Stx2-producing *E. coli* non-O157 strain was isolated from a deer scat specimen. The animal origin of the coyote and river otter scat specimens were definitively identified by partial DNA sequencing of mitochondrial cytochrome b (*4*).

To assess strain relatedness, we compared STEC O157 isolates from the case-patients, water, deer scat, and coyote scat by using pulsed-field gel electrophoresis (PFGE) and multilocus variable-number tandem-repeat analysis (MLVA) (*5*). PFGE patterns for *Xba*I-digested genomic DNA were highly similar among all isolates; only slight variations were found in the lower-sized bands (Figure). PFGE patterns for genomic DNA samples digested with *Bln*I also demonstrated a high degree of similarity (data not shown). Furthermore, MLVA profiles were identical for the case-patient, water, and deer scat isolates; only the coyote scat isolate differed from the main profile by 2 repeats at a single locus (VNTR_3).

This study provides laboratory evidence linking STEC O157 infections with the ingestion of recreational water that was probably contaminated by wildlife scat. Wild ruminants, including deer and elk, are known carriers of STEC and have been connected to outbreaks of human infections (*6*–*9*). We detected STEC in 50% of deer scat specimens collected from the stream bank. One of these specimens, found 1.5 miles upstream of the exposure site, contained an *E. coli* O157 isolate that was highly similar by molecular subtyping to case-patient and water isolates. These findings support the likelihood that feces from deer carrying STEC were the source of water contamination or, at the very least, contributed to the persistence of STEC in the water. It is unknown whether the STEC detected in coyote and river otter scat represents carriage or transitory colonization within these animals.

The common risk factor among the case-patients in this STEC O157 outbreak was exposure to a natural stream within a city park. After the outbreak was recognized, signs warning of bacterial contamination were posted along the stream. No further STEC O157 infections attributed to stream water exposure were reported.

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Diphtheria in Mayotte, 2007–2015

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Epidemiology of diphtheria in the southwestern Indian Ocean is poorly documented. We analyzed 14 cases of infection with toxigenic *Corynebacterium diphtheriae* reported during 2007–2015 in Mayotte, a French department located in this region. Local control of diphtheria is needed to minimize the risk for importation of the bacterium into disease-free areas.

Diphtheria due to toxigenic *Corynebacterium diphtheriae* occurs sporadically across Europe, mostly in persons who emigrated from disease-endemic countries (*1*,*2*). Systemic toxic effects occur in cutaneous diphtheria but less commonly than in pharyngeal or laryngeal diphtheria. *C. diphtheriae* is a well-recognized cause of chronic, nonhealing skin ulcers in the tropics (*3*,*4*). In France, diphtheria has been a reportable disease since 1945. A vaccination program began in the late 1940s; no cases were reported during 1990–2001, and 13 cases were reported, all in visitors or immigrants to France, since 2002. However, the epidemiology of diphtheria in the southwestern Indian Ocean region, where several French departments are located, is poorly documented.

Mayotte, a French department, is an island of the Comoros archipelago (Figure) and an attractive destination for migrants from the area. In 2012, Mayotte had 212,645 inhabitants, at least 40% foreign born (*5*). We analyzed all cases of infection with toxigenic *C. diphtheriae* reported in Mayotte since 2007 to evaluate the potential risk for dissemination of diphtheria inside and outside this area.

During 2007–2015, local hospitals reported 14 cases of toxigenic *C. diphtheriae* infection to the local health authorities: 1 case in an infant with severe respiratory symptoms who died from multiple organ failure and 13 cutaneous diphtheria cases in patients who survived. Most of the patients (11/14) were male, and the median age was 11 years (range 2 months–39 years). Eight patients had recently (most within 1 month) emigrated from neighboring islands.

Patients' medical history was usually unknown, but vaccination status was available for 12 patients: 7 had received ≥3 doses of diphtheria vaccine according to the vaccination schedule of France (*6*) and 5 were unvaccinated (the infant, 2 children, and 2 adults). All cutaneous infections occurred in patients with preexisting wounds. Eleven patients had chronic diphtheria infections (1–36 months) with

co-infections of other pathogens such as *Staphylococcus aureus* and *Streptococcus pyogenes*. All patients received antimicrobial drug therapy with surgical debridement if necessary. In the absence of signs of toxin dissemination, none of the patients with cutaneous diphtheria received diphtheria antitoxin.

Diagnosis was established by isolation of *C. diphtheriae* and PCR detection of the toxin gene. All 14 isolates were sent to the National Reference Centre (Paris, France) to confirm the identification of the clinical isolates (all positive for *C. diphtheriae tox* gene), determine their biotypes (2 variant *gravis* and 12 variant *mitis*), and characterize their toxin production using the Elek test (positive for 9 isolates). All isolates were sensitive to a large spectrum of antimicrobial drugs, except for fosfomycin. All investigations were conducted by local health authorities to implement control measures (throat swab specimens and antimicrobial prophylaxis of close contacts), according to national recommendations (*7*). Among 168 identified close contacts, we observed 8 asymptomatic respiratory carriers of toxigenic *C. diphtheriae*.

Figure. Location of Mayotte in the southwestern Indian Ocean. Maps created by using IGN-GEOFLA (http://professionnels.ign.fr/geofla) and Esri Data and Maps 10 http://www.esri.com/).

Poor health status and substandard living conditions are risk factors associated with transmission of diphtheria. Cutaneous diphtheria has been shown to be more contagious than respiratory diphtheria (*8*). Furthermore, cutaneous diphtheria may be clinically indistinguishable from other common skin lesions in the tropics and underdiagnosed in cases of co-infection or confusion with the normal skin flora.

Due to its geographic location and given its low socioeconomic status, the population of Mayotte remains exposed to diphtheria. Absence of widespread circulation of toxigenic *C. diphtheriae* cannot be ruled out because of living conditions, the population's difficulties in accessing care, frequency of skin infections, and complexity of microbiological analyses of cutaneous samples. The high coverage for diphtheria vaccination in young children as estimated in a 2010 survey (95% for those 24–59 months of age) is likely to have contributed to the absence of respiratory cases and of systemic complications (*9*). Another possible explanation is that a high rate of skin infections caused by *C. diphtheriae* may result in early development or boosting of natural immunity against the disease (*10*).

Several elements are in favor of a persisting risk: pockets of nonvaccinated populations (many children and teenagers and most adults) in the local context of illegal immigration from neighboring countries pose a risk of severe disease and of persistence of bacterium transmission. The finding that several isolates were producing the toxin is another element, as is the possibility of transmission from a cutaneous lesion to the throat of a close contact. Of note, >20 nontoxigenic isolates (in which PCRs did not detect the *tox* gene) were collected from cutaneous wounds during the same period. In accordance with national guidelines, no control measures were implemented around these cases.

Because human migrations across the area seem inevitable, improving sanitary conditions such as access to clean drinking water and maintaining high immunity levels in the population through vaccination are critical for local prevention and control of diphtheria. Use of these tools will minimize the risk of importation of *C. diphtheriae* into disease-free areas.

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Haemophilus influenzae Type a Meningitis in Immunocompetent Child, Oman, 2015

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Meningitis caused by *Haemophilus influenzae* type b (Hib) was eliminated in Oman after the introduction of Hib vaccine in 2001. However, a case of *H. influenzae* type a meningitis was diagnosed in a child from Oman in 2015, which highlights the need to monitor the incidence of invasive non-Hib *H. influenza*e disease.

Haemophilus influenzae can be encapsulated (serotypes a–f) or unencapsulated, nontypeable (NTHi) (*1*). By the end of 2014, all countries in the Eastern Mediterranean Region had introduced *H. influenzae* type b (Hib) vaccine into their immunization programs; in Oman, where it was introduced in 2001, it led to an elimination of Hib meningitis (*2*,*3*). However, Hib vaccine does not cross-protect against other serotypes.

A previously healthy 17-month-old girl with G6PD deficiency was admitted to Nizwa Hospital, Nizwa, Oman, in August 2015 with a 1-day history of fever and lethargy and frequent vomiting and refusal of food for 6–8 hours before admission. She had no history of rash, head trauma, drug ingestion, travel abroad, or contact with animals. Her vaccination record was up to date. Her 3 older siblings were healthy. On examination, she was irritable and febrile (temperature 39°C), with tachypnea, tachycardia, and photophobia. On lung auscultation, a few crackles were heard on the right side. The rest of her physical examination, including a bedside undilated fundoscopic examination, was unremarkable. Blood tests, cerebrospinal fluid examination, and neuroimaging studies were conducted (Table). Results of renal and liver function and metabolic screening tests and serum calcium, troponin T, immunoglobulins, and total complement levels were within reference limits. Diagnostic test results were negative for respiratory viruses including influenza A(H1N1) and Middle East respiratory syndrome coronavirus and for herpes simplex virus types 1 and 2. A chest radiograph showed right middle lobe haziness suggestive of pneumonitis.

The patient was treated with intravenous ceftriaxone. Blood culture revealed *H. influenzae* type a (Hia), which was serotyped by slide agglutination and determined to

Table. Results of sequential laboratory tests and CT scans of the head during the clinical course of Hia meningitis in a child admitted to Nizwa Hospital, Oman, August 2015*

	Hospitalization day								
	1								
Test type	Adm	Adm + 12	2	8	10	14	18	26	27
Blood									
Leukocytes, × 10^3 cells/μL†	2.24	8.71	10.52	13.08		12.32	8.48	4.56	
Neutrophils, × 10^3 cells/μL‡	0.78	6.04	7.66	4.92		5.51	2.50	0.82	
Lymphocytes, × 10^3 cells/μL§	1.22	2.02	2.38	5.98		5.21	5.12	2.89	
Platelets, × 10^3/μL¶	158.30	79.12	41.13	419.90		644.70	525.80	279.20	
Hemoglobin, g/dL#	10.18	9.30	9.13	9.06		8.47	9.22	10.38	
CRP, mg/L**	79.30		483.40	248.50			33.70	35.30	
Blood culture	Hia			NG				NG	
CSF									
Leukocytes, cells/mm^3	2,970				390			65	
Neutrophils, %	91				10			12	
Lymphocytes, %	9				90			88	
Protein, mg/dL††	190.79				100.34			27.11	
Glucose, mmol/L‡‡	1.13				2.24			2.83	
Glucose CSF:blood	0.26				0.56			0.70	
Gram stain	NM				NM			NM	
Culture	NG				NG			NG	
CT scan of the head									
	Unremarkable			Mild ventricular dilation with bilateral subdural effusion					Complete disappearance of subdural effusion; persistence of mild ventricular dilation

*Adm, at admission; Adm + 12, 12 h after admission; CRP, C-reactive protein; CSF, cerebrospinal fluid; CT, computed tomography; Hia, *Haemophilus influenzae* type a; NA, not applicable; NG, no growth; NM, no microorganisms.
†Reference range 4.5–14.5 × 10^3 cells/μL.
‡Reference range 1.4–9.0 × 10^3 cells/μL.
§Reference range 1.9–9.8 × 10^3 cells/μL.
¶Reference range 150–450 × 10^3/μL.
#Reference range 11.5–15.5 g/dL.
**Reference range 0–5 mg/L.
††Reference range 15–45 mg/dL.
‡‡Reference range 2.2–3.9 mmol/L.

be β-lactamase negative, with susceptibility to all tested antimicrobial drugs. The patient had a protracted clinical course, characterized by continued photophobia, intermittent fever (38–39°C), and subdural effusion. After 10 days of ceftriaxone treatment, her drug therapy was changed to intravenous ampicillin, administered for 2 weeks. Her condition gradually improved; she became afebrile by day 21 after admission and was well at discharge on day 27. Results of vision and hearing screening tests 1 month after discharge and 1 year later were unremarkable.

Several case studies have documented prolonged clinical courses of Hia meningitis, with sequelae reported in some children (*4*,*5*; online Technical Appendix Table, https://wwwnc.cdc.gov/EID/article/23/7/17-0311-Techapp1.pdf). Hia meningitis is strikingly reminiscent of Hib meningitis, manifesting as a serious illness mostly in otherwise healthy children 6–24 months of age (*1*,*4*,*5*). Hia has been reported to be the most virulent among encapsulated *H. influenzae* after Hib; the genetic structure of virulent Hia strains closely resembles that of virulent Hib strains with respect to the duplicated arrangement of the capsule locus and, in some cases, partial deletion of the IS*1016-bexA* gene locus (*5*–*7*; online Technical Appendix Table). An active hospital-based surveillance study for meningitis during 1996–2007 in Salvador, Brazil, reported that Hia and Hib meningitis occurred mainly among children <5 years of age; case-fatality rates were higher than those for meningitis caused by types e and f and NTHi strains, which occurred in older age groups and tended to have a better prognosis (*6*). The study observed an association between IS*1016-bexA* deletion and poor clinical outcome of Hia meningitis.

Since Hib vaccine implementation, concerns have arisen about serotype replacement and emergence of virulent non-b *H. influenzae* (*5*,*6*; online Technical Appendix Table). With documentation of 3 cases (including the case reported here) of Hia meningitis in the Eastern Mediterranean Region within <2 years (*8*), more than a decade after Hib vaccine implementation, it is crucial to monitor meningitis in children within the region, complemented by laboratory characterization of incoming specimens by molecular methods for rapid, accurate information on all

H. influenzae serotypes and NTHi (*1*; https://www.cdc.gov/meningitis/lab-manual/full-manual.pdf; online Technical Appendix Table). In Oman, it is mandatory to report cases of Hib meningitis within 24 hours of laboratory diagnosis, and those caused by other serotypes and NTHi within 1 week, to the Department of Communicable Disease Surveillance and Control, Ministry of Health. Evidence of capsule-deficient variants of Hia that cannot be differentiated from NTHi by conventional methods (*7*) and recurrent invasive diseases (*9*,*10*) and outbreaks caused by Hia (*9*; online Technical Appendix Table) emphasize the necessity for continued surveillance, strong laboratory support, and local epidemiologic studies on non-b *H. influenzae* disease.

Hia meningitis has been reported mainly in the indigenous peoples of Canada, Alaska (USA), and Australia; in the Navajo and White Mountain Apache tribes in the southwestern United States; and in Utah (USA), Brazil, the Gambia, East Africa, and Papua New Guinea. Sporadic cases have been reported in the rest of the world (*1*,*10*; online Technical Appendix Table). The reasons behind the high rates of invasive Hia disease among indigenous children remain unclear (*1*). In Canada, where invasive non-b *H. influenzae* disease has been included in the list of nationally reportable diseases (http://diseases.canada.ca/notifiable/diseases-list) since 2007, a public health–driven initiative has been established to provide a better characterization of the epidemiology of invasive Hia disease and develop a candidate vaccine against Hia (online Technical Appendix Table).

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Importation of Zika Virus from Vietnam to Japan, November 2016

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We report a case of Zika virus infection that was imported to Japan by a traveler returning from Vietnam. We detected

Zika virus RNA in the patient's saliva, urine, and whole blood. In the Zika virus strain isolated from the urine, we found clearly smaller plaques than in previous strains.

Zika virus has been documented in Southeast Asia since the 1940s; however, the prevalence and geographic extent of Zika virus disease in Asia remain unclear (*1*). In Vietnam, 219 cases of Zika virus infection were reported in 2016 and 13 new cases in 2017 (*2*). We report a case of Zika virus infection imported from Vietnam to Japan, diagnosed after PCR amplification of Zika virus RNA in the patient's saliva, urine, and whole blood.

A 40-year-old man came to the National Center for Global Health and Medicine in Tokyo, Japan, in the middle of November 2016 with fever and a rash. In early November 2016, he had traveled to Ho Chi Minh City, Vietnam, where he stayed for 10 days. During his return to Japan, he developed fever and a diffuse rash on his face, trunk, arms, and legs. He went to the hospital on the day after his return and reported having been bitten by mosquitoes in Ho Chi Minh City. Upon arrival at the hospital, he had no fever (temperature 36.8°C); physical examination revealed conjunctivitis and a maculopapular rash on his face, trunk, and extremities. Results of laboratory tests showed leukopenia (2,250 cells/μL; reference 3,500–8,500 cells/

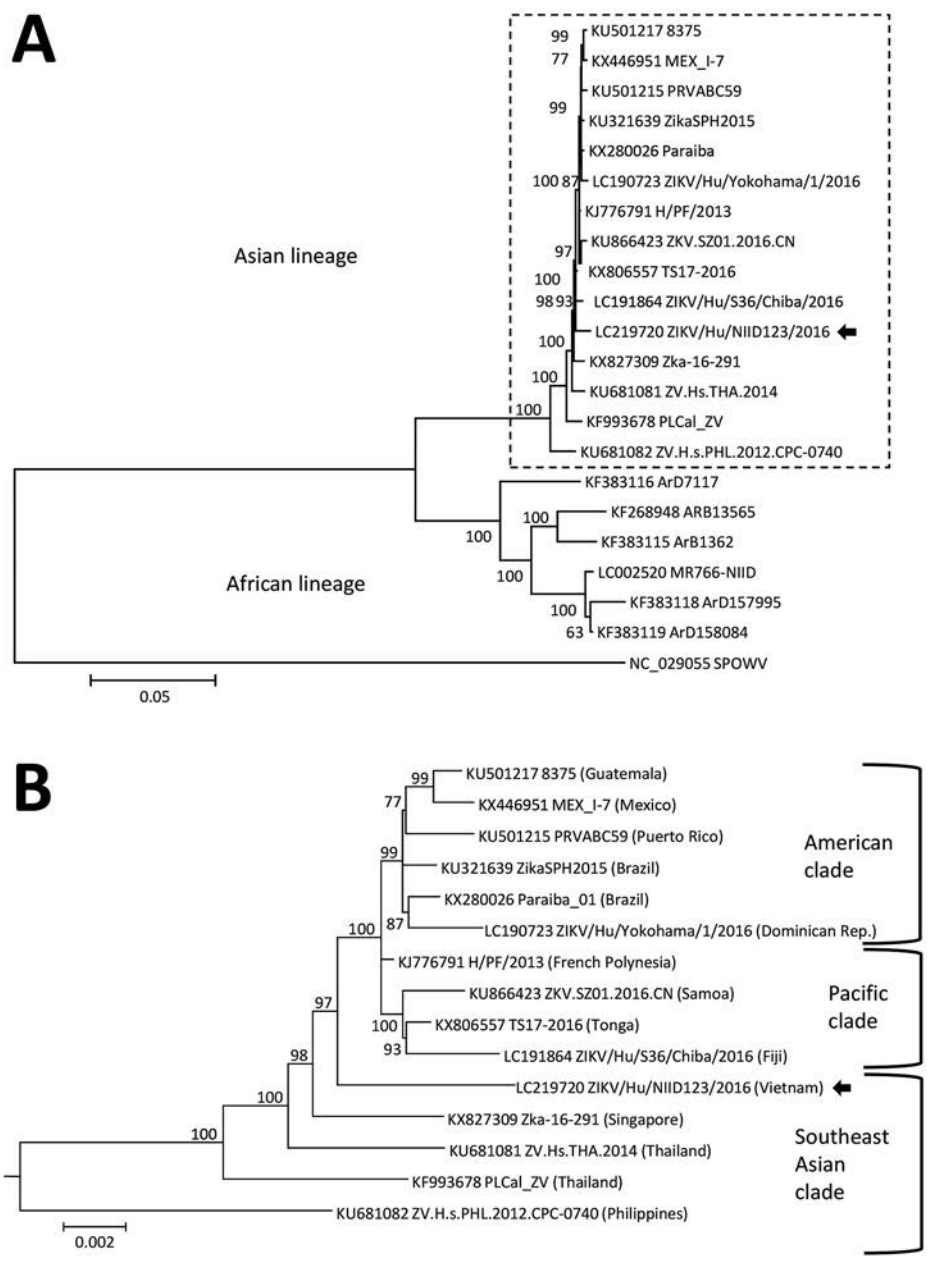

Figure. Phylogenetic analysis of the Zika virus sequence derived from a patient returning to Japan from Vietnam in November 2016. The phylogenetic tree was based on a nearly complete genome and constructed by using the maximum-likelihood method (MEGA 7.0, http://www.kumarlab.net/publications). The sequence derived from the patient is indicated with an arrow. A) The phylogenetic tree based on a nearly full-length region. B) The expanded Asian lineage branch (dotted box in panel A). Scale bars indicate nucleotide substitutions per site.

μL) and a platelet count within reference range. We performed a rapid dengue test (Dengue Duo NS-1 Ag + Ab combo; SD Bioline, Standard Diagnostics Inc., Gyeonggi-do, South Korea); results were negative for nonstructural protein 1, IgM, and IgG. We performed real-time reverse transcription PCR (RT-PCR) amplification using Zika virus primers and probes with urine, saliva, whole blood, serum (obtained 4 days after symptom onset), and semen samples (obtained 6 days after symptom onset). We detected Zika virus RNA in the urine (cycle threshold [C_t] 32.0), saliva (C_t 39.1), and whole blood (C_t 38.1) samples. However, we did not detect Zika virus RNA in the serum or semen samples. We diagnosed Zika virus infection in this patient; his symptoms resolved without treatment within 7 days after he initially sought care.

We successfully isolated the infectious Zika virus from the urine specimen. We amplified the nearly complete genome (10,696 bases; GenBank accession no. LC219720) of the Zika virus from the isolate using RT-PCR and subsequently sequenced it. BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that the isolate was an Asian lineage virus (*3*), sharing 99.3% sequence identity with the Zika virus strain isolated in French Polynesia in 2013 (H/PF/2013; GenBank accession no. KJ776791), 98.9% identity with the strain isolated in Fiji in 2016 (ZIKV/Hu/S36/Chiba/2016; GenBank accession no. LC191864), 99.0% identity with the strain isolated in Puerto Rico in 2015 (PRVABC59; GenBank accession no. KU501215), and 88.5% identity with the strain isolated in Uganda in 1947 (MR766-NIID; GenBank accession no. LC002520). The phylogenetic tree that we constructed using the complete coding region of the Zika virus genome suggested that the sequence belonged to the Southeast Asian clade of the Asian lineage (Figure). The plaque size of the isolated strain in Vero cells was obviously smaller than that of the Asian strain ZIKV/Hu/S36/Chiba/2016 (Pacific clade) (online Technical Appendix, https://wwwnc.cdc.gov/EID/article/23/7/17-0519-Techapp1.pdf).

A previous study showed that Zika virus RNA could be detected more easily in urine than in serum a few days after disease onset (*4*). Other reports showed that Zika virus RNA could be detected for a longer period in whole blood than that in urine and serum (*5*) and that Zika virus RNA could be detected more easily in saliva than in plasma and urine during the first week after symptom onset (*6*). In the case we report, during RT-PCR analysis of the patient's serum, urine, saliva, whole blood, and semen samples, the urine sample showed the lowest C_t, indicating that the urine sample had the highest concentration of Zika virus RNA. However, we could not detect the Zika virus genome in serum and semen samples.

Our phylogenetic analysis suggested that the Asian lineage can be divided into 3 clades (Southeast Asian, Pacific, and American), and the strain we isolated belonged to the Southeast Asian clade. A previous study indicated that a strain isolated in Singapore in 2016 (ZKA-16-291; GenBank accession no. KX827309) also belonged to the Southeast Asian branch, and it was distinct from the isolates obtained in the Americas (*7*). Therefore, it is possible that the isolate from the current case was the strain that is already circulating in Vietnam and was not imported from South America. Our isolate formed smaller plaques in Vero cells than those observed with the other Asian lineage ZIKV/Hu/S36/Chiba/2016 strain (Pacific clade). We also confirmed that the plaque sizes of the PRVABC59 (American clade) and MR766-NIID (African lineage) strains resemble that of ZIKV/Hu/Chiba/S36/2016 (data not shown), suggesting that the Southeast Asian clade Zika virus strains might have a lower cytotoxicity and replicative ability than the American clade and African lineage.

In conclusion, the replicative ability of Zika virus might differ by region and thus influences endemic potential. Further studies are necessary to validate these findings.

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Case of Nigeria-Acquired Human African Trypanosomiasis in United Kingdom, 2016

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Human African trypanosomiasis has not been reported in Nigeria since 2012. Nevertheless, limitations of current surveillance programs mean that undetected infections may persist. We report a recent case of stage 2 trypanosomiasis caused by *Trypanosoma brucei gambiense* acquired in Nigeria and imported into the United Kingdom.

Human African trypanosomiasis (HAT), known as African sleeping sickness, is a protozoal infection, the West African form of which is caused by *Trypanosoma brucei gambiense*. We report a case of imported *T. brucei gambiense* HAT, acquired in Nigeria, where no cases have been reported since 2012 (*1*).

The case-patient, a 58-year-old Nigerian woman, lived near Warri, in Delta State, Nigeria. She traveled infrequently to towns within Delta State, across the Niger River into Bayelsa State, and to larger cities in Nigeria, but never outside Nigeria. She reported no history of tsetse fly bites.

In January 2016, the patient experienced leg tremors and lethargy. These symptoms persisted until arrival in the United Kingdom in May 2016. Over the next 2 months, increasing malaise and unsteadiness in walking developed. In August 2016, the patient was admitted to a regional hospital with confusion and drowsiness. She was febrile at admission but had no lymphadenopathy; neurologic examination revealed no neck stiffness or photophobia, but did show poor coordination with slow cognitive processes.

Laboratory investigations revealed microcytic anemia with a C-reactive protein level of 13 mg/L (reference value <5 mg/L) and a total serum IgM of 13.7 g/L (reference range 0.5–2.0 g/L). A blood film was negative for malaria. Confirmatory assays after positive screening assay results for HIV and syphilis antibodies showed the original results to be false positive.

Cerebrospinal fluid (CSF) examination revealed 331 leukocytes/mm^3, 99% lymphocytes; CSF protein level 0.82 g/L (reference range 0.23–0.38 g/L); and glucose level was >50% plasma glucose. Results of CSF PCR for herpesviruses, enterovirus, and JC virus were negative. Results of GeneXpert (Cepheid, Buckinghamshire, UK) tests of CSF and mycobacterial culture were negative. Magnetic resonance imaging of the brain showed, on T2 weighted and flair images, bilateral diffuse hyperintensities within white matter located in the periventricular regions, basal ganglia, cerebellum, and brainstem.

Treatment with ceftriaxone, acyclovir, antituberculous treatment and prednisolone was stopped at 14 days because of a lack of clinical improvement and drug-induced transaminitis.

The patient's lethargy, intermittent confusion, and periods of somnolence became more severe. Examination in August 2016 revealed intention tremor in all limbs and myoclonic jerks. Her case was discussed with the Imported Fever Service at Public Health England. A serum sample was sent to the Hospital of Tropical Diseases in London, UK for *T. brucei gambiense* indirect fluorescent antibody testing (IFAT), which showed a positive result (titer 1:400). The patient was transferred to this hospital.

Repeat CSF examination revealed a protein level of 1.14 g/L, CSF glucose level of 2.3 mmol/L (serum 5.5 mmol/L), and 1,140 leukocytes/mm^3 (90% mononuclear). No trypanosomes were seen in the buffy coat of peripheral blood or CSF. CSF total IgM of 1.98 mg/L (reference range 0–0.9 mg/L) and IgG of 306 mg/L (reference range 10–40 mg/L) were markedly raised (*2*).

T. brucei gambiense IFAT results for CSF (titer 1:4) and blood (titer 1:400) were positive. DNA extracted from CSF was positive for trypanosomes of the subgenus *Trypanozoon* by PCR (*3)* and confirmed as *T. brucei gambiense* group 1 by diagnostic PCR with *TgsGP* primers (*4*). The result of immune trypanolysis was negative

when *T. brucei gambiense* variable antigen type LiTat 1.3 was used but positive when using LiTat 1.5 (*4*). The patient had a titer of 1:32 in the CATT/*T. brucei gambiense* test (*5*,*6*).

We administered nifurtimox/eflornithine combination therapy, according to World Health Organization guidelines, for treatment of stage 2 *T. brucei gambiense*. By the end of treatment, the patient's cognitive function, myoclonus, tremor, and ataxia had all improved, and she was discharged.

A posttreatment CSF examination revealed 4 leukocytes/mm^3 and a protein level of 1.17 g/L. Six months after discharge, the patient had made a full functional recovery. Repeat CSF examination revealed 4 leukocytes/mm^3 and a protein level of 0.63 g/L, and MRI of the brain showed improvement. The CSF *T. brucei gambiense* IFAT titer declined to 1:2.

No cases of HAT in Nigeria have been reported to the World Health Organization since 2012 (*1*). The last reported patients were from the Abraka region, in Delta State, ≈50 km from this case-patient's residence (*7*). We do not have a clear exposure time for this patient, which is likely to have been within 2–3 years before her hospitalization, although we have reported a case-patient with an exposure ≥29 years prior to illness onset; the emerging scientific consensus is that trypanotolerance may exist among some infected patients, leading to delay in diagnosis and premature confidence of disease elimination (*8*,*9*).

A clue to diagnosis, alongside clinical and travel history, was a high serum IgM level, a prominent feature in *T. brucei gambiense*–infected patients, as a result of polyclonal B-cell activation (*10*). False-positive antibody test results specific for pathogens have been reported; multiple positive antibody tests may aid the clinician in considering HAT as a differential diagnosis (*10*).

This case shows that HAT transmission continues in Nigeria and surveillance in Delta state is warranted. There may be a role for additional diagnostic tools if we are to achieve the goal of eliminating HAT by 2030.

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We thank Jose Ramon Franco Minguell, the HAT Control and Surveillance Programme, and the World Health Organization for releasing the nifurtimox/eflornithine combination therapy medication and for providing advice on the manuscript.

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Mycobacterium chimaera Isolates from Heater–Cooler Units, United Kingdom

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To the Editor: In their recent article, Svensson et al. provided results of an investigation into contamination of heater–cooler units (HCU) used in open-chest surgery with *Mycobacterium chimaera* in Denmark (*1*). We write to provide further information on the UK isolates included in their study.

The authors performed whole-genome sequencing (WGS) of 5 isolates sampled from HCUs in hospitals in Denmark, 1 from a Maquet HCU and 4 from Sorin 3T HCUs. They compared these sequences with publicly available WGS data for 31 *M. chimaera* isolates sampled in the United Kingdom, United States, and Ireland and reported, "*M. chimaera* sequences from the UK HCU water samples were genetically nearly identical to the US and Denmark isolates" (*1*). Without knowledge of the source of the 8 UK HCU isolates, and given the high genetic similarity found between Sorin 3T HCU isolates, the authors concluded that the UK isolates probably originated from Sorin 3T HCUs (*1*).

The WGS data for the UK isolates were generated as part of our investigation into the *M. chimaera* outbreak in the United Kingdom, which included national case finding, aerobiological investigations, environmental sampling, and phylogenetic analysis (*2*). We performed WGS for 246 *M. chimaera* isolates, including multiple isolates from 15 cardiothoracic surgery patients, 143 control patients without history of cardiothoracic surgery, and 11 HCUs. We found close phylogenetic clustering of isolates from cardiothoracic patients and HCUs. All HCU isolates for which WGS data were generated originated from Sorin 3T HCUs, including the 8 UK isolates analyzed by Svensson et al., confirming their inference of a shared origin. We have added this information to the corresponding US National Center for Biotechnology Information Short Read Archive BioProject (accession no. PRJNA324238).

Our investigation also identified a mechanism of aerosol release through controlled laboratory assessment of a decommissioned HCU. Nationwide risk quantification identified a low but increasing risk of *M. chimaera* infection. A UK-wide patient notification exercise is underway (*3*,*4*).

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Outbreaks and Surveys: A Dilogy

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GIDEON Guide to Outbreaks, 1,818 pages, 47,454 references, ISBN: 978-1-4988-1276-4 (e-book), https://www.gideononline.com/ebooks/outbreaks

GIDEON Guide to Surveys, 3,705 pages, 48,430 references, ISBN: 978-1-4988-1274-0 (e-book), https://www.gideononline.com/ebooks/surveys

Pages: 1,811 and 3,682; Price: $99.99 each

For medical academics and health workers, the task of efficiently gathering published data concerning outbreaks and epidemics can be overwhelming and time-consuming, always at the risk of excluding crucial information. As of February 2017, a single source has compiled a staggering amount of medical information into a comprehensive collation of infectious disease outbreaks and surveys.

GIDEON Guide to Outbreaks was "written" by a computer program that converts database entries (https://www.gideononline.com) into e-book format. The guide describes 21,365 pandemics, epidemics, and case-clusters summarized in 5,120 tables, each devoted to a specific disease–country grouping (e.g., Salmonellosis–Albania …Salmonellosis–Zimbabwe). Charts detail outbreak year, region (e.g., city, province), affected population, setting, number of cases, number of deaths, pathogen, syndrome, vehicle/source, and electronically linked references. Where relevant, chapters begin with a listing of global and regional pandemics or unusually extensive or iconic events (i.e., the Typhoid Mary outbreaks, the Black Death). To facilitate searching within the book, introductory charts summarize total outbreaks, earliest recorded outbreaks, links to specific countries, and indexing terms (including foreign language disease designations).

The format of GIDEON Guide to Surveys is similar to that of the Outbreaks book; it chronicles all published studies of disease prevalence (e.g., hookworms in Tanzania, gonorrhea in the United States) and seroprevalence (HIV in India, toxoplasmosis in Brazil). The current edition includes 53,917 studies.

Charts in Outbreaks group events by year, and charts in Surveys list entries by demographic group (e.g., blood donors, children, STD patients). Sorting these tables by user-selected parameters (region, case numbers) is not possible in the e-book format, but the option is available in the online version of the program.

Both books are updated yearly. Both incorporate data for zoonotic diseases among animals, but not to the extent allotted to human illness. The list of diseases and their references (many from PubMed) included in these charts is exhaustive and includes such illnesses and conditions as Spondweni virus infection, coenurosis, philophthalmosis, and Wesselsbron disease. On the other hand, these books do not address healthcare-associated infections or infections caused by a growing list of bacteria of >1,600 taxa associated with human disease (e.g., *Acinetobacter*, *Klebsiella*, enterococci). The most useful features of these 2 e-books are their exhaustive and up-to-date content, intuitive organization, and encyclopedic format.

It would be helpful if relatively major events were displayed in bold text. For example, Outbreaks lists 2 outbreaks of trichinosis that occurred in France in 1985, one of 21 and the other of 1,073 cases, without special visual treatment or comment. Those of us accustomed to standard books written in lines of text will find these sources difficult to "read."

Berger notes that GIDEON Guide to Outbreaks and GIDEON Guide to Surveys are written for use by workers, educators, and students in the fields of infectious diseases and geographic medicine. I am certain that both will prove useful to readers of Emerging Infectious Diseases.

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Correction: Vol. 23, No. 4

The number of semen samples with high Zika virus levels was incorrectly stated in the abstract of Presence and Persistence of Zika Virus RNA in Semen, United Kingdom, 2016 (B. Atkinson et al.). Zika virus RNA was detected at high levels in 12 (52.2%) samples and was not detected in 10 (43.5%) samples from 43 patients. The article has been corrected online (https://wwwnc.cdc.gov/eid/article/23/4/16-1692_article).

Fabergé firm, Russian, 1842–1917, *Imperial Red Cross Easter Egg*, 1915 (detail)**.** Egg: enamel, silver-gilt, gold; screen: enamel, gold, mother-of-pearl, watercolor, ivory; 3 in x 2 3/8 in/7.7 cm x 6 cm. © Virginia Museum of Fine Arts, Richmond, Virginia, USA. Bequest of Lillian Thomas Pratt. Photo: Katherine Wetzel. © Virginia Museum of Fine Arts.

Elaborate Details, Hidden Surprises

Byron Breedlove and Terence Chorba

In 1885, Russian Emperor Alexander III commissioned the first Imperial Easter egg from the House of Fabergé as a gift for his wife, Maria Feodorovna. Upon Alexander's death in 1894, his son, Emperor Nikolai II, continued this Imperial family tradition, annually requesting one egg for his mother and another for his wife. The skilled artisans who created these treasures worked under the auspices of master craftsman Peter Carl Fabergé, head of the firm that still bears his family name.

Fabergé operated with creative freedom, and his craftsmen had a treasure chest of riches at their disposal. Diamonds, pearls, sapphires, rubies, gold, guilloché enamel, aquamarine, lapis lazuli, ivory, mother of pearl, and platinum were commonly used embellishments. Each of these unique 50 *objets d'art* created during 1885–1916 shared a single feature: a concealed surprise. These sequestered miniature wonders include a rotating globe, a replica of Gatchina Palace, a four-leaf clover, a gold watch, Peter the Great's monument on the Neva River, miniature portraits, a mechanical swan, an 18th century carriage that took 14 months to complete, and a jeweled elephant automaton (rediscovered in 2015 and reunited with its egg in 2017).

Fabergé expert Géza von Habsburg describes Fabergé eggs as being "the absolute summit of craftsmanship. They are unbelievably made. They were the sort of apogee of

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what Fabergé was able to do, and he lavished everything he could on them." The whereabouts of 43 of the 50 Imperial Fabergé eggs are known, and 5, including the Imperial Red Cross Easter Egg featured on this month's cover, are found in the Virginia Museum of Fine Arts.

This egg, created in 1915 during World War I, honors the efforts of the Empress Alexandra Feodorovna, who served as head of the Russian branch of the International Red Cross. Feodorovna and her older daughters had trained to become nurses, and she had the Winter Palace set up to function as a makeshift hospital for the growing number of wounded soldiers. Because Fabergé recognized that the mood in Russia was growing more solemn and grave as the war progressed, he focused on the royal family's service instead of showcasing its lavishness during this time of wartime austerity.

The Fabergé Research Site offers this description for the egg, which was created by workmaster Henrik Wigström: "Opalescent white guilloche enamel covers a chased silver ground on this egg. Two opposing red enamel crosses bear the dates 1914 and 1915. A Russian inscription, in stylized gold enamel script in a band around the egg reads: 'Greater love hath no man than this, that a man lay down his life for his comrades.' On the top of the egg is the crown and monogram of the Dowager Empress Marie in silver, while at the bottom is a six-petal rosette."

Concealed inside this egg is a hinged, folding screen that displays 5 miniature portraits of the tsar's mother, sister, daughters, and cousin wearing Red Cross uniforms. Each portrait is encased in white enamel, mounted in gold, and backed with mother of pearl. The screen is signed by craftsman Vassilii Zuiev.

At this time, the fall of the Romanov dynasty was nearing. The Imperial Fabergé eggs, festooned with jewels and fine metals and containing bejeweled hidden surprises, remain as delicate reminders.

Within microbiological realms, intracellular pathogens offer an analogous melding of elaborate detail and hidden surprises. Honed through evolution rather than hours logged on an artisan's workbench, intracellular pathogens comprise an ancient, diverse group of microbes that by dint of moving into the intracellular environment have mastered living within the hostile realm of host macrophages.

Intracellular pathogens are classified into two groups: obligate and facultative. Obligate intracellular pathogens cannot survive outside of their host cells and include viruses, some bacteria (*Chlamydiae*, *Rickettsia* spp., *Coxiella burnetti*, *Mycobacterium leprae*), and protozoa. Facultative intracellular pathogens survive and replicate outside of their host cells and include many bacteria (*Legionella pneumophila*, *Rickettsia rickettsii*, *Mycobacterium tuberculosis*, *Listeria monocytogenes*, *Salmonella* spp., *Shigella* spp., invasive *Escherichia coli*, *Neisseria* spp., and *Brucella* spp.) and fungi (*Histoplasma capsulatum*).

Intracellular pathogens sequestered within the walls of their host cells commonly cause granulomatous lesions and are not delightful surprises, unlike the surprises ensconced inside the Fabergé Imperial Eggs. In addition to those previously noted, a partial roll call of intracellular pathogens includes *Yersinia pestis*, *Cryptococcus neoformans*, *Burkholderia pseudomallei*, and *Plasmodium* spp. Such pathogens are clearly not rarities, cause a wide range of infections in animals, and are major contributors to human morbidity and mortality globally.

During World War I and its wake, an estimated 16 to 22 million military combatants and civilians died. Professor Francis Cox of Gresham College has noted the difficulty of obtaining accurate figures and states that "it is almost impossible to make any meaningful comparisons between the numbers of those who died in combat and those who died from disease as a direct result of combat." The Red Cross emblazoned on this 1915 Fabergé Imperial egg reminds us that healing and medicine are intrinsically intertwined with conflict. As Professor Cox has written, ". . . all over the world in every combat zone, big or small, diseases still have the potential to hold the upper hand and . . . we are complicit in their survival and spread."

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EMERGING INFECTIOUS DISEASES®

Upcoming Issue

- Zika Virus Infection in Patient with No Known Risk Factors, Utah, USA, 2016
- Characteristics of Dysphagia in Infants with Microcephaly due to Congenital Zika Virus Infection
- *Bartonella quintana*, an Unrecognized Cause of Infective Endocarditis in Children in Ethiopia
- Genomic Characterization of Recrudescent *Plasmodium malariae* after Treatment with Artemether/Lumefantrine
- Human Infection with Highly Pathogenic Avian Influenza A(H7N9) Virus, China
- Molecular Characterization of *Corynebacterium diphtheriae* Outbreak Isolates, South Africa, March–June 2015
- High Infection Rates for Adult Macaques after Intravaginal or Intrarectal Inoculation with Zika Virus
- Characterization of Fitzroy River Virus and Serologic Evidence of Human and Animal Infection
- Maguari Virus Associated with Human Disease
- Real-Time Evolution of Zika Virus Disease Outbreak, Roatán, Honduras
- Serologic Evidence of Powassan Virus Infection in Patients with Suspected Lyme Disease
- Density-Dependent Prevalence of *Francisella tularensis* in Fluctuating Vole Populations, Northwestern Spain
- Occupational Exposures to Ebola Virus in Ebola Treatment Center, Conakry, Guinea
- Serologic Evidence of Scrub Typhus in the Peruvian Amazon
- Seroprevalence of *Baylisascaris procyonis* Infection in Humans, Santa Barbara County, California, USA, 2014–2016
- Preliminary Epidemiology of Human Infections with Highly Pathogenic Avian Influenza A(H7N9) Virus, China, 2017
- West Nile Virus Outbreak in Houston and Harris County, Texas, 2014
- Genesis of Influenza A(H5N8) Viruses
- Global Spread of Norovirus Genogroup II Genotype 17 Kawasaki 308, 2014–2016
- Clonal Expansion of New Penicillin-Resistant Clade of Serogroup W, Clonal Complex 11 *Neisseria meningitidis*, Western Australia
- Outcomes for 2 Children after Peripartum Acquisition of Zika Virus Infection, French Polynesia, 2013–2014
- Scrub Typhus Outbreak in a Remote Primary School, Bhutan, 2014
- Mucus-Activatable Shiga Toxin Genotype stx2d in *Escherichia coli* O157:H7
- *mcr-1* and *bla* in *Escherichia coli* ST744 after Meropenem and Colistin Therapy, Portugal

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 1–3, 2017
Emerging and Re-emerging Viruses Symposia
Arlington, VA, USA
http://cell-symposia.com/emerging-viruses-2017/

October 3–7, 2017
ID Week
San Diego, CA, USA
http://www.idweek.org/

November 5–9, 2017
ASTMH
American Society for Tropical Medicine and Hygiene
66th Annual Meeting
The Baltimore Convention Center
Baltimore, MD, USA
http://www.astmh.org/

December 5–8, 2017
6th National Congress of Tropical Medicine and International Symposium on HIV/aids Infection
9th National Congress of Microbiology and Parasitology
80th Anniversary of the Institute of Tropical Medicine Pedro Kourí
Havana, Cuba
http://microbiologia2017.sld.cu/index.php/microbiologia/2017

February 1–3, 2018
8th Advances in Aspergillosis
Lisbon, Portugal
www.AAA2018.org

March 1–4, 2018
18th International Congress on Infectious Diseases (ICID)
Buenos Aires, Argentina
http://www.isid.org/icid/

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Article Title

Clonal Clusters and Virulence Factors of Group C and G *Streptococcus* Causing Severe Infections, Manitoba, Canada, 2012–2014

CME Questions

1. Your patient is a 67-year-old man with group C and G *Streptococcus* (GCGS) bacteremia from a skin infection. According to the retrospective study by Lother and colleagues, which of the following statements about clinical features of patients with GCGS bacteremia is most accurate?

A. Younger patients were more likely to have GCGS bacteremia
B. An estimated 10% of patients had necrotizing fasciitis or pharyngitis
C. Comorbid conditions were common, predominantly cardiovascular disease (47%) and diabetes mellitus (43%)
D. Approximately half of patients had bacteremia from skin and soft tissue infection, and only 7% had primary bacteremia

2. According to the retrospective study by Lother and colleagues, which of the following statements about outcomes in patients with GCGS bacteremia is correct?

A. Disease burden was lower than previously reported, suggesting decreased virulence of recently emerging strains
B. Half of patients were admitted to an intensive care unit (ICU)
C. In-hospital all-cause mortality rate was 10%
D. Vasopressors were needed in 19% of patients, and ventilator support was needed in 17%

3. According to the retrospective study by Lother and colleagues, which of the following statements about genetic determinants of GCGS bacteremia is correct?

A. Seven unrelated genetic lineages were more likely to cause invasive disease
B. Virulence factors *cbp, fbp, speG, sicG, gfbA,* and *bca* clustered clonally into the clades more likely to cause invasive disease
C. Invasive infection could be attributed to a single genetic determinant
D. A specific cluster within clade E organisms was associated with polymicrobial bacteremia with *Staphylococcus aureus*

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to http://www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers.

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Only one answer is correct for each question. Once you successfully answer all post-test questions, you will be able to view and/or print your certificate. For questions regarding this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please go to https://www.ama-assn.org. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the AMA PRA CME credit certificate, and present it to your national medical association for review.

Article Title

Risk Factors for *Legionella longbeachae* Legionnaires' Disease, New Zealand

CME Questions

1. You are seeing a 40-year-old woman with a 1-week history of cough with hemoptysis and fever. She is an avid gardener, and you consider whether she might have Legionnaires' disease. What should you consider regarding the background of Legionnaires' disease?

A. The mortality rate among inpatients is approximately 10%
B. It is often overdiagnosed
C. The urinary antigen test detects all *Legionella* species
D. Approximately 95% of cases resolve with no intervention

2. Which of the following health conditions was most strongly associated with *L. longbeachae* pneumonia in the current study?

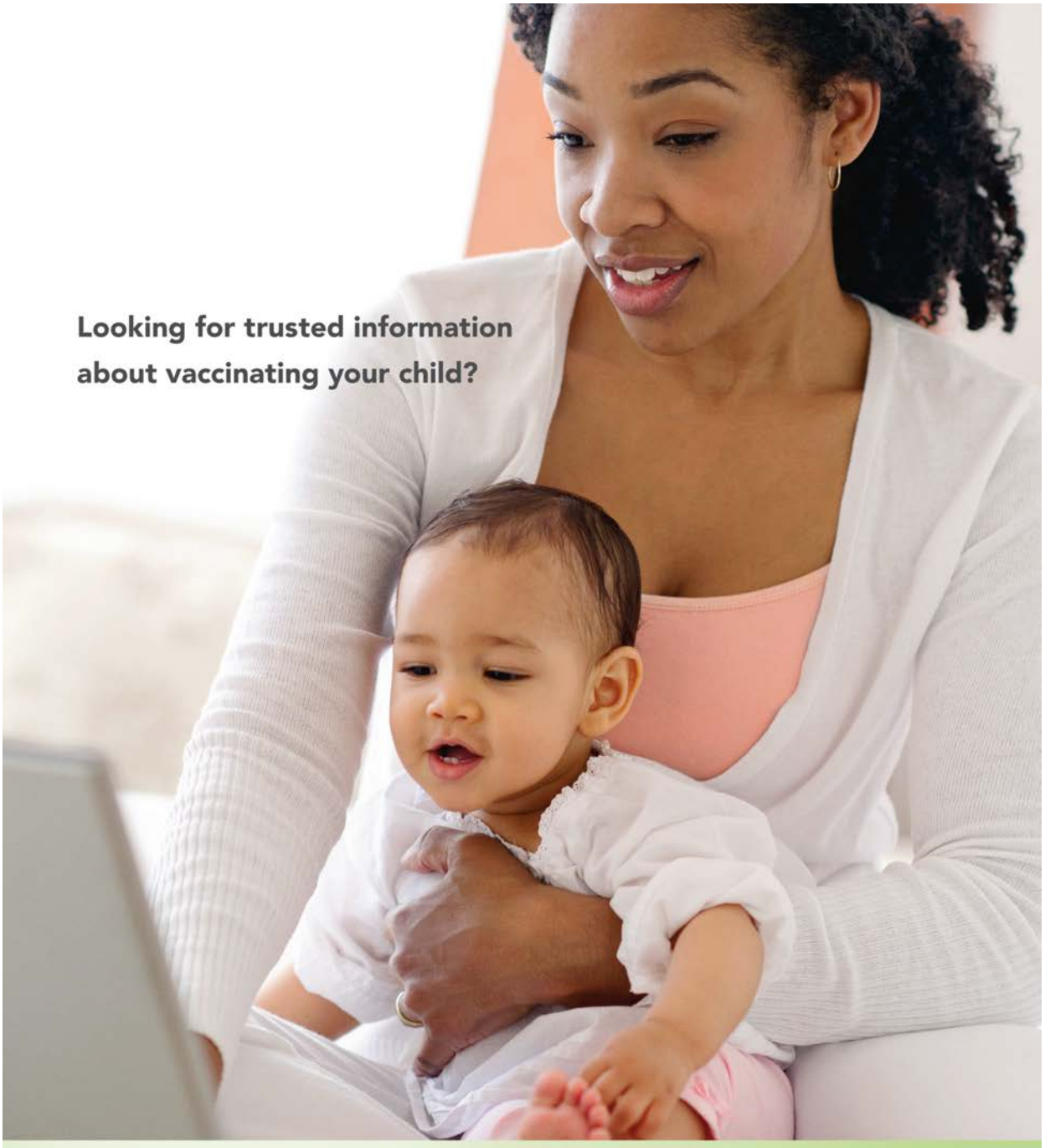
A. HIV/AIDS
B. Congestive heart failure
C. Pulmonary fibrosis
D. Chronic obstructive pulmonary disease (COPD)

3. Which of the following statements regarding variables associated with a higher risk for *L. longbeachae* infection in the current study is most accurate?

A. Female sex and younger age were risk factors for infection
B. There was a dose-response effect of smoking in increasing the risk for infection
C. Homemade compost was associated with the highest risk for infection
D. Hand-to-face contact with compost did not increase the risk for infection

4. Which of the following methods was most proven to reduce the risk for infection with *L. longbeachae* in the current study?

A. Hand hygiene only
B. Hand hygiene and wearing gloves
C. Wearing gloves only
D. Wearing gloves and/or a mask



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Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.

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