EMERGING INFECTIOUS DISEASES®

CDC
Centers for Disease Control and Prevention

Antimicrobial Resistance

July 2019

Amie Esslinger (b. 1984), *Hydro Vents and Other Difficult Places* (2016). Acrylic, ink, paper, gel medium, thread, 30 in x 22 in/76 cm x 56 cm. Digital image courtesy of the artist.

EMERGING INFECTIOUS DISEASES®

Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop H16-2, Atlanta, GA 30329-4027, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

EMERGING INFECTIOUS DISEASES®

Antimicrobial Resistance

July 2019

On the Cover

Amie Esslinger (b. 1984), *Hydro Vents and Other Difficult Places* (2016). Acrylic, ink, paper, gel medium, thread, 30 in x 22 in/76 cm x 56 cm. Digital image courtesy of the artist.

About the Cover p. 1440

Synopses

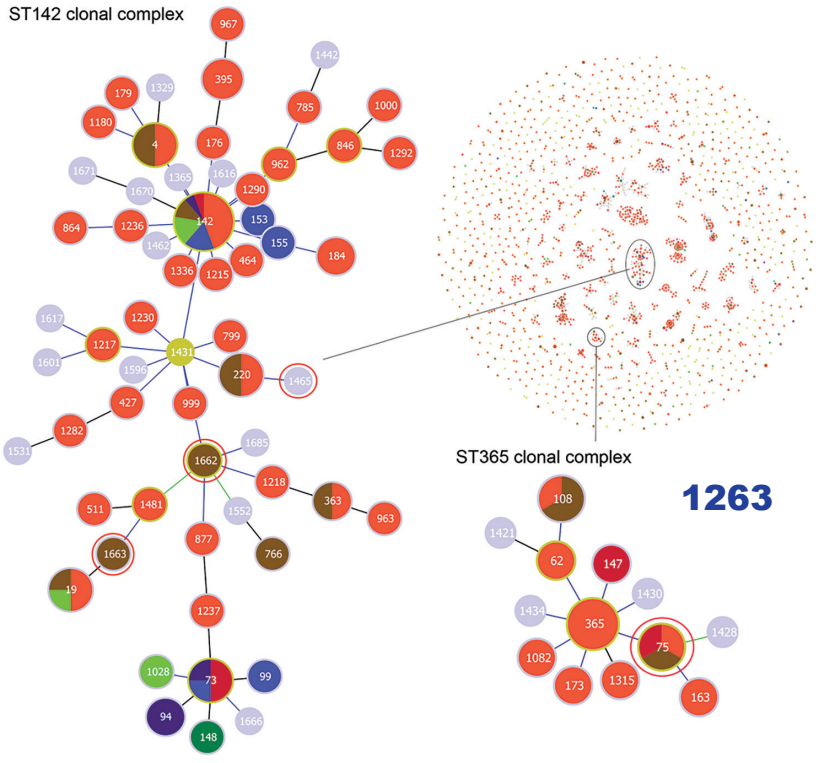
Research

EMERGING INFECTIOUS DISEASES®

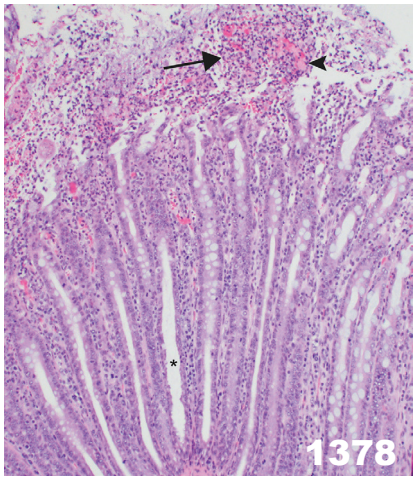
July 2019

Historical Review

Dispatches



Research Letters



EMERGING INFECTIOUS DISEASES®

July 2019

The World Unseen:
Intersections of Art and Science
May 20–August 30, 2019

Amie Esslinger, *Collisions*, mixed media, 2016

The World Unseen: Intersections of Art and Science gathers the work of 10 international artists who draw upon microbiology, biotechnology, anatomy, and texts in their investigations of microbes and cells, DNA, history of disease and science, the body, and beauty. Through paintings, drawings, installations, and videos, these artists ponder the humanistic and scientific implications of knowing and seeing what we normally cannot see.

DAVID J. SENCER
CDC MUSEUM
History·Legacy·Innovation

The David J. Sencer CDC Museum, a Smithsonian Affiliate, uses award-winning exhibitions, dynamic educational programming, and physical and web archives to educate visitors about the value of public health, while presenting the rich heritage and vast accomplishments of CDC.

Hours

Monday–Wednesday: 9 a.m.–5 p.m.
Thursday: 9 a.m.–7 p.m.
Friday: 9 a.m.–5 p.m.
Closed weekends and federal holidays

Location

1600 Clifton Road, NE
Atlanta, GA
30329-4021
Phone (404) 639-0830

Admission and parking free
Vehicle inspection required
Government-issued photo ID required for adults over the age of 18
Passport required for non-U.S. citizens

Bacillus cereus–Attributable Primary Cutaneous Anthrax-Like Infection in Newborn Infants, India

Lahari Saikia,[1] Navonil Gogoi, Partha Pratim Das, Arunjyoti Sarmah, Kumari Punam, Bipanchi Mahanta, Simi Bora, Reeta Bora

During March 13–June 23, 2018, anthrax-like cutaneous lesions attributed to the *Bacillus cereus* group of organisms developed in 12 newborns in India. We traced the source of infection to the healthcare kits used for newborn care. We used multilocus sequence typing to characterize the 19 selected strains from various sources in hospital settings, including the healthcare kits. This analysis revealed the existence of a genetically diverse population comprising mostly new sequence types. Phylogenetic analysis clustered most strains into the previously defined clade I, composed primarily of pathogenic bacilli. We suggest that the synergistic interaction of nonhemolytic enterotoxin and sphingomyelinase might have a role in the development of cutaneous lesions. The infection was controlled by removing the healthcare kits and by implementing an ideal housekeeping program. All the newborns recovered after treatment with ciprofloxacin and amikacin.

The *Bacillus cereus* group includes ecologically diverse gram-positive and endospore-forming bacilli that are ubiquitous in the environment. The prominent members of this group are *B. wiedmanii*, *B. anthracis*, *B. cereus* sensu lato, *B. cereus* sensu stricto, *B. thuringiensis*, *B. weihenstephanensis*, *B. mycoides*, *B. pseudomycoides*, *B. cytotoxicus*, and *B. toyonensis*. Because the endospores of these species can resist extreme environmental conditions and thermal treatments, they are difficult to eliminate from processing chains of healthcare products and from clinical settings (*1*). The pathogenic potential of the *B. cereus* group varies from strains used as probiotics in animal feed to lethal and highly toxic strains (*2*,*3*). Thus, determining the degree to which pathogenic strains can be distinguished from nonpathogenic strains is essential.

B. cereus is well-known as a foodborne pathogen. In recent years, this bacterium was reported to cause several systemic and local nongastrointestinal infections in immunocompromised and immunocompetent persons (*4,5*). Specific populations, including intravenous drug abusers and patients with postsurgical or posttraumatic wounds, are at risk for these infections (*6,7*). In addition, numerous cases of fulminant infections similar to anthrax have been reported in healthy persons (*8,9*). Skin lesions of *B. anthracis* infection begin with a papule, which eventually becomes serosanguinous and develops a black eschar similar to some of the *B. cereus* skin lesions described by Henrickson et al. (*10*). Infections caused by *B. cereus* in newborns have been reported occasionally (*11,12*). We describe a cluster of 12 cases of severe anthrax-like cutaneous infections in otherwise healthy newborns attributed to the *B. cereus* group.

Materials and Methods

Case Study and Investigation

The Assam Medical College & Hospital (AMCH) is a tertiary care hospital in Dibrugarh, Assam, in northeastern India. During March 13–June 23, 2018, extensive cutaneous vesicles or bullous lesions, mostly on the face, neck, and arm, developed in 12 newborns (8 boys, 4 girls); gas gangrene–like lesions eventually developed in 2 of the infants (Figure 1). All had been born healthy. All 12 newborns had a positive indication of sepsis. The initial clinical diagnosis was early-onset sepsis with staphylococcal scalded skin syndrome. Retrospectively, when records of these cases were analyzed, blood cultures were sterile or had growth of coagulase-negative *Staphylococcus aureus*.

This investigation focused on the labor room and the attached baby room because skin lesions developed within a few hours after delivery. Samples from exposed healthcare products, including the healthcare kits that contained items used during delivery, were cultured in nutrient broth and incubated at 37°C for 24 h. Upon confirmation of visible growth in nutrient broth, the samples were subcultured in blood and nutrient agar and incubated at 37°C for 24 h. Hand swab samples from hospital staff

Author affiliation: Assam Medical College & Hospital, Dibrugarh, India

DOI: https://doi.org/10.3201/eid2507.181493

[1]Current affiliation: Gauhati Medical College & Hospital, Guwahati, India.

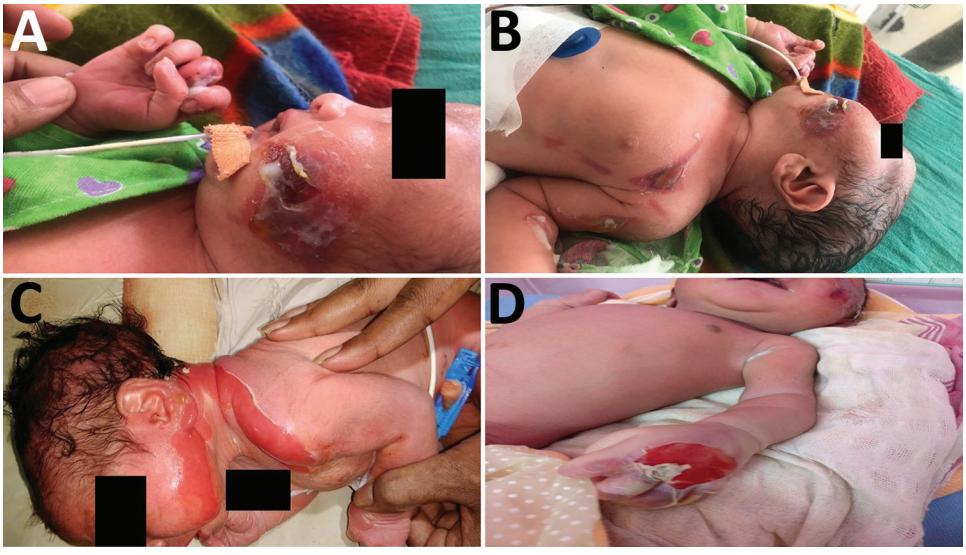


Figure 1. Newborn infants with cutaneous lesions mostly on face (A), left upper chest (B), neck (C), and hand (D), Assam Medical College & Hospital, Dibrugarh, India, 2018. This outbreak was later determined to have been caused by *Bacillus cereus*.

and the environment were cultured both aerobically and anaerobically. We obtained samples from the skin, armpit, and umbilical cord stump of newborns just after delivery at 2-day intervals. All samples from infants, staff, and the environment showed substantial growth of *Bacillus* species. All the bacteriology work was conducted in a Biosafety Level 3 laboratory. The institutional ethics committee (human) of AMCH approved this study.

Intervention

On May 19, 2018, after confirmation of *B. cereus* group in the healthcare kits, the infection control officer from the AMCH Department of Microbiology advised using these kits only after they were autoclaved in a validated steam autoclave and terminal cleaning (i.e., extensive cleaning of all detachable objects in the room, cleaning of air duct surfaces in the ceiling, and thorough cleaning of everything downward to the floor) of the labor and the attached baby room was performed. However, on June 23, the same type of lesion developed in another newborn. When *B. cereus* outbreaks occur, obtaining control is difficult because these bacilli can survive long periods in the environment and are resistant to many commonly used sanitizing agents (*13*). After the June 23 case, an extensive terminal cleaning of the unit was done, along with staff training on appropriate housekeeping practices. All the instruments and containers were autoclaved, and surfaces were cleaned in 2 steps: first, with alkaline detergents, then with a disinfectant (D-125, Microgen, http://microgenindia.co). Beds were manually cleaned with detergent and water followed by heat treatment. Rooms were fogged with a sporicidal disinfectant containing hydrogen peroxide and silver nitrate (ECOSHIELD; Johnson & Johnson, https://www.jnj.com). All the healthcare kits were removed, and staff were advised to discontinue their use. Since June 23, 2018, no additional cases have been reported.

Identification of *B. cereus* Group

Based on the colony characteristics, β-hemolysis in blood agar, motility, production of lecithinase in egg yolk media, inability to ferment mannitol, and penicillin resistance, the isolates were designated as *B. cereus*. We subjected 4 representative strains to sequencing using the universal primer PF (5′-AGAGTTTGATCATGGCTCAG-3′) and PR (5′-GGACTACCAGGGTATCTAAT-3′) for the 16s rRNA gene (*14*).

PCR Detection of Toxin-Encoding Genes

We performed PCR detection for toxins (*cytK*, *nheA*, *nheB*, *nheC*, *hblA*, *hblC*, *hblD*, *entFM*, *pi-plc*, and *sph*) and plasmid-encoding *B. anthracis* virulence factors (*cap*, *lef pag*, and *cya*) encoding genes. The primer sequences used for PCR are listed in the Appendix Table (https://wwwnc.cdc.gov/EID/article/25/7/18-1493-App1.pdf).

Multilocus Sequence Typing Data Analysis

We selected 19 strains for molecular characterization. We used the *B. cereus* multilocus sequence typing (MLST) website (https://pubmlst.org/bcereus) that contains the partial sequences of the 7 housekeeping genes (*glp*, *gmk*, *ilv*, *pta*, *pur*, *pyc*, and *tpi*) (*15*). We conducted Sanger sequencing using Genetic Analyzer 3500 (Applied Biosystems, https://www.thermofisher.com). We compared the allele sequences with those available in the MLST database for assignment of allele numbers and sequence type (ST). We submitted all new alleles, MLST profiles (STs), and isolates to the MLST database. We obtained the population snapshot of the 1,795 STs available in the MLST database using goeBURST implemented in Phyloviz 2.0 using the default single-locus variant level (sharing at least 6/7 alleles) (*16*). The goeBURST Full MST (minimum spanning tree; http://www.phyloviz.net/goeburst) was done to identify BURST groups (BGs) among the STs identified in this study.

Phylogenetic Analysis

The concatenated MLST sequences available at the MLST database were used for constructing maximum-likelihood trees. We used RAxML version 8 (*17*) implemented in RDP4 version 4.66 (*18*) with the GTRCAT model and a bootstrap resampling of 1,000 replicates.

Diversity and Recombination Analysis

We calculated the length of each MLST locus, number of alleles, average nucleotide diversity (π), and number of polymorphic sites using DnaSP version 6.11.01 (*19*) based on the allelic sequences of the STs. We calculated the ratio of nonsynonymous to synonymous substitutions (dN/dS) to determine the selective pressure at each locus using the Nei and Gojobori method in START2 (*20*). The parameters dN and dS indicated average nonsynonymous and synonymous substitutions per site, respectively.

We conducted phylogenetic network analysis using Splits Tree version 4 (*21*) to identify lineages and recombination events within and across the lineages. We constructed the Splits Tree networks based on the concatenated sequences of STs using the neighbor-net algorithm with bootstrap resampling of 1,000 replicates. The resulting networks were analyzed using pairwise homoplasy index (PHI) test implemented in Splits Tree. A p value <0.05 indicated significant evidence of recombination.

We evaluated the linkage disequilibrium for the allelic data using LIAN version 3.7 (http://guanine.evolbio.mpg.de/cgi-bin/lian/lian.cgi.pl/query) (*22*). The standardized index of association (I_A^S) used for estimating linkage disequilibrium between alleles of the 7 MLST loci was calculated using the Monte Carlo method with 10,000 burn-in iterations. The I_A^S values >0 and p<0.05 indicated significant linkage disequilibrium.

Results

Population Structure and BURST Group

MLST identified 14 STs among the selected 19 strains, including 5 predefined STs (ST75, ST127, ST266, ST380,

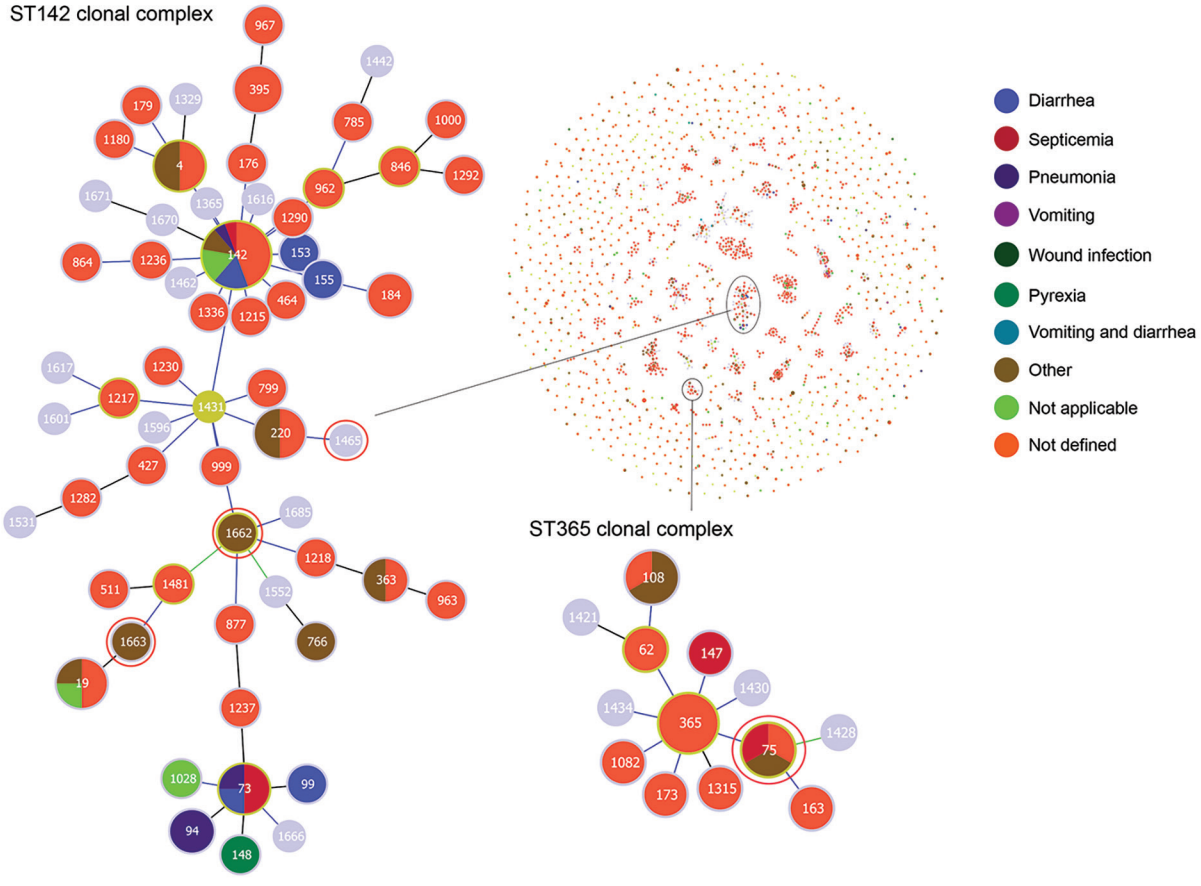


Figure 2. Population snapshot obtained using goeBURST (http://www.phyloviz.net/goeburst) of the 1,795 STs available to date in the *Bacillus cereus* multilocus sequence typing database overlaid by isolate data of human diseases. Each circle represents an ST. Size of the circle is logarithmically proportionate to the number of isolates represented by a given ST. Two ST clonal complexes are enlarged; STs highlighted in red circles were identified during investigation of an outbreak at Assam Medical College & Hospital, Dibrugarh, India, 2018. ST, sequence type.

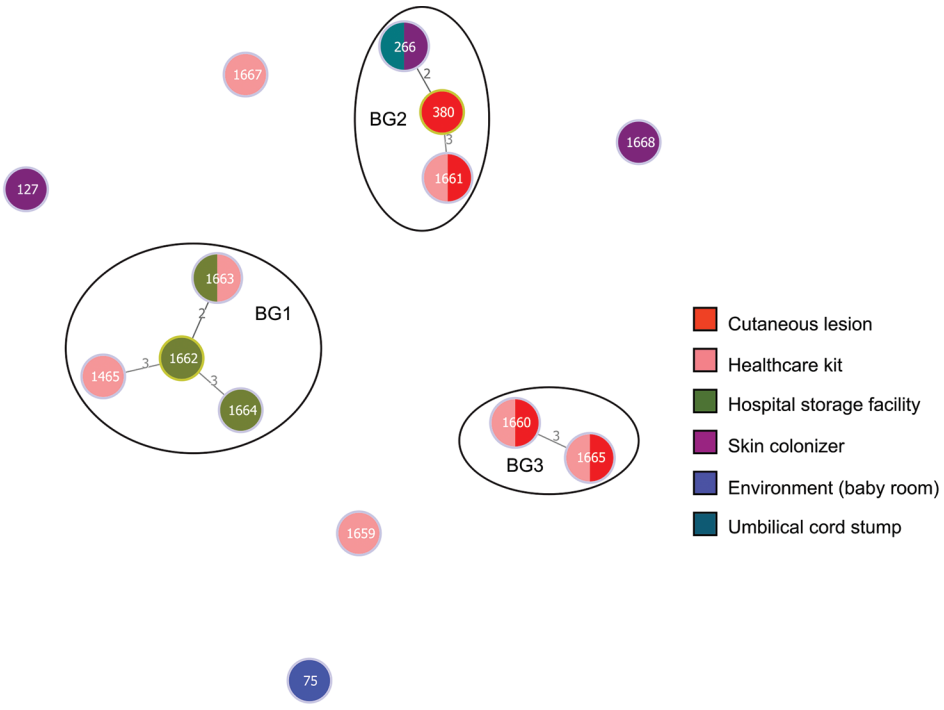


Figure 3. goeBURST full MST analysis (http://www.phyloviz.net/goeburst) at triple-locus variant level illustrating the evolutionary relationship within the 3 BGs identified during investigation of an outbreak at Assam Medical College & Hospital, Dibrugarh, India, 2018. Each circle represents a sequence type (ST), and its size is logarithmically proportionate to the number of isolates represented by that ST. The numbers over the line connecting 2 STs indicate the number of allele difference between them. The colors of the circles indicate the source of isolation. BG, BURST group.

and ST1465) and 9 new STs (ST1659, ST1660, ST1661, ST1662, ST1663, ST1664, ST1665, ST1667 and ST1668). Most (64.3%) identified STs were new.

The *B. cereus* MLST database clusters the available 1,795 STs into 10 major clonal complexes (CCs); a CC is a group of STs defined by goeBURST using the stringent group definition of single-locus variant level. The population snapshot obtained using goeBURST identified 3 STs (ST1465, ST1662, and ST1663) from the ST142 CC and 1 ST (ST75) from ST365 CC among the 14 STs identified in this study (Figure 2). The remaining STs were not a part of these major CCs. With a less stringent group definition of double-locus variant (DLV, sharing at least 5/7 alleles) or triple-locus variant (TLV, sharing at least 4/7 alleles) level, all of the STs in a goeBURST group cannot be considered as members of a single CC and hence referred to as BGs in our study. The goeBURST full MST analysis using the 14 STs from this study identified 3 BGs at TLV level (sharing at least 4/7 alleles) (Figure 3). The BG1 with ST1465, ST1662, ST1664, and ST1665 comprised strains from storage facilities and healthcare kits with ST1662 assigned as the putative primary founder. The BG2 included STs 266, 380, and 1661 with ST380 assigned as the putative primary founder. The BG2 consisted of strains from skin colonizers, umbilical cord stumps, healthcare kits, and cutaneous lesions. The doubleton BG3 consisted of ST1660 and ST1665 with isolates recovered from healthcare kits and cutaneous lesions. We identified 5 singletons (not linked to any other ST): ST75, ST127, ST1659, ST1662, and ST1668.

Phylogenetic Origin of the Strains

The taxonomic identification of the 14 STs found in this study was done by constructing a phylogenetic tree with 18 *B. cereus* group species type strains (Figure 4). To illustrate the virulence potential of the strains, we constructed a phylogenetic tree (Figure 5) using the 14 STs from this study along with 38 STs representing 55 virulent isolates of *B. cereus* based on the selection made by Hoffmaster et al. (*23*). The STs clustered into 3 phylogenetic clades and were named to be consistent with previously defined phylogenetic clades by Priest et al. (*24*). Among the 14 STs we identified, 10 STs representing 14 strains were assigned to clade I, which comprised primarily pathogenic bacilli and mostly represented strains from healthcare kit and cutaneous lesion (*24*). Out of the 10 STs, 3 STs clustered into the cereus III/clade I lineage, 3 STs into a new cluster/clade I represented by ST144, 1 ST in cereus I/clade I lineage, and 2 STs in cereus II (emetic)/clade I lineage. None of the clade I–designated 10 STs grouped into the cereus IV lineage. Clade II consisted of 4 STs representing 5 strains grouped in tolworthi/clade II lineage and were representing strains mostly from the environment. None of the 14 STs identified in this study clustered in clade III (others).

Distribution of Enterotoxins, Sphingomyelinase, and Phosphatidylinositol Phospholipase C Encoding Genes

The *cytK*, *sph*, and *pi-plc* genes encoding cytotoxin K, sphingomyelinase (SMase), and phosphatidylinositol phospholipase C (Pi-Plc), respectively, were commonly detected

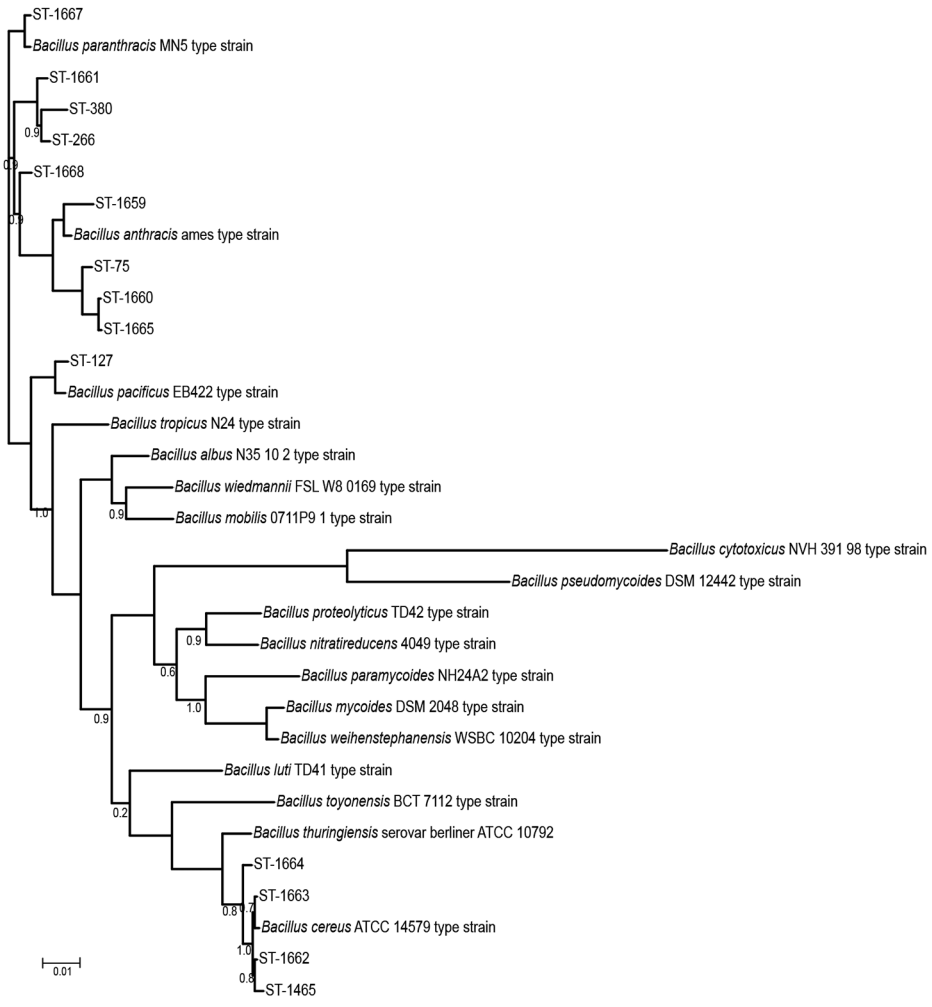


Figure 4. Phylogenetic relatedness of the 14 STs identified during investigation of an outbreak at Assam Medical College & Hospital, Dibrugarh, India, 2018, with 18 *Bacillus cereus* group species type strains. Scale bar indicates nucleotide substitutions per site.

in 73.7% (n = 14) of the 19 selected strains, followed by *nheA* and *nheC* in 68.4% (n = 13), *nheB* in 52.6% (n = 10), and *entFM* in 42.1% (n = 8) (Table 1). Of the 14 strains previously designated to clade I, the *nheABC* gene complex (*nheA*, *nheB*, and *nheC*) encoding the nonhemolytic enterotoxin (Nhe) was detected in 8 strains and *entFM* gene encoding enterotoxin FM was also detected in 8 strains. None of the clade II–assigned strains were detected positive for the *nheABC* gene complex and the *entFM* gene. The *hbl* gene complex (*hblA*, *hblC*, and *hblD*) encoding hemolysin BL (HBL) was found in only 1 strain designated to clade II, whereas none of the clade I–assigned strains harbored this complex (Table 2). Both *hblA* and *hblC* were detected in 1 each of the 19 strains. None of the strains were positive for genes encoding *B. anthracis* plasmid-mediated virulence factors (Table 1).

Sequence and Allelic Diversity

Sequence alignment of each of the 7 MLST loci showed no insertion or deletion with sizes ranging from 348 bp (*pur*) to 504 bp (*gmk*). The number of alleles at each locus ranged from 4 (*gmk*) to 11 (*ilv* and *tpi*). The dN/dS values indicate selective pressure on protein-coding genes; dN/dS >1 indicates positive and dN/dS <1 negative selective pressure. The dN/dS ratio for each locus varied from 0.0076 (*ilv*) to 0.0547 (*pur*), indicating strong negative/purifying selective pressure on these genes (Table 3).

Recombination Analysis

The Splits Tree network for the 14 STs representing the 19 selected strains identified 2 lineages among them (Figure 6). Lineage 1 comprised STs 75, 127, 266, 380, 1660, 1659, 1661, 1665, 1667, and 1668 previously designated to clade I. The STs 1465, 1662, 1663, and 1664 previously designated to clade II were in lineage 2. We observed extensive reticulations across the lineages and within lineage 1. The PHI test also provided significant evidence of recombination for the whole population (14 STs) and lineage 1 ($p<0.05$). However, the I_A^S values, for estimation of linkage disequilibrium, differed significantly from 0 for the entire population, as well as for the lineages, indicating the

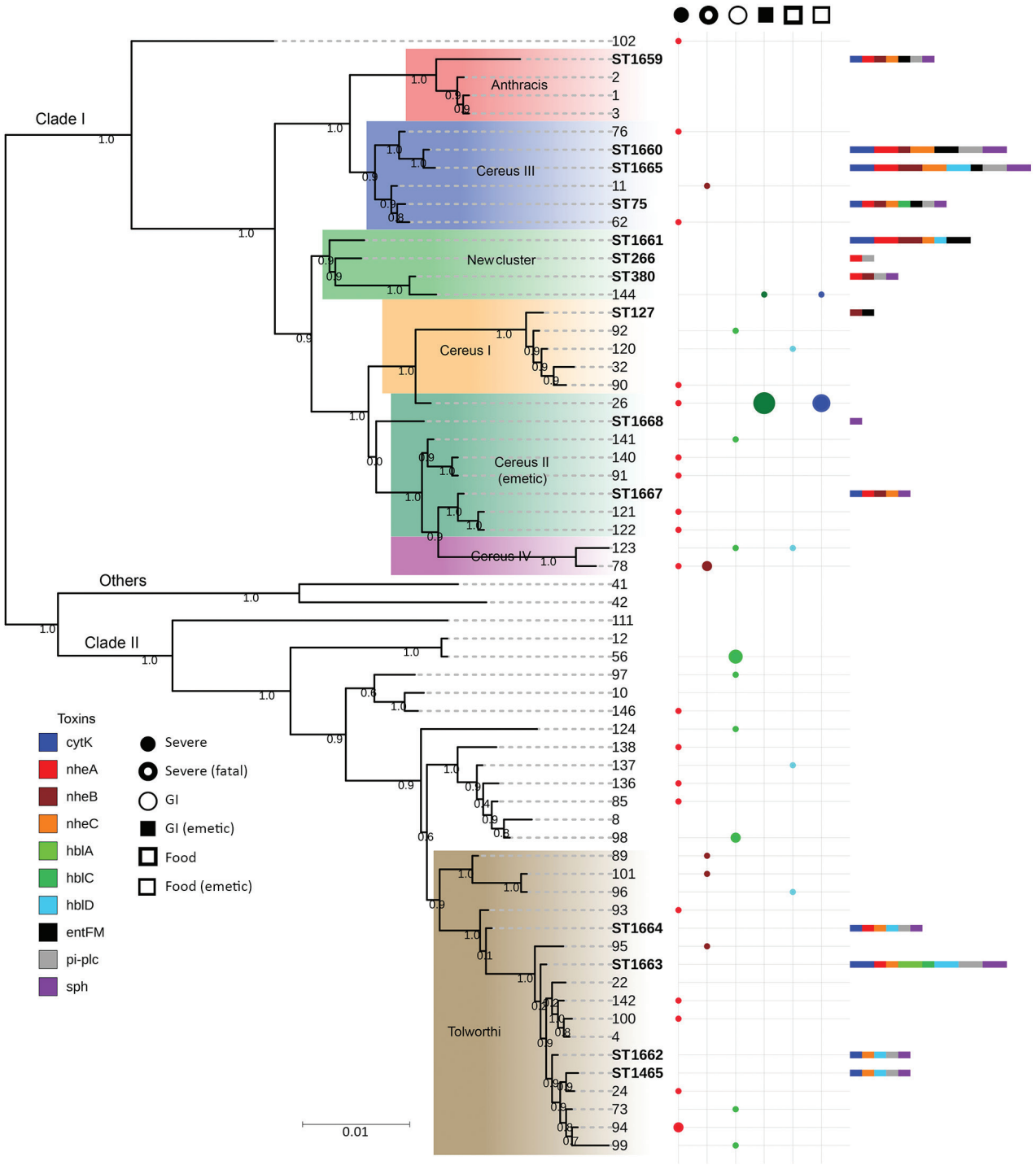


Figure 5. Maximum-likelihood tree constructed on the basis of concatenated sequences of the 51 *Bacillus cereus* STs illustrating the phylogenetic relatedness of the 14 STs identified during investigation of an outbreak at Assam Medical College & Hospital, Dibrugarh, India, 2018, and the 37 STs representing clinical isolates. The bootstrap support values for the nodes are indicated in decimals. The STs represented in bold letters are identified in this study. The color gradient boxes represent the various lineages found within the clades. The colored circle plot represents the number of isolates from various human diseases assigned to a particular ST, and each color represents a different human disease. The size of each circle is proportionate to the number of isolates. The multicolored bar indicates the number of toxin-encoding genes found in isolates represented by an ST and each color represents different toxin-encoding genes. The length of each colored bar is proportionate to the number of isolates positive for that toxin-encoding gene. GI, gastrointestinal; ST, sequence type. Scale bar indicates nucleotide substitutions per site.

Table 1. Toxin-encoding gene profile of 19 selected *Bacillus cereus* strains, Assam Medical College & Hospital, Dibrugarh, Assam, India, 2018*

Strain	Source	ST	Clade	*cytK*	*nheABC*			*hblCDA*			*entFM*	*pi-plc*	*sph*
					nheA	*nheB*	*nheC*	*hblA*	*hblC*	*hblD*			
AMCER1	Healthcare kit	1659	I	+	+	+	+	–	–	–	+	+	+
AMCER2	Healthcare kit	1660	I	+	+	–	+	–	–	–	+	+	+
AMCER3	Cutaneous lesion	1660	I	+	+	+	+	–	–	–	+	+	+
AMCER4	Healthcare kit	1661	I	+	+	+	+	–	–	+	+	–	–
AMCER5	Cutaneous lesion	1661	I	+	+	+	–	–	–	–	+	–	–
AMCER6	Healthcare kit	1465	II	+	–	–	+	–	–	+	–	+	+
AMCER7	Healthcare kit	1667	I	+	+	+	+	–	–	–	–	–	+
AMCER8	Hospital storage facility	1662	II	+	–	–	+	–	–	+	–	+	+
AMCER9	Hospital storage facility	1663	II	+	+	–	+	+	–	+	–	+	+
AMCER11	Healthcare kit	1663	II	+	–	–	–	+	+	+	–	+	+
AMCER10	Hospital storage facility	1664	II	+	+	–	+	–	–	+	–	+	+
AMCER12	Healthcare kit	1665	I	+	+	+	+	–	–	+	+	+	+
AMCER15	Cutaneous lesion	1665	I	+	+	+	+	–	–	+	–	+	+
AMCER13	Umbilical cord stump	266	I	–	–	–	–	–	–	–	–	+	–
AMCER14	Environment	75	I	+	+	+	+	–	+	–	+	+	+
AMCER18	Skin colonizer	266	I	–	+	–	–	–	–	–	–	+	–
AMCER19	Cutaneous lesion	380	I	–	+	+	+	–	–	–	–	+	+
AMCER20	Skin colonizer	1668	I	–	–	–	–	–	–	–	–	–	+
AMCER21	Skin colonizer	127	I	–	–	+	–	–	–	–	+	–	–

*ST, sequence type; +, positive; –, negative.

existence of linkage disequilibrium between the loci or a clonal population structure (Table 4).

Discussion

MLST data analysis identified a genetically diverse population of 14 STs representing the 19 selected *B. cereus* strains because most of the STs were identified as new. Population snapshot using goeBURST illustrated the rare occurrences of clinical cases among the *B. cereus* group. Among the 14 STs, 3 were from the ST142 CC and 1 from the ST365 CC, indicating evolutionary descent from worldwide clones. The goeBURST Full MST analysis of the 14 STs identified only 3 BGs even at the TLV level; the rest were identified as singletons, which again illustrates the high genetic diversity. Most of the strains isolated from healthcare kits and cutaneous lesions were represented by new STs, suggesting that greater diversity was possibly because of adaptation to the new niche.

The phylogenetic origin of the 19 strains was determined to investigate whether any of the 14 STs representing these strains clustered into the previously described clade I, composed primarily of pathogenic bacilli (*24*). The clade I–designated 10 STs were distributed into various lineages of clade I and were closely related to the Anthracis lineage, as described by Hoffmaster et al. (*23*). Among them, ST1659, a new ST representing a strain isolated from a healthcare kit, had the closest relationship to the Anthracis lineage and shared the same *gmk*, *pta*, *pur*, and *pyc* alleles with the Ames anthracis strain (ST1). The ST75 isolate from ST365 CC, which has been reported earlier for representing a severe septicemic *B. cereus* strain, shared the same *gmk* and *pta* alleles with the *B. anthracis* strains (*25*).

Several studies have demonstrated that *B. cereus* isolates closely related to *B. anthracis* are of clinical rather than environmental origin (*26,27*). All the clade I–designated strains were negative for genes encoding *B. anthracis* virulence factors. Alternatively, these factors might not be necessary for severe nongastrointestinal infections because isolates from severe cases have been reported to be negative for plasmids (*8*). Most of the clade I–assigned STs represented strains recovered from healthcare kits, suggesting these strains might be responsible for cutaneous lesions. Three clade II–assigned STs were from ST142 CC, comprising mostly foodborne isolates with potential to cause foodborne illness (*28*). Hence, the potential role of the strains represented by these 3 STs in the development of cutaneous lesions is arguable.

Phylogenetic network analysis using Splits Tree identified 2 lineages among the 14 STs identified in this study. All the clade I–designated STs clustered in lineage 1, whereas all the clade II–designated STs clustered in lineage 2. Extensive reticulations in Splits Tree networks and PHI

Table 2. Interclade variability of *Bacillus cereus* toxin–encoding genes and gene complexes, Assam Medical College & Hospital, Dibrugarh, Assam, India, 2018

PCR characterization	Phylogenetic group, %*	
	Clade I	Clade II
pi-plc	9 (64.3)	5 (100)
sph	9 (64.3)	5 (100)
cytK	9 (64.3)	5 (100)
entFM	8 (57.1)	0
nheABC	8 (57.1)	0
hblCDA	0	1 (20)
Total	14	5

*The percentage of each cell corresponds to the number of strains within a given clade positive for the encoding gene. Strains were classified as positive for a gene complex if all the genes in a given complex were detected by PCR.

Table 3. Sequence and allelic diversity of the 7 multilocus sequence type loci of *Bacillus cereus*, Assam Medical College & Hospital, Dibrugarh, Assam, India, 2018

Locus	Size, bp	Guanine + cytosine content, %	Allele	Polymorphic site	π	dN/dS
Glp	372	38.3	10	18	0.01607	0.0312
Gmk	504	38.2	4	24	0.02546	0.0214
Ilv	393	45.1	11	58	0.06283	0.0076
Pta	414	40.5	8	21	0.02088	0.0077
Pur	348	38.5	9	42	0.05388	0.0098
Pyc	363	40.6	8	48	0.05460	0.0301
Tpi	435	44.1	11	18	0.01492	0.0547

test provided significant evidence of recombination across the lineages and within lineage 2. Even though the I_A^S and dN/dS values predicted an overall clonal population structure, the high genetic diversity and recombination might have enabled the population to enhance fitness and survive.

To evaluate the interclade variability of toxin-encoding genes, we performed PCR detection of these genes. The *nheABC* gene complex and *entFM* gene were detected only among the clade I–designated strains, whereas none of the clade II–identified strains were positive for the *nheABC* gene complex and the *entFM* gene. The Nhe enterotoxin is considered to be the major virulence factor in *B. cereus* diarrheal disease (*29*). The synergistic interaction of Nhe and SMase in in vitro cytotoxicity has been demonstrated (*30*). All the members of the *nheABC* gene complex are required to form functional transmembrane pores for the entry of SMase into the epithelial cell membrane, otherwise inaccessible, and could result in cell membrane destabilization, as well as cell apoptosis through the ceramide intracellular signaling pathway (*31,32*). This case might be valid in our study because the clade I–designated strains harbored the *nheABC* gene complex, as well as the *sph* gene. Several findings have suggested that enterotoxin FM might be a potential cell wall peptidase involved in mutant bacterial shape, impairment in motility, and adhesion

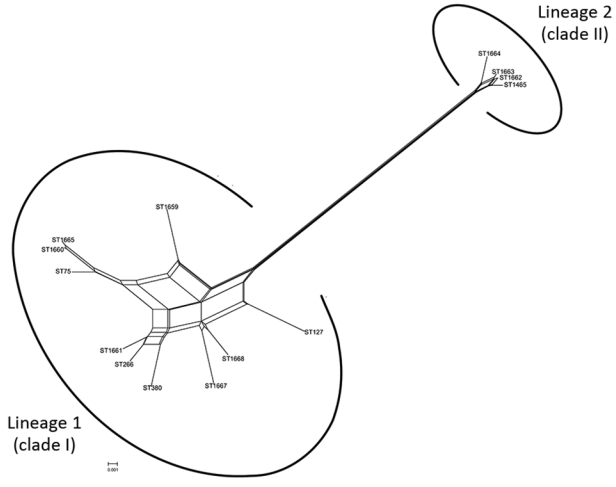


Figure 6. Phylogenetic network analysis using Splits Tree (*21*) identified 2 lineages among the whole population of 14 STs, Assam Medical College & Hospital, Dibrugarh, Assam, India, 2018. ST, sequence type.

to eukaryotic cells and thus might be responsible for the virulence of the clade I–assigned strains because most of them harbored this gene (*33*). The *hblA* gene encoding the binding subunit C component and the *hblC* gene encoding the L_2 lytic component were sparsely detected among the 19 strains. The tripartite HBL enterotoxin requires all its components for maximum enterotoxic activity (*34*). Moreover, the *cytK* and *hbl* enterotoxin genes are often absent in *B. cereus* strains isolated from disease outbreaks, which argues against its potential role to elicit disease (*35,36*).

In this investigation, only 12 newborns were infected, even though the kits also were used for other newborns. Thus, development of nongastrointestinal infections in newborns is complex and might depend on factors such as the number of spores exposed, the presence of a virulent and avirulent cluster of microorganisms, toxin expression and interaction, and host conditions. Moreover, seasonal variation of increased count and germination of *B. cereus* spores in spring and summer have been reported (*37–39*). In this outbreak, lesions occurred during April in 6 newborns, May in 4, and March and June in 1 each. However, a thorough investigation is needed to understand the complexity of these infections. Our findings, along with previous reports, reinforce the idea that the members of the *B. cereus* group are underestimated emerging pathogens that can be involved in fatal nosocomial infections.

The cutaneous infections attributed to the *B. cereus* group in most of the cases in this study occurred in the exposed areas of the skin because they are often in contact with the environment and are prone to microscopic skin abrasions (*39*). The spores from the healthcare kits might have invaded the skin of newborns through these microscopic skin abrasions formed during baby cleaning (*39*). Moreover, vernix caseosa, a waxy substance covering the skin of newborns, often requires cleaning and might also be the cause of microscopic skin abrasions. Once in contact with skin, spores germinate and enterotoxin production occurs (*39*). In addition, toxicoinfection can occur because the kits contain all the items required to conduct labor, including contaminated gloves. Among the infants in this report, duration of labor ranged from 6 to 10 hours, so germination and toxin production might have occurred

Table 4. Estimation of linkage disequilibrium of *Bacillus cereus*, Assam Medical College & Hospital, Dibrugarh, Assam, India, 2018

Group	Linkage disequilibrium	
	I_A^S	p value
Lineage 1	0.3800	<0.05
Lineage 2	0.3471	<0.05
Whole population	0.4573	<0.05

in the birth canal and accounted for the initial lesions that later extended from contact with the cleaning linens inside the kit and led to additional spore germination and toxin production.

Clinically, the lesions started as bullous or ruptured bullous lesions with extensive and rapidly spreading cellulitis. Two lesions eventually developed into gas gangrene–like infections as reported previously (*4,40*). However, in all 12 cases, blood culture was negative for *B. cereus*. Henrickson et al. reported the primary cutaneous infections caused by *B. cereus* in the absence of positive blood cultures (*10*,*39*). Extensive soft tissue involvement with gas gangrene infections in the first few newborns might have resulted from the initial use of β-lactam antimicrobial drugs because the existence of β-lactamase in sporulated *Bacillus* species has been predicted (*41*). All newborns recovered after treatment with ciprofloxacin and amikacin.

In conclusion, *B. cereus* primary cutaneous infection in newborns without bacteremia can occur from contaminated environments in hospitals. Bullous lesions or cellulitis during or just after delivery should be included in the differential diagnosis, and caution should be taken in initiating β-lactam antimicrobial drug treatment.

Acknowledgments

We thank Biswajit Borkotoky for performing the initial Sanger sequencing of the 16s rRNA gene. We thank all the staff of Obstetrics and Gynecology, AMCH, for their cooperation and hospital administration for supporting this investigation.

About the Author

At the time of this study, Dr. Saikia was a professor in the Department of Microbiology, Gauhati Medical College & Hospital, Guwahati, India. She is currently a professor in the Department of Microbiology, Gauhati Medical College & Hospital, Guwahati, India. Her primary research interests are clinical microbiology, epidemiology of infectious diseases, healthcare-associated infections, and antimicrobial resistance.

References

1. Mazas M, López M, Martínez S, Bernardo A, Martin R. Heat resistance of *Bacillus cereus* spores: effects of milk constituents and stabilizing additives. J Food Prot. 1999;62:410–3. http://dx.doi.org/10.4315/0362-028X-62.4.410
2. Hong HA, Duc H, Cutting SM. The use of bacterial spore formers as probiotics. FEMS Microbiol Rev. 2005;29:813–35. http://dx.doi.org/10.1016/j.femsre.2004.12.001
3. Miller RA, Jian J, Beno SM, Wiedmann M, Kovac J. Intraclade variability in toxin production and cytotoxicity of *Bacillus cereus* group type strains and dairy-associated isolates. Appl Environ Microbiol. 2018;84:e02479–17. http://dx.doi.org/10.1128/AEM.02479-17
4. Darbar A, Harris IA, Gosbell IB. Necrotizing infection due to *Bacillus cereus* mimicking gas gangrene following penetrating trauma. J Orthop Trauma. 2005;19:353–5.
5. Henrickson KJ. A second species of *Bacillus* causing primary cutaneous disease. Int J Dermatol. 1990;29:19–20. http://dx.doi.org/10.1111/j.1365-4362.1990.tb03748.x
6. Brett MM, Hood J, Brazier JS, Duerden BI, Hahné SJM. Soft tissue infections caused by spore-forming bacteria in injecting drug users in the United Kingdom. Epidemiol Infect. 2005;133:575–82. http://dx.doi.org/10.1017/S0950268805003845
7. Akesson A, Hedström SA, Ripa T. *Bacillus cereus*: a significant pathogen in postoperative and post-traumatic wounds on orthopaedic wards. Scand J Infect Dis. 1991;23:71–7. http://dx.doi.org/10.3109/00365549109023377
8. Hoffmaster AR, Ravel J, Rasko DA, Chapman GD, Chute MD, Marston CK, et al. Identification of anthrax toxin genes in a *Bacillus cereus* associated with an illness resembling inhalation anthrax. Proc Natl Acad Sci U S A. 2004;101:8449–54. http://dx.doi.org/10.1073/pnas.0402414101
9. Miller JM, Hair JG, Hebert M, Hebert L, Roberts FJ Jr, Weyant RS. Fulminating bacteremia and pneumonia due to *Bacillus cereus.* J Clin Microbiol. 1997;35:504–7.
10. Henrickson KJ, Shenep JL, Flynn PM, Pui CH. Primary cutaneous *Bacillus cereus* infection in neutropenic children. Lancet. 1989;1:601–3. http://dx.doi.org/10.1016/S0140-6736(89)91621-8
11. Gray J, George RH, Durbin GM, Ewer AK, Hocking MD, Morgan MEI. An outbreak of *Bacillus cereus* respiratory tract infections on a neonatal unit due to contaminated ventilator circuits. J Hosp Infect. 1999;41:19–22. http://dx.doi.org/10.1016/S0195-6701(99)90032-4
12. Patrick CC, Langston C, Baker CJ. *Bacillus* species infections in neonates. Rev Infect Dis. 1989;11:612–5. http://dx.doi.org/10.1093/clinids/11.4.612
13. Sagripanti JL, Bonifacino A. Comparative sporicidal effects of liquid chemical agents. Appl Environ Microbiol. 1996;62:545–51.
14. Ehresmann C, Stiegler P, Fellner P, Ebel JP. The determination of the primary structure of the 16S ribosomal RNA of *Escherichia coli*. 2. Nucleotide sequences of products from partial enzymatic hydrolysis. Biochimie. 1972;54:901–67. http://dx.doi.org/10.1016/S0300-9084(72)80007-5
15. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11:595. http://dx.doi.org/10.1186/1471-2105-11-595
16. Francisco AP, Vaz C, Monteiro PT, Melo-Cristino J, Ramirez M, Carriço JA. PHYLOViZ: phylogenetic inference and data visualization for sequence based typing methods. BMC Bioinformatics. 2012;13:87. http://dx.doi.org/10.1186/1471-2105-13-87
17. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics. 2014;30:1312–3. http://dx.doi.org/10.1093/bioinformatics/btu033
18. Martin DP, Murrell B, Golden M, Khoosal A, Muhire B. RDP4: detection and analysis of recombination patterns in virus genomes. Virus Evol. 2015;1:vev003.
19. Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R. DnaSP, DNA polymorphism analyses by the coalescent and other methods. Bioinformatics. 2003;19:2496–7. http://dx.doi.org/10.1093/bioinformatics/btg359

20. Jolley KA, Feil EJ, Chan MS, Maiden MC. Sequence type analysis and recombinational tests (START). Bioinformatics. 2001; 17:1230–1. http://dx.doi.org/10.1093/bioinformatics/ 17.12.1230
21. Huson DH, Bryant D. Application of phylogenetic networks in evolutionary studies. Mol Biol Evol. 2006;23:254–67. http://dx.doi.org/10.1093/molbev/msj030
22. Haubold B, Hudson RR. LIAN 3.0: detecting linkage disequilibrium in multilocus data. Linkage analysis. Bioinformatics. 2000;16:847–8. http://dx.doi.org/10.1093/bioinformatics/16.9.847
23. Hoffmaster AR, Novak RT, Marston CK, Gee JE, Helsel L, Pruckler JM, et al. Genetic diversity of clinical isolates of *Bacillus cereus* using multilocus sequence typing. BMC Microbiol. 2008;8:191. http://dx.doi.org/10.1186/1471-2180-8-191
24. Priest FG, Barker M, Baillie LWJ, Holmes EC, Maiden MCJ. Population structure and evolution of the *Bacillus cereus* group. J Bacteriol. 2004;186:7959–70. http://dx.doi.org/10.1128/JB.186.23.7959-7970.2004
25. Barker M, Thakker B, Priest FG. Multilocus sequence typing reveals that *Bacillus cereus* strains isolated from clinical infections have distinct phylogenetic origins. FEMS Microbiol Lett. 2005;245:179–84. http://dx.doi.org/10.1016/j.femsle.2005.03.003
26. Hill KK, Ticknor LO, Okinaka RT, Asay M, Blair H, Bliss KA, et al. Fluorescent amplified fragment length polymorphism analysis of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis* isolates. Appl Environ Microbiol. 2004;70:1068–80. http://dx.doi.org/10.1128/AEM.70.2.1068-1080.2004
27. Helgason E, Okstad OA, Caugant DA, Johansen HA, Fouet A, Mock M, et al. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl Environ Microbiol. 2000;66:2627–30. http://dx.doi.org/10.1128/AEM.66.6.2627-2630.2000
28. Cardazzo B, Negrisolo E, Carraro L, Alberghini L, Patarnello T, Giaccone V. Multiple-locus sequence typing and analysis of toxin genes in *Bacillus cereus* food-borne isolates. Appl Environ Microbiol. 2008;74:850–60. http://dx.doi.org/10.1128/AEM.01495-07
29. Moravek M, Dietrich R, Buerk C, Broussolle V, Guinebretière MH, Granum PE, et al. Determination of the toxic potential of Bacillus cereus isolates by quantitative enterotoxin analyses. FEMS Microbiol Lett. 2006;257:293–8. http://dx.doi.org/10.1111/j.1574-6968.2006.00185.x
30. Doll VM, Ehling-Schulz M, Vogelmann R. Concerted action of sphingomyelinase and non-hemolytic enterotoxin in pathogenic *Bacillus cereus*. PLoS One. 2013;8:e61404. http://dx.doi.org/10.1371/journal.pone.0061404
31. Kolesnick RN, Goñi FM, Alonso A. Compartmentalization of ceramide signaling: physical foundations and biological effects. J Cell Physiol. 2000;184:285–300. http://dx.doi.org/10.1002/1097-4652(200009)184:3<285::AID-JCP2>3.0.CO;2-3
32. Haug TM, Sand SL, Sand O, Phung D, Granum PE, Hardy SP. Formation of very large conductance channels by *Bacillus cereus* Nhe in Vero and GH(4) cells identifies NheA + B as the inherent pore-forming structure. J Membr Biol. 2010;237:1–11. http://dx.doi.org/10.1007/s00232-010-9298-6
33. Tran S-L, Guillemet E, Gohar M, Lereclus D, Ramarao N. CwpFM (EntFM) is a *Bacillus cereus* potential cell wall peptidase implicated in adhesion, biofilm formation, and virulence. J Bacteriol. 2010;192:2638–42. http://dx.doi.org/10.1128/JB.01315-09
34. Beecher DJ, Macmillan JD. Characterization of the components of hemolysin BL from *Bacillus cereus*. Infect Immun. 1991; 59:1778–84.
35. Ehling-Schulz M, Guinebretiere MH, Monthán A, Berge O, Fricker M, Svensson B. Toxin gene profiling of enterotoxic and emetic *Bacillus cereus*. FEMS Microbiol Lett. 2006;260:232–40. http://dx.doi.org/10.1111/j.1574-6968.2006.00320.x
36. Ehling-Schulz M, Svensson B, Guinebretiere MH, Lindbäck T, Andersson M, Schulz A, et al. Emetic toxin formation of *Bacillus cereus* is restricted to a single evolutionary lineage of closely related strains. Microbiology. 2005;151:183–97. http://dx.doi.org/10.1099/mic.0.27607-0
37. Kmiha S, Aouadhi C, Klibi A, Jouini A, Béjaoui A, Mejri S, et al. Seasonal and regional occurrence of heat-resistant spore-forming bacteria in the course of ultra-high temperature milk production in Tunisia. J Dairy Sci. 2017;100:6090–9. http://dx.doi.org/10.3168/jds.2016-11616
38. Cheng VCC, Chen JHK, Leung SSM, So SYC, Wong S-C, Wong SCY, et al. Seasonal outbreak of *Bacillus* bacteremia associated with contaminated linen in Hong Kong. Clin Infect Dis. 2017;64(suppl_2):S91–7.
39. Bottone EJ. *Bacillus cereus*, a volatile human pathogen. Clin Microbiol Rev. 2010;23:382–98. http://dx.doi.org/10.1128/CMR.00073-09
40. Gröschel D, Burgress MA, Bodey GP Sr. Gas gangrene-like infection with *Bacillus cereus* in a lymphoma patient. Cancer. 1976;37:988–91. http://dx.doi.org/10.1002/1097-0142(197602)37:2<988::AID-CNCR2820370256>3.0.CO;2-Y
41. Saz AK. An introspective view of penicillinase. J Cell Physiol. 1970;76:397–403. http://dx.doi.org/10.1002/jcp.1040760318

Address for correspondence: Lahari Saikia, Gauhati Medical College & Hospital, Department of Microbiology, Guwahati, Assam, India; email: mdrlamch@yahoo.com

Prescribing Patterns for Treatment of *Mycobacterium avium* Complex and *M. xenopi* Pulmonary Disease in Ontario, Canada, 2001–2013

Sarah K. Brode, Hannah Chung, Michael A. Campitelli, Jeffrey C. Kwong, Alex Marchand-Austin, Kevin L. Winthrop, Frances B. Jamieson, Theodore K. Marras

Medscape EDUCATION ACTIVITY

In support of improving patient care, this activity has been planned and implemented by Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is jointly accredited by the Accreditation Council for Continuing Medical Education (ACCME), the Accreditation Council for Pharmacy Education (ACPE), and the American Nurses Credentialing Center (ANCC), to provide continuing education for the healthcare team.

Medscape, LLC designates this Journal-based CME activity for a maximum of 1.00 ***AMA PRA Category 1 Credit(s)™***. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

Successful completion of this CME activity, which includes participation in the evaluation component, enables the participant to earn up to 1.0 MOC points in the American Board of Internal Medicine's (ABIM) Maintenance of Certification (MOC) program. Participants will earn MOC points equivalent to the amount of CME credits claimed for the activity. It is the CME activity provider's responsibility to submit participant completion information to ACCME for the purpose of granting ABIM MOC credit.

All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test with a 75% minimum passing score and complete the evaluation at http://www.medscape.org/journal/eid; and (4) view/print certificate. For CME questions, see page 1442.

Release date: June 14, 2019; Expiration date: June 14, 2020

Learning Objectives

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

- Identify the percentage of older adults with MAC-PD and Mx-PD in Ontario, Canada, who received antimicrobial therapy and factors associated with receipt of treatment, according to a retrospective cohort study
- Determine the percentage of older adults with MAC-PD and Mx-PD in Ontario, Canada, who received guideline-recommended treatment and compare this percentage with that in other countries, according to a retrospective cohort study
- Ascertain the percentage of older adults with MAC-PD and Mx-PD in Ontario, Canada, who received treatment that could lead to macrolide resistance and factors associated with receipt of such treatment, according to a retrospective cohort study

CME Editor

P. Lynne Stockton Taylor, VMD, MS, ELS(D), Technical Writer/Editor, Emerging Infectious Diseases. *Disclosure: P. Lynne Stockton Taylor, VMD, MS, ELS(D), has disclosed no relevant financial relationships.*

CME Author

Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. *Disclosure: Laurie Barclay, MD, has disclosed no relevant financial relationships.*

Authors

Disclosures: ***Sarah Brode, MD, MPH; Hannah Chung, MPH; Michael Campitelli, MPH; Jeff Kwong, MD; Alex Marchand-Austin, MD, MSc;*** *and* ***Frances B. Jamieson, MD,*** *have disclosed no relevant financial relationships.* ***Kevin Winthrop, MD, MPH,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for Insmed Incorporated, Johnson & Johnson Pharmaceutical Research & Development L.L.C., Paratek Pharmaceuticals, RedHill Biopharma Inc.; received grants for clinical research from Insmed Incorporated.* ***Theodore Marras, MD,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for Insmed Incorporated, RedHill Biopharma Inc.; served as a speaker or a member of a speakers bureau for AstraZeneca Pharmaceuticals LP, Novartis Pharmaceuticals Corporation; received grants for clinical research from Insmed Incorporated.*

Author affiliations: West Park Healthcare Centre, Toronto, Ontario, Canada (S.K. Brode); ICES, Toronto (S.K. Brode, H. Chung, M.A. Campitelli, J.C. Kwong); University of Toronto, Toronto (S.K. Brode, J.C. Kwong, F.B. Jamieson, T.K. Marras); University Health Network and Sinai Health System, Toronto (S.K. Brode, T.K. Marras); Toronto Western Family Health Team, Toronto (J.C. Kwong); Public Health Ontario, Toronto (J.C. Kwong, A. Marchand-Austin, F.B. Jamieson); Oregon Health and Science University, Portland, Oregon, USA (K.L. Winthrop)

DOI: https://doi.org/10.3201/eid2507.181817

Surveys suggest that clinicians diverge from guidelines when treating *Mycobacterium avium* complex (MAC) pulmonary disease (PD). To determine prescribing patterns, we conducted a cohort study of adults ≥66 years of age in Ontario, Canada, with MAC or *Mycobacterium xenopi* PD during 2001–2013. Using linked laboratory and health administrative databases, we studied the first treatment episode (≥60 continuous days of ≥1 of a macrolide, ethambutol, rifamycin, fluoroquinolone, linezolid, inhaled amikacin, or, for *M. xenopi*, isoniazid). Treatment was prescribed for 24% MAC and 15% of *M. xenopi* PD patients. Most commonly prescribed was the recommended combination of macrolide, ethambutol, and rifamycin, for 47% of MAC and 36% of *M. xenopi* PD patients. Among MAC PD patients, 20% received macrolide monotherapy and 33% received regimens associated with emergent macrolide resistance. Although the most commonly prescribed regimen was guidelines-recommended, many regimens prescribed for MAC PD were associated with emergent macrolide resistance.

Nontuberculous mycobacteria (NTM) pulmonary disease (PD) is increasing in North America (*1*–*3*). The 2 most common causes of NTM PD in Ontario, Canada, are *Mycobacterium avium* complex (MAC) and *M. xenopi* (*1*). Treatment guidelines detailing evidence-based treatment regimens for MAC PD have been published; the first-line recommendation is a 3-drug combination of a macrolide, ethambutol, and a rifamycin (hereafter referred to as standard triple therapy) (*4*). Although there are no evidence-based treatment regimens for *M. xenopi* PD, expert-supported regimens have been suggested (*4*). Physician surveys suggest that, when treating MAC PD, clinicians frequently diverge from guideline recommendations (*5*,*6*). However, population-based data on treatment practices for MAC PD or *M. xenopi* PD are lacking. Our study objective was to examine antimicrobial drug prescribing patterns for MAC PD or *M. xenopi* PD in older Ontario residents.

Methods

Our retrospective cohort study used population-based linked laboratory and health administrative databases in Ontario, Canada, described previously (*7*). These datasets were linked by using unique encoded identifiers and analyzed at ICES (Toronto, Ontario, Canada). Ontario is Canada's most populous province; the population in 2013 was 13.5 million residents. Ontario has a single-payer healthcare system that provides universal access to medically necessary inpatient and outpatient services and prescription drugs to adults ≥65 years of age. Ontario also has a reference mycobacteriology laboratory that processes >95% of NTM specimens for the province (*8*).

Our study cohort consisted of adults ≥66 years of age with incident MAC PD or *M. xenopi* PD, defined according to American Thoracic Society/Infectious Diseases Society of America (ATS/IDSA) microbiological criteria (*4*), during 2001–2013; observations ended December 31, 2014. The date of diagnosis was defined as the date of collection of the first positive culture sample. To avoid confusion regarding the species for which the treatment was intended, we excluded patients who met ATS/IDSA microbiological criteria for infection with >1 NTM species during follow-up. We also excluded patients who died within 1 year of NTM PD diagnosis (and in a sensitivity analysis those who died within 2 years of diagnosis) and patients who had culture-confirmed tuberculosis (TB) after NTM PD diagnosis. We looked back 3 years before the study period to find pre-existing isolation of NTM and *M. tuberculosis* complex; we excluded patients for whom NTM had been isolated during the 3-year look-back period and *M. tuberculosis* complex within 18 months of NTM PD diagnosis.

We studied the first treatment episode after NTM PD diagnosis, defined as ≥60 continuous days of treatment (either daily or intermittent) with ≥1 drug or class commonly used to treat MAC PD or *M. xenopi* PD (macrolide, ethambutol, rifamycin, fluoroquinolone, linezolid, inhaled amikacin, or, for *M. xenopi* PD, isoniazid), started within 1 year of any culture positive for the causative NTM species/complex and ended at the time of a ≥60 day treatment interruption. To allow for patients who refilled their prescriptions late, we defined treatment as continuous if they filled their next prescription for the same antimicrobial drug class within 1.5 times the number of days supplied in their last prescription. We also examined antimicrobial drug treatment given in the first 18 months after the start of the first treatment episode (i.e., not ending at a treatment interruption of ≥60 days) to capture breaks in therapy and switches between regimens (each defined as lasting ≥60 days).

We collected data about patient demographics and underlying conditions at the time of NTM PD diagnosis, prescribing physician specialty, treatment details, and medication use and healthcare use associated with asthma and chronic obstructive pulmonary disease (COPD) at the time of NTM PD diagnosis (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1817-App1.pdf) (*9*–*16*). To compare patient characteristics, we used analysis of variance for continuous variables and χ^2 tests for categorical variables. To determine patient characteristics associated with initial prescription of macrolide monotherapy for ≥60 continuous days versus other regimens, we also performed bivariate and multivariable logistic regression analyses among MAC PD patients; included variables were selected a priori on the basis of clinical relevance. We used SAS version 9.4 (SAS Institute, https://www.sas.com) for all analyses and considered a 2-sided p value of ≤0.05 to be significant. This study was approved by research ethics boards at University Health Network and Public Health Ontario.

Results

Of the 3,163 patients with MAC PD and 1,048 with *M. xenopi* PD, we excluded 329 (10.4%) MAC PD and 120 (11.4%) *M. xenopi* PD patients because they also met microbiological criteria for infection with another species of NTM PD or had TB. Treatment was received by 688 (24.2%) of the 2,834 patients with exclusively MAC PD and 142 (15.3%) of the 928 with exclusively *M. xenopi* PD. A sensitivity analysis limited to patients who survived ≥2 years after NTM PD diagnosis indicated that treatment was received by 622/2533 (24.6%) of MAC PD patients and 114/785 (14.5%) of *M. xenopi* PD patients. Compared with MAC PD patients who did not receive treatment, those who did receive treatment were younger (mean age 75.6 vs. 76.9 years); more likely to be female (59.4 vs. 54.8%); more likely to reside in neighborhoods in the higher income quintile and rural settings; more likely to have bronchiectasis, COPD, and interstitial lung disease; and less likely to have diabetes mellitus, chronic kidney disease, and lung cancer (Table 1). Compared with *M. xenopi* PD patients who did not receive treatment, those who did receive treatment were more likely to have COPD (83.1 vs 63.0%) (Table 1).

The median time from NTM PD diagnosis to start of the first treatment episode was 77 (interquartile range [IQR] 28–239) days for MAC PD and 79 (IQR 40–199) days for *M. xenopi* PD patients. Among MAC PD patients who received treatment, the most commonly prescribed drug in the first treatment episode was a macrolide (87.1%), followed by ethambutol (70.2%), a rifamycin (58.6%), and a fluoroquinolone (33.7%) (Table 2). These drugs were prescribed with similar frequency for *M. xenopi* PD patients. No linezolid was prescribed. Isoniazid, assessed for *M. xenopi* PD disease only, was rarely prescribed (≤5 [≤3.5%] patients). Amikacin, as recorded in our databases, was dispensed for inhalation and was rarely used; for ≤5 (≤0.7%) MAC PD and 0 *M. xenopi* PD patients, inhaled amikacin for ≥60 days was prescribed.

Table 1. Baseline characteristics of patients who did and did not receive treatment for MAC PD and *M. xenopi* PD, Ontario, Canada, 2001–2013*

	MAC PD			*M. xenopi* PD		
Characteristic	Treated, n = 688†	Untreated, n = 2,146	p value	Treated, n = 142†	Untreated, n = 786	p value
Sex			0.031			0.248
F	409 (59.4)	1,175 (54.8)		61 (43.0)	380 (48.1)	
M	279 (40.6)	971 (45.2)		81 (57.0)	407 (51.8)	
Age, mean ± SD	75.6 ± 5.94	76.9 ± 6.65	<0.001	75.1 ± 5.92	76.1 ± 6.50	0.077
Income quintile			0.018			0.932
1 (lowest)	142 (20.6)	550 (25.6)		32 (22.5)	164 (20.9)	
2	146 (21.2)	428 (19.9)		33 (23.2)	178 (22.6)	
3	124 (18.0)	425 (19.8)		26 (18.3)	131 (16.7)	
4	117 (17.0)	354 (16.5)		24 (16.9)	157 (20.0)	
5 (highest)	155–159 (22.5–23.1)	382 (17.8)		22–27 (15.5–19.01)	153–158 (19.5–20.1)	
Missing	≤5 (≤0.7)	7 (0.3)		≤5 (≤3.5)	≤5 (≤0.7)	
Residency‡			<0.001			0.288
Rural	37 (5.4)	53 (2.5)		≤5 (≤3.5)	8 (1.0)	
Suburban	89 (12.9)	159 (7.4)		9–14 (6.3–9.9)	38 (4.8)	
Urban	562 (81.7)	1,934 (90.1)		129 (90.8)	740 (94.1)	
ADGs, mean ± SD	10.4 ± 3.49	10.4 ± 3.67	0.775	11.3 ± 3.52	10.8 ± 3.77	0.117
Underlying conditions§						
Asthma	265 (38.5)	751 (35.0)	0.094	64 (45.1)	311 (39.6)	0.219
Bronchiectasis	169 (24.6)	335 (15.6)	<0.001	19 (13.4)	90 (11.5)	0.511
Chronic kidney disease	40 (5.8)	199 (9.3)	0.004	12 (8.5)	66 (8.4)	0.983
COPD	462 (67.2)	1,209 (56.3)	<0.001	118 (83.1)	474 (60.3)	<0.001
Cystic fibrosis	≤5 (≤0.7)	≤5 (≤0.2)	0.327	≤5 (≤3.5)	≤5 (≤0.6)	0.172
Diabetes mellitus	121 (17.6)	518 (24.1)	<0.001	28 (19.7)	206 (26.2)	0.101
GERD	139 (20.2)	429 (20.0)	0.903	31 (21.8)	158 (20.1)	0.638
HIV infection	≤5 (≤0.7)	≤5 (≤0.2)	0.087	0	0	NA
Interstitial lung disease	81 (11.8)	138 (6.4)	<0.001	14 (9.9)	62 (7.9)	0.430
Lung cancer	19 (2.8)	135 (6.3)	<0.001	9 (6.3)	74 (9.4)	0.237
Prior TB	≤5 (≤0.7)	17 (0.8)	0.161	≤5 (≤3.5)	12 (1.5)	0.915
Rheumatoid arthritis	27 (3.9)	79 (3.7)	0.770	6 (4.2)	31 (3.9)	0.875

*Values are no. (%) except as indicated. According to privacy regulations, values representing <6 persons are reported as ≤5, and data are presented as a range of values for categorical variables where back-calculation is possible. ADGs, aggregated diagnostic groups (from the adjusted clinical group case mix system); COPD, chronic obstructive pulmonary disease; GERD, gastroesophageal reflux disease; MAC, *Mycobacterium avium* complex;, *M. xenopi*; *Mycobacterium xenopi*; NA, not applicable; PD, pulmonary disease; TB, tuberculosis.

†Treated defined as ≥60 continuous days of treatment with ≥1 drugs/classes commonly used to treat MAC or *M. xenopi* PD (macrolide, ethambutol, rifamycin, fluoroquinolone, linezolid, inhaled amikacin, or, for *M. xenopi*, isoniazid), started within 1 y of any positive culture for the causative nontuberculous mycobacteria species/complex.

‡Derived from rural index for Ontario group, a measure of rurality designed for Ontario (*9*).

§Defined according to inpatient and outpatient diagnostic codes in databases of hospital discharges and physicians' services claims, respectively; see Appendix (https://wwwnc.cdc.gov/EID/article/25/7/18-1817-App1.pdf) for definitions. Definitions have been validated for all underlying conditions with the exception of bronchiectasis and interstitial lung disease.

We examined the proportion of patients for whom particular regimens had ever been prescribed for ≥60 days during their first treatment episode. Among patients with treated MAC PD, the guidelines-recommended standard triple regimen (macrolide/ethambutol/rifamycin) was the most commonly prescribed (47.4%), followed by nonmacrolide monotherapy (29.2%) and macrolide monotherapy (20.5%) (Table 2). Drug regimens associated with development of macrolide-resistant MAC (macrolide monotherapy, macrolide/fluoroquinolone, and macrolide/rifamycin) (*17*,*18*) were prescribed for 224/688 (32.6%) of MAC PD patients. Among *M. xenopi* PD patients who received treatment, standard triple therapy was prescribed for 35.9%, followed by nonmacrolide monotherapy for 28.2%, and macrolide monotherapy for 23.9% (Table 2).

The flow of antimicrobial drug treatment during the first 18 months (i.e., regimen sequence, duration, and transitions) revealed that, among MAC PD patients who

Table 2. Proportion of patients with MAC PD and *M. xenopi* PD who had ever received each antimicrobial drug and select drug combinations and duration during first treatment episode, Ontario, Canada, 2001–2013*

	MAC PD, n = 688		*M. xenopi* PD, n = 142	
Treatment†	No. (%)	Mean duration ± SD, d	No. (%)	Mean duration ± SD, d
Individual drug				
Macrolide				
Any	599 (87.1)	447 ± 367	120 (84.5)	359 ± 312
Clarithromycin	318 (46.2)	369 ± 364	66 (46.5)	319 ± 367
Azithromycin	354 (51.5)	424 ± 348	66 (46.5)	335 ± 244
Rifamycin				
Any	403 (58.6)	437 ± 366	69 (48.6)	349 ± 228
Rifampin	384 (55.8)	434 ± 363	61 (43.0)	351 ± 233
Rifabutin	30 (4.4)	323 ± 285	9 (6.3)	294 ± 217
Ethambutol	483 (70.2)	456 ± 357	84 (59.2)	363 ± 284
Fluoroquinolone				
Any	232 (33.7)	369 ± 353	63 (44.4)	312 ± 189
Moxifloxacin	82 (11.9)	318 ± 382	27 (19.0)	251 ± 195
Levofloxacin	56 (8.1)	226 ± 239	11 (7.7)	240 ± 282
Ciprofloxacin	137 (19.9)	328 ± 333	36 (25.4)	283 ± 181
Gatifloxacin	≤5 (≤0.7)	126 ± 163	0	NA
Norfloxacin	8 (1.2)	197 ± 288	0	NA
Linezolid	0	NA	0	NA
Isoniazid	NA	NA	≤5 (≤3.5)	160 ± 110
Drug regimen				
Standard triple: macrolide + ethambutol + rifamycin ± others	326 (47.4)	369 ±269	51 (35.9)	241 ± 173
Macrolide + ethambutol	91 (13.2)	315 ± 283	11 (7.7)	159 ± 83
Macrolide + rifamycin	49 (7.1)	284 ± 392	10 (7.0)	251 ± 208
Macrolide + fluoroquinolone	65 (9.4)	267 ± 278	20 (14.1)	228 ± 153
Other macrolide-containing combinations‡	115 (16.7)	346 ±276	31 (21.8)	295 ± 156
Nonmacrolide combination§	63 (9.2)	258 ± 298	19 (13.4)	198 ± 187
Macrolide monotherapy	141 (20.5)	262 ± 358	34 (23.9)	330 ± 509
Nonmacrolide monotherapy	201 (29.2)	206 ± 226	40 (28.2)	253 ± 207
No. drugs given, mean ± SD	2.5 ± 0.9		2.4 ± 1.0	
No. drugs given				
1	142 (20.6)		37 (26.1)	
2	121 (17.6)		24 (16.9)	
3	372 (54.1)		69 (48.6)	
4	53 (7.7)		12 (8.5)	
No. switched regimens¶				
0	401 (58.3)		85 (59.9)	
1	177 (25.7)		33 (23.2)	
2	72 (10.5)		14 (9.9)	
≥3	38 (5.5)		10 (7.0)	
Maximum no. drugs used at any 1 time, mean ± SD	2.4 ± 0.9		2.3 ± 0.9	

*MAC, *Mycobacterium avium* complex; M. *xenopi*, *Mycobacterium xenopi*; PD, pulmonary disease.
†≥60 continuous d of treatment with ≥1 drugs/classes commonly used to treat MAC PD or *M. xenopi* PD (macrolide, ethambutol, rifamycin, fluoroquinolone, linezolid, inhaled amikacin, or for *M. xenopi* PD, isoniazid), started within 1 y of any positive culture for the causative nontuberculous mycobacteria species/complex. Patients may have received >1 drug from an antibiotic class, and/or>1antibiotic regimen during their first treatment episode, so values do not add up to 100%. According to privacy regulations, values representing <6 persons are reported as ≤5, and data are presented as a range of values for categorical variables where back-calculation is possible.
‡≥2 drugs excluding: macrolide + fluoroquinolone, macrolide + EMB, macrolide + rifamycin.
§≥2 drugs (e.g., ethambutol, a rifamycin, a fluoroquinolone (or for *M. xenopi* PD, isoniazid), without macrolide.
¶Change in treatment lasting ≥60 d, in the first treatment episode.

received treatment, the most common starting regimen was standard triple therapy (290/688; 42.1%) (Figure), prescribed for a mean (± SD) of 315 (± 167) days (median 334; IQR 151–467 days) before a regimen switch or discontinuation. Among MAC PD patients for whom the initial regimen was associated with development of macrolide resistance, these regimens were prescribed for the following mean (± SD) durations before a switch or discontinuation: macrolide monotherapy, 230 (± 167) days; macrolide/fluoroquinolone, 216 (± 147) days; and macrolide/rifamycin, 197 (± 139) days (Figure). For a large minority of MAC PD patients, therapy was switched during the first treatment episode; ≥1 regimen was switched for 41.7% (Table 2; Figure). Among MAC PD patients who received treatment, 50.2% received treatment for ≥12 months before discontinuation. Among *M. xenopi* PD patients who received treatment, 31.0% initially received standard triple therapy and 40.1% underwent ≥1 regimen switch (Table 2).

Among MAC PD patients who received treatment, for their first regimen, the specialties of the main prescribing physicians varied. The prescriber was a pulmonologist for 55.7%, an infectious diseases specialist for 10.0%, an internal medicine specialist for 7.4%, a family physician/general practitioner for 12.3%, and another specialist or of unknown specialty for 14.5% (Table 3).

According to bivariate analyses, patients with MAC PD whose initial regimen was macrolide monotherapy were more likely than those whose initial regimen was anything else to have asthma or COPD , to have received a long-acting bronchodilator or oral corticosteroid in the prior year, to have visited an emergency department or been hospitalized in the prior 2 years for an asthma or COPD exacerbation, to have received oxygen at home, and to have received pulmonary function tests in the previous 5 years (Table 4). However, according to adjusted analyses, only use of oral corticosteroids in the prior year was significantly associated with a starting regimen of macrolide monotherapy (adjusted odds ratio 2.01, 95% CI 1.16–3.50).

Discussion

In this population-based study of treatment practices for MAC PD and *M. xenopi* PD in adults ≥66 years of age, we found that a minority of patients received antimicrobial therapy: 24% of MAC PD patients and 15% of

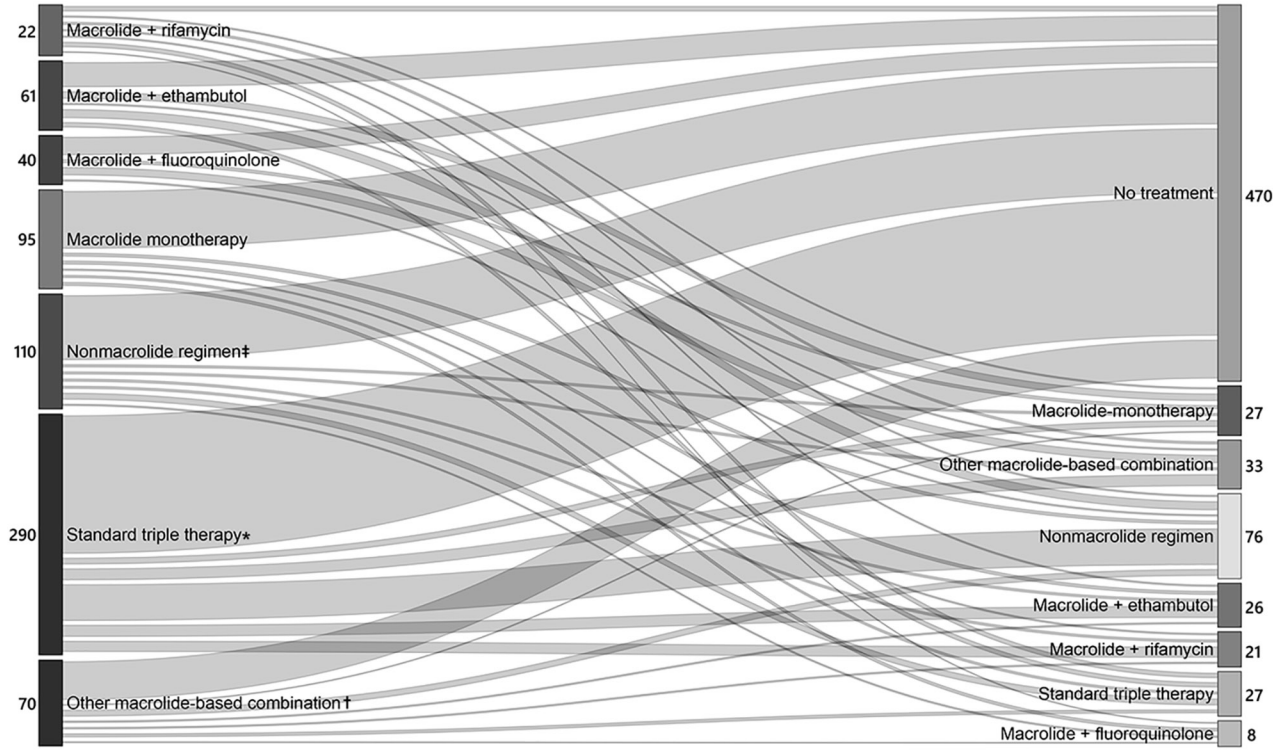


Figure. Flow of therapy for 688 patients with *Mycobacterium avium* complex pulmonary disease, depicting transition between first and second regimens during first 18 months of treatment, Ontario, Canada, 2001–2013. Values are the number of patients receiving each treatment regimen in each epoch of therapy. An epoch is defined as ≥60 days of the therapy. The width of the lines is proportional to the number of patients receiving and transitioning between each regimen. Mean (± SD) duration of treatment, in days, for each starting regimen is as follows: standard triple therapy 315 (± 167), other macrolide containing combination 331 (± 157), macrolide-ethambutol 274 (± 172), macrolide monotherapy 230 (±167), nonmacrolide containing regimen 176 (± 178), macrolide-fluoroquinolone 216 (± 147), macrolide-rifamycin 197 (± 139). *Macrolide, ethambutol, and a rifamycin, ± other drugs; †macrolide + ≥2 additional drugs (other than standard triple therapy); ‡ethambutol, a rifamycin, or fluoroquinolone, either alone or in combination.

Table 3. Initial treatment regimen, by prescriber specialty, for 688 patients with *Mycobacterium avium* complex pulmonary disease, Ontario, Canada, 2001–2013*

	Specialty, no. (%) patients				
Regimen	Respirology, n = 383 (55.7)	ID, n = 69 (10.0)	GIM, n = 51 (7.4)	FP/GP, n = 85 (12.3)	Other/unknown, n = 100 (14.5)
Standard triple therapy	166 (43.3)	37 (53.6)	22 (43.1)	34 (40.0)	31 (31.0)
Macrolide monotherapy	55 (14.4)	7 (10.1)	≤5 (≤9.8)	14 (16.5)	15 (15.0)
Macrolide + rifamycin or fluoroquinolone	38 (9.9)	≤5 (≤7.2)	≤5 (≤9.8)	6 (7.1)	8 (8.0)
Other	124 (32.4)	20–25 (29.0–36.1)	20 (39.2)	31 (36.5)	46 (46.0)

*Includes the regimen dispensed for at least the first 60 d of treatment. According to privacy regulations, values representing<6 persons are reported as ≤5, and data are presented as a range of values for categorical variables where back-calculation is possible. GIM, general internal medicine; FP/GP, family practice/general practice; ID, infectious diseases.

M. xenopi PD patients. During the first treatment episode, the most commonly prescribed regimen, initially and overall, was standard triple therapy. However, it is concerning that many MAC PD patients received ≥60 days of treatment with regimens associated with macrolide resistance, a situation that is extremely difficult to treat and associated with high mortality rates (*17–19*). Macrolide monotherapy was prescribed for 20% of MAC PD patients, and other regimens associated with facilitating macrolide resistance (macrolide/fluoroquinolone, macrolide/rifamycin) were also frequently prescribed. Although standard triple therapy was prescribed initially for 42% and ever (during the first treatment episode) for 47% of patients, regimens that facilitate macrolide resistance were prescribed initially for 23% and ever for 33%. Treatment flow was complex, and switches between regimens were common.

In our study, the proportion of patients with MAC PD who received antimicrobial drug treatment (24%) was lower than that described by others. Studies from South Korea (*20*) and Germany (*21*) reported treatment rates within 3 years of diagnosis of 65% for MAC PD and 74% for NTM PD patients. In Oregon, USA, treatment was initiated within 2 years of diagnosis for 54% of NTM PD patients (*22*). According to physician survey studies, antimicrobial drug treatment was received by 55% of MAC PD patients in the United States (*5*), 68% of NTM PD patients from 5 countries in the European Union (*6*), and 43% of NTM PD patients in Japan (*6*). The reasons why a relatively small proportion of MAC PD patients in our study received treatment are probably many. First, our definition of NTM PD (based on microbiological criteria only) probably more often misclassified patients from whom NTM were repeatedly isolated as having disease, compared with the Oregon study, which reviewed all diagnostic criteria (*22*), and the Germany study, which used diagnostic codes (*21*). Second, our study was population based and thereby included the full spectrum of disease severity and physician expertise, compared with specialty clinic-based studies (*20*), which probably comprise patients with more severe disease and physicians who may be more likely to treat NTM PD because of greater experience. Third, we included only adults ≥66 years of age; older patients may be less likely to receive treatment, as was noted among the MAC PD patients in this study and has been described by others (*20*). Some patients may not have been prescribed treatment because of a limited life expectancy resulting from underlying conditions; however, when we performed a sensitivity analysis limited to patients who survived ≥2 years after NTM PD diagnosis, we found no notable change in the proportion who received treatment. Fourth, we required 1 positive culture for the causative NTM within 1 year of treatment initiation and ≥60 continuous days of dispensed prescriptions, which were not requirements for the other studies. Of note, the proportion of patients who received treatment in our study was similar to the 18% of NTM PD patients who received treatment at 4 integrated US healthcare delivery systems (*23*), in which patients were identified by using a combination of culture results and codes from the International Classification of Diseases, Ninth Revision.

We found associations between baseline characteristics and receipt of MAC PD treatment. Income distribution was significantly associated with treatment; patients residing in neighborhoods in lower income quintiles seemed less likely to receive treatment. Although Canada provides universal access to medically necessary health services, including prescription drugs for adults ≥65 years of age, socioeconomic disparities in access to specialist care have been observed (*24*) and may play a role. We also found that patients living in urban settings were less likely to receive treatment; this finding is somewhat surprising in that others have shown that urban patients are more likely than rural patients to receive ambulatory care, including specialist care, for other chronic medical conditions (*25*,*26*). Whether a disparity in the proportion of patients with true disease exists when comparing patients with MAC isolates from urban versus rural settings is not clear.

We found prescription of standard triple therapy for MAC PD to be more common (47% ever received it and 42% received it as initial therapy) than that reported in the United States (13% ever received) (*5*), the European Union (9% for >6 months) (*6*), and Germany (19%) (*21*) but similar to that reported in Japan (42% for >6 months) (*6*). The possible reasons for these differences include differences in study methods, prescribing physician specialty,

Table 4. Characteristics of MAC PD patients according to initial treatment regimen, Ontario, Canada, 2001–2013*

Characteristic	Macrolide monotherapy, n = 95†	Other regimen, n = 593	Unadjusted OR (95% CI)	p value	Adjusted OR (95% CI)‡	p value
Sex						
F	52 (54.7)	357 (60.2)	0.80 (0.52–1.24)	0.314	0.92 (0.57–1.48)	0.738
M	43 (45.3)	236 (39.8)	Referent	NA	Referent	NA
Age, mean ± SD	76.21 ± 6.67	75.52 ± 5.81	1.02 (0.98–1.06)	0.292	1.03 (0.99–1.07)	0.123
Income quintile						
1 (lowest)	19 (20.0)	123 (20.7)	Referent	0.682	NA	NA
2	22 (23.2)	124 (20.9)	1.15 (0.59–2.23)	0.470	NA	NA
3	13 (13.7)	111 (18.7)	0.76 (0.36–1.61)	0.945	NA	NA
4	16 (16.8)	101 (17.0)	1.03 (0.50–2.10)	0.752	NA	NA
5 (highest)	21–25 (22.1–26.3)	134 (22.6)	1.11 (0.58–2.14)	0.986	NA	NA
Missing data	≤5 (≤2.1)	0	NA		NA	NA
Residency§						
Rural	≤5 (≤2.1)	35 (5.9)	0.33 (0.08–1.40)	0.132	NA	NA
Suburban	8–12 (8.4–12.6)	79 (13.3)	0.73 (0.36–1.47)	0.378	NA	NA
Urban	83 (87.4)	479 (80.8)	Referent	NA	NA	NA
ADGs, mean ± SD	10.45 ± 3.90	10.36 ± 3.42	1.01 (0.95–1.07)	0.805	NA	NA
Underlying conditions¶						
Asthma	47 (49.5)	218 (36.8)	1.68 (1.09–2.60)	0.019	1.22 (0.72–2.07)	0.451
Bronchiectasis	27 (28.4)	142 (23.9)	1.26 (0.78–2.05)	0.347	1.19 (0.71–1.99)	0.504
Chronic kidney disease	7 (7.4)	133 (5.6)	1.35 (0.58–3.15)	0.486	NA	NA
COPD	75 (78.9)	387 (65.3)	2.00 (1.19–3.36)	0.009	1.47 (0.81–2.66)	0.208
Diabetes mellitus	21 (22.1)	100 (16.9)	1.40 (0.82–2.38)	0.214	NA	NA
GERD	21 (22.1)	118 (19.9)	1.14 (0.68–1.93)	0.619	NA	NA
Interstitial lung disease	14 (14.7)	67 (11.3)	1.36 (0.73–2.53)	0.335	NA	NA
Lung cancer	≤5 (≤2.1)	17 (2.9)	0.73 (0.17–3.21)	0.676	NA	NA
Rheumatoid arthritis	≤5 (≤2.1)	25 (4.2)	0.49 (0.11–2.10)	0.335	NA	NA
Drug exposure within 1 y#						
Short-acting BD	49 (51.6)	271 (45.7)	1.27 (0.82–1.95)	0.287	0.69 (0.38–1.26)	0.225
Long-acting BD	52 (54.7)	248 (41.8)	1.68 (1.09–2.60)	0.019	1.16 (0.56–2.39)	0.694
ICS	55 (57.9)	287 (48.4)	1.47 (0.95–2.27)	0.087	0.89 (0.42–1.87)	0.754
OCS	38 (40.0)	130 (21.9)	2.37 (1.51–3.74)	<0.001	2.01 (1.16–3.50)	0.013
Methylxanthine	9 (9.5)	28 (4.7)	2.11 (0.96–4.63)	0.062	1.52 (0.64–3.57)	0.340
ED visit/hospitalization for asthma or COPD within 2 y#	22 (23.2)	88 (14.8)	1.73 (1.02–2.93)	0.042	0.92 (0.48–1.77)	0.799
Prior/current home oxygen therapy	12 (12.6)	31 (5.2)	2.62 (1.30–5.31)	0.007	1.83 (0.84–3.98)	0.128
PFTs within 5 y#	78 (82.1)	416 (70.2)	1.95 (1.12–3.39)	0.018	1.52 (0.82–2.78)	0.180
Pulmonologist prescriber	55 (57.9)	328 (55.3)	1.11 (0.72–1.72)	0.638	1.03 (0.66–1.63)	0.889

*Values are no. (%) except as indicated. According to privacy regulations, values representing <6 persons are reported as ≤5, and data are presented as a range of values for categorical variables where back-calculation is possible. ADGs, aggregated diagnostic groups (from the adjusted clinical group case mix system); BD, bronchodilator; COPD, chronic obstructive pulmonary disease; ED, emergency department; GERD, gastroesophageal reflux disease; ICS, inhaled corticosteroid; MAC, *Mycobacterium avium* complex; NA, not applicable; NTM, nontuberculous mycobacteria; OCS, oral corticosteroid; OR, odds ratio; PD, pulmonary disease; PFTs, pulmonary function tests.
†Macrolide monotherapy was the first antibiotic regimen given after NTM PD diagnosis, and was considered ≥60 d with no companion drugs of interest.
‡Variables were selected for inclusion in the multivariable model a priori, based on clinical relevance.
§Derived from rural index for Ontario group, a measure of rurality designed for Ontario (*9*).
¶Defined according to inpatient and outpatient diagnostic codes in databases of hospital discharges and physicians' services claims, respectively. Definitions have been validated for all underlying conditions with the exception of bronchiectasis and interstitial lung disease.
#Before NTM PD diagnosis.

financial coverage for medications, and familiarity with ATS/IDSA guidelines. Regarding physician specialties, the proportion of MAC PD patients receiving treatment from a pulmonologist in our study (57%) was similar to that in Japan (54%) (*6*) but higher than that in the European Union (29%) (*6*) and in the United States (37%) (*5*). Pulmonologists may be more aware of the ATS/IDSA guidelines than are other specialists. However, pulmonologists in Ontario (43%) seemed more likely than those in the United States (18%) to prescribe standard triple therapy for MAC PD (*5*), which may result from different patient populations and medication coverage. We included only adults ≥66 years of age because this population has comprehensive medication coverage. Pulmonologists in the United States may prescribe nonstandard antimicrobial drug regimens for patients who do not have prescription drug coverage because of cost.

In our study, 20% of MAC PD patients who received treatment were prescribed ≥60 days of macrolide monotherapy, 9% ≥60 days of macrolide/fluoroquinolone, and 7% ≥60 days of macrolide/rifamycin therapy. Findings of studies of physicians in the United States (*5*) and Germany (*21*) were similar. These regimens are associated with development of macrolide resistance (*17*,*18*); resistance developed in 20% of 59 patients who received macrolide monotherapy for 4 months compared with 4%

of 303 patients who received standard triple therapy (*17*). In our study, among 95 MAC PD patients whose initial regimen was macrolide monotherapy, the mean duration was 230 days; macrolide/fluoroquinolone and macrolide/rifamycin regimens were given for similar durations. Therefore, the regimen duration was long enough to constitute a risk for macrolide resistance.

For some patients, these drugs may have been prescribed for other conditions. This possibility applies especially to macrolide monotherapy, which may have been prescribed to treat exacerbations of asthma, COPD, or bronchiectasis and may not have been prescribed to treat MAC per se. This possibility is supported by our analyses; bivariate analysis indicated that presence of asthma and COPD were associated with receipt of macrolide monotherapy versus another regimen. Although bronchiectasis was not associated with receipt of macrolide monotherapy, our databases contain no validated bronchiectasis definition, and the definition we used (1 physician billing claim or hospitalization with bronchiectasis diagnosis) seems to be of very low sensitivity, given the small number of patients with NTM assigned a code for bronchiectasis. Also, some treatments for asthma/COPD (long-acting bronchodilators, oral corticosteroids, home oxygen), as well as emergency department visits/hospitalizations for asthma/COPD, were associated with prescription of macrolide monotherapy. According to multivariable analyses, the only variable associated with prescription of macrolide monotherapy was receipt of oral corticosteroids ≤1 year before MAC PD diagnosis, which is consistent with the possibility that macrolide monotherapy was prescribed to prevent asthma/COPD exacerbations. It is possible that some patients for whom macrolide monotherapy was prescribed for asthma, COPD, or bronchiectasis did not have clinical or radiologic findings of MAC PD. However, these patients did fulfill microbiological criteria for MAC PD and had ≥1 positive culture within 1 year before filling the prescription. No data describe the risk of inducing macrolide resistance in persons with positive sputum cultures who do not meet full diagnostic criteria for MAC PD. However, the fact that one fifth of patients in our study who received treatment and met microbiological criteria for MAC PD received this regimen for ≥60 days is concerning. Given the increasing use of macrolides for asthma, COPD, and bronchiectasis, further research into the safety of these drugs in patients with NTM isolation is needed.

Few data exist regarding prescribing patterns for patients with *M. xenopi* PD. A retrospective study of 136 patients in France who had *M. xenopi* PD meeting full ATS/IDSA diagnostic criteria found that 59% received treatment (*27*) compared with 15% in our study. That study found that patients' initial treatment regimens contained an average of 4 drugs among rifamycins (88%), ethambutol (75%), isoniazid (66%), clarithromycin (30%), and fluoroquinolones (21%). In our study, patients received fewer drugs (mean 2.4 ± 1.0 SD) and were more likely to receive a macrolide (84%) or a fluoroquinolone (44%) and less likely to receive a rifamycin (49%), ethambutol (59%), or isoniazid (≤4%). The difference between prescribing patterns for *M. xenopi* PD in France versus Ontario may be partially explained by the periods of the studies (1983–2003 in France vs. 2001–2013 in Ontario); evidence supporting the efficacy of macrolides for treating *M. xenopi* infection emerged in the mid-1990s (*28*–*31*). Also, the study in France was not restricted to older adults and included only patients at 1 of 13 hospitals, which may have limited them to more severe cases. Another possible explanation relates to differences in the more geographically proximal treatment guidelines; the 1999 British Thoracic Society guidelines recommended treatment with rifampin and ethambutol ± isoniazid (*32*), whereas the 1997 and 2007 ATS/IDSA guidelines recommend a regimen of clarithromycin, rifampin, and ethambutol (*4*,*28*), ± isoniazid ± moxifloxacin (*4*).

For MAC PD and *M. xenopi* PD patients who received treatment, regimen switches were common (42% of MAC PD patients underwent ≥1 regimen switch). Others have reported similar findings; in 2 case series of MAC PD patients who received standard triple therapy, regimens were switched for 46%–71% of patients receiving daily therapy and 3%–21% of patients receiving intermittent therapy (*33*,*34*). We did not study daily versus intermittent therapy and are not able to draw conclusions regarding tolerability of different drugs or combinations. However, our finding of frequent regimen switches suggests that drug intolerance was common and may partially explain the frequent use of regimens not recommended in treatment guidelines.

Our study has several limitations. We based our definition of NTM PD on microbiological criteria alone and therefore probably misclassified some patients as having true disease, possibly contributing to the observation that a low proportion of patients received treatment. We defined treatment as ≥60 days of an antimicrobial drug of interest being dispensed; this definition may not capture some patients in whom there was an intent to treat, such as patients who had medications prescribed but never dispensed and patients who received treatment for NTM PD but stopped taking the medication in <60 days. Because we included only adults ≥66 years of age, our findings may not apply to younger patients. Last, we were not able to study the use of clofazimine or injectable aminoglycosides because the relevant information is not contained in our databases. This omission may have caused us to erroneously label some patients as having received an inappropriate regimen when the regimen was strengthened by clofazimine or an aminoglycoside. However, because

clofazimine is not approved for use in Canada and was difficult to access during the study period, we think that these patients are probably few.

The use of inhaled amikacin seemed to be rare. We also excluded patients who met diagnostic criteria for NTM PD associated with >1 species. The proportion was small, combined with exclusions for TB amounting to 10.4% for MAC PD and 11.1% for *M. xenopi* PD. Given the species distribution of NTM in Ontario (MAC PD and *M. xenopi* PD comprising the overwhelming majority of treated NTM episodes), the very high similarity between MAC PD and *M. xenopi* PD treatments, and our lack of data for intravenous treatments needed to analyze *M. abscessus* therapy, we elected to exclude these patients.

In summary, the most commonly prescribed regimen for MAC PD and *M. xenopi* PD in Ontario was standard triple therapy. This finding is somewhat reassuring; however, a large minority of patients with MAC PD received regimens that may lead to macrolide resistance. Physicians who treat patients with NTM PD should take care to follow established treatment guidelines for management of this condition.

Acknowledgment

We thank IMS Brogan Inc. for use of their Drug Information Database.

This study was supported by ICES and Public Health Ontario, which are funded by annual grants from the Ontario Ministry of Health and Long-Term Care. The opinions, results, and conclusions reported in this article are those of the authors and are independent from the funding sources. No endorsement by ICES or the Ontario Ministry of Health and Long-Term Care is intended or should be inferred. Parts of this material are based on data and/or information compiled and provided by the Canadian Institute of Health Information. However, the analyses, conclusions, opinions, and statements expressed herein are those of the authors, and not necessarily those of the Institute.

About the Author

Dr. Brode is an assistant professor of medicine at the University of Toronto, the physician lead of the TB Program at West Park Healthcare Centre (Toronto), and a staff pulmonologist and a clinician in the NTM and TB Programs at Toronto Western Hospital (Toronto). Her research focuses on epidemiology, treatment, and outcomes for patients with TB and NTM infection.

References

1. Marras TK, Mendelson D, Marchand-Austin A, May K, Jamieson FB. Pulmonary nontuberculous mycobacterial disease, Ontario, Canada, 1998–2010. Emerg Infect Dis. 2013;19:1889–91. http://dx.doi.org/10.3201/eid1911.130737
2. Henkle E, Hedberg K, Schafer S, Novosad S, Winthrop KL. Population-based incidence of pulmonary nontuberculous mycobacterial disease in Oregon 2007 to 2012. Ann Am Thorac Soc. 2015;12:642–7. http://dx.doi.org/10.1513/AnnalsATS.201412-559OC
3. Adjemian J, Olivier KN, Seitz AE, Holland SM, Prevots DR. Prevalence of nontuberculous mycobacterial lung disease in U.S. Medicare beneficiaries. Am J Respir Crit Care Med. 2012;185:881–6. http://dx.doi.org/10.1164/rccm.201111-2016OC
4. Griffith DE, Aksamit T, Brown-Elliott BA, Catanzaro A, Daley C, Gordin F, et al.; ATS Mycobacterial Diseases Subcommittee; American Thoracic Society; Infectious Disease Society of America. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416. http://dx.doi.org/10.1164/rccm.200604-571ST
5. Adjemian J, Prevots DR, Gallagher J, Heap K, Gupta R, Griffith D. Lack of adherence to evidence-based treatment guidelines for nontuberculous mycobacterial lung disease. Ann Am Thorac Soc. 2014;11:9–16. http://dx.doi.org/10.1513/AnnalsATS.201304-085OC
6. van Ingen J, Wagner D, Gallagher J, Morimoto K, Lange C, Haworth CS, et al.; NTM-NET. Poor adherence to management guidelines in nontuberculous mycobacterial pulmonary diseases. Eur Respir J. 2017;49:1601855. http://dx.doi. org/10.1183/13993003.01855-2016
7. Brode SK, Jamieson FB, Ng R, Campitelli MA, Kwong JC, Paterson JM, et al. Risk of mycobacterial infections associated with rheumatoid arthritis in Ontario, Canada. Chest. 2014;146:563–72. http://dx.doi.org/10.1378/chest.13-2058
8. Al Houqani M, Jamieson F, Chedore P, Mehta M, May K, Marras TK. Isolation prevalence of pulmonary nontuberculous mycobacteria in Ontario in 2007. Can Respir J. 2011;18:19–24. http://dx.doi.org/10.1155/2011/865831
9. Kralj B. Measuring "rurality" for purposes of health-care planning: an empirical measure for Ontario. Ontario Medical Review. 2000;10:33–52.
10. Hux JE, Ivis F, Flintoft V, Bica A. Diabetes in Ontario: determination of prevalence and incidence using a validated administrative data algorithm. Diabetes Care. 2002;25:512–6. http://dx.doi.org/10.2337/diacare.25.3.512
11. Lopushinsky SR, Covarrubia KA, Rabeneck L, Austin PC, Urbach DR. Accuracy of administrative health data for the diagnosis of upper gastrointestinal diseases. Surg Endosc. 2007;21:1733–7. http://dx.doi.org/10.1007/s00464-006-9136-1
12. Widdifield J, Bombardier C, Bernatsky S, Paterson JM, Green D, Young J, et al. An administrative data validation study of the accuracy of algorithms for identifying rheumatoid arthritis: the influence of the reference standard on algorithm performance. BMC Musculoskelet Disord. 2014;15:216. http://dx.doi.org/10.1186/1471-2474-15-216
13. Reid RJ, MacWilliam L, Verhulst L, Roos N, Atkinson M. Performance of the ACG case-mix system in two Canadian provinces. Med Care. 2001;39:86–99. http://dx.doi.org/10.1097/00005650-200101000-00010
14. Gershon AS, Wang C, Guan J, Vasilevska-Ristovska J, Cicutto L, To T. Identifying patients with physician-diagnosed asthma in health administrative databases. Can Respir J. 2009;16:183–8. http://dx.doi.org/10.1155/2009/963098
15. Gershon AS, Wang C, Guan J, Vasilevska-Ristovska J, Cicutto L, To T. Identifying individuals with physician diagnosed COPD in health administrative databases. COPD. 2009;6:388–94. http://dx.doi.org/10.1080/15412550903140865
16. Fleet JL, Dixon SN, Shariff SZ, Quinn RR, Nash DM, Harel Z, et al. Detecting chronic kidney disease in population-based administrative databases using an algorithm of hospital encounter and physician claim codes. BMC Nephrol. 2013;14:81. http://dx.doi.org/10.1186/1471-2369-14-81

17. Griffith DE, Brown-Elliott BA, Langsjoen B, Zhang Y, Pan X, Girard W, et al. Clinical and molecular analysis of macrolide resistance in *Mycobacterium avium* complex lung disease. Am J Respir Crit Care Med. 2006;174:928–34. http://dx.doi.org/10.1164/rccm.200603-450OC
18. Morimoto K, Namkoong H, Hasegawa N, Nakagawa T, Morino E, Shiraishi Y, et al.; Nontuberculous Mycobacteriosis Japan Research Consortium. Macrolide-resistant *Mycobacterium avium* complex lung disease: analysis of 102 consecutive cases. Ann Am Thorac Soc. 2016;13:1904–11. http://dx.doi.org/10.1513/AnnalsATS.201604-246OC
19. Moon SM, Park HY, Kim SY, Jhun BW, Lee H, Jeon K, et al. Clinical characteristics, treatment outcomes, and resistance mutations associated with macrolide-resistant *Mycobacterium avium* complex lung disease. Antimicrob Agents Chemother. 2016;60:6758–65. http://dx.doi.org/10.1128/AAC.01240-16
20. Hwang JA, Kim S, Jo KW, Shim TS. Natural history of *Mycobacterium avium* complex lung disease in untreated patients with stable course. Eur Respir J. 2017;49:1600537. http://dx.doi.org/10.1183/13993003.00537-2016
21. Diel R, Jacob J, Lampenius N, Loebinger M, Nienhaus A, Rabe KF, et al. Burden of non-tuberculous mycobacterial pulmonary disease in Germany. Eur Respir J. 2017;49:1602109. http://dx.doi.org/10.1183/13993003.02109-2016
22. Henkle E, Novosad SA, Shafer S, Hedberg K, Siegel SAR, Ku J, et al. Long-term outcomes in a population-based cohort with respiratory nontuberculous mycobacteria isolation. Ann Am Thorac Soc. 2017;14:1120–8. http://dx.doi.org/10.1513/AnnalsATS.201610-801OC
23. Prevots DR, Shaw PA, Strickland D, Jackson LA, Raebel MA, Blosky MA, et al. Nontuberculous mycobacterial lung disease prevalence at four integrated health care delivery systems. Am J Respir Crit Care Med. 2010;182:970–6. http://dx.doi.org/10.1164/rccm.201002-0310OC
24. Dunlop S, Coyte PC, McIsaac W. Socio-economic status and the utilisation of physicians' services: results from the Canadian National Population Health Survey. Soc Sci Med. 2000 51:123–33.
25. Tran C, Wijeysundera HC, Qui F, Tu JV, Bhatia RS. Comparing the ambulatory care and outcomes for rural and urban patients with chronic ischemic heart disease: a population-based cohort study. Circ Cardiovasc Qual Outcomes. 2014;7:835–43. http://dx.doi.org/10.1161/CIRCOUTCOMES.114.001076
26. Benchimol EI, Kuenzig ME, Bernstein CN, Nguyen GC, Guttmann A, Jones JL, et al.; Canadian Gastro-Intestinal Epidemiology Consortium. Rural and urban disparities in the care of Canadian patients with inflammatory bowel disease: a population-based study. Clin Epidemiol. 2018;10:1613–26. http://dx.doi.org/10.2147/CLEP.S178056
27. Andréjak C, Lescure FX, Pukenyte E, Douadi Y, Yazdanpanah Y, Laurans G, et al.; Xenopi Group. *Mycobacterium xenopi* pulmonary infections: a multicentric retrospective study of 136 cases in north-east France. Thorax. 2009;64:291–6. http://dx.doi.org/10.1136/thx.2008.096842
28. Medical Section of the American Lung Association. Diagnosis and treatment of disease caused by nontuberculous mycobacteria. Am J Respir Crit Care Med. 1997;156:S1–25.
29. Klemens SP, Cynamon MH. Activities of azithromycin and clarithromycin against nontuberculous mycobacteria in beige mice. Antimicrob Agents Chemother. 1994;38:1455–9. http://dx.doi.org/10.1128/AAC.38.7.1455
30. Roche B, Rozenberg S, Cambau E, Desplaces N, Dion E, Dubourg G, et al. Efficacy of combined clarithromycin and sparfloxacin therapy in a patient with discitis: due to *Mycobacterium xenopi*. Rev Rhum Engl Ed. 1997;64:64–5.
31. Schmitt H, Schnitzler N, Riehl J, Adam G, Sieberth HG, Haase G. Successful treatment of pulmonary *Mycobacterium xenopi* infection in a natural killer cell–deficient patient with clarithromycin, rifabutin, and sparfloxacin. Clin Infect Dis. 1999;29:120–4. http://dx.doi.org/10.1086/520140
32. Subcommittee of the Joint Tuberculosis Committee of the British Thoracic Society. Management of opportunist mycobacterial infections: Joint Tuberculosis Committee Guidelines 1999. Thorax. 2000;55:210–8. http://dx.doi.org/ 10.1136/thorax.55.3.210
33. Wallace RJ Jr, Brown-Elliott BA, McNulty S, Philley JV, Killingley J, Wilson RW, et al. Macrolide/azalide therapy for nodular/bronchiectatic *Mycobacterium avium* complex lung disease. Chest. 2014;146:276–82. http://dx.doi.org/10.1378/chest.13-2538
34. Jeong BH, Jeon K, Park HY, Kim SY, Lee KS, Huh HJ, et al. Intermittent antibiotic therapy for nodular bronchiectatic *Mycobacterium avium* complex lung disease. Am J Respir Crit Care Med. 2015;191:96–103. http://dx.doi.org/10.1164/rccm.201408-1545OC

Address for correspondence: Sarah K. Brode, Toronto Western Hospital, 7E-453, 399 Bathurst St, Toronto, ON M5T 2S8, Canada; email: sarah.brode@uhn.ca

Carbapenem-Resistant *Pseudomonas aeruginosa* at US Emerging Infections Program Sites, 2015

Maroya Spalding Walters, Julian E. Grass, Sandra N. Bulens, Emily B. Hancock, Erin C. Phipps, Daniel Muleta, Jackie Mounsey, Marion A. Kainer, Cathleen Concannon, Ghinwa Dumyati, Chris Bower, Jesse Jacob, P. Maureen Cassidy, Zintars Beldavs, Karissa Culbreath, Walter E. Phillips, Jr., Dwight J. Hardy, Roberto L. Vargas, Margret Oethinger, Uzma Ansari, Richard Stanton, Valerie Albrecht, Alison Laufer Halpin, Maria Karlsson, J. Kamile Rasheed, Alexander Kallen

Pseudomonas aeruginosa is intrinsically resistant to many antimicrobial drugs, making carbapenems crucial in clinical management. During July–October 2015 in the United States, we piloted laboratory-based surveillance for carbapenem-resistant *P. aeruginosa* (CRPA) at sentinel facilities in Georgia, New Mexico, Oregon, and Tennessee, and population-based surveillance in Monroe County, NY. An incident case was the first *P. aeruginosa* isolate resistant to antipseudomonal carbapenems from a patient in a 30-day period from any source except the nares, rectum or perirectal area, or feces. We found 294 incident cases among 274 patients. Cases were most commonly identified from respiratory sites (120/294; 40.8%) and urine (111/294; 37.8%); most (223/280; 79.6%) occurred in patients with healthcare facility inpatient stays in the prior year. Genes encoding carbapenemases were identified in 3 (2.3%) of 129 isolates tested. The burden of CRPA was high at facilities under surveillance, but carbapenemase-producing CRPA were rare.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (M.S. Walters, J.E. Grass, S.N. Bulens, U. Ansari, R. Stanton, V. Albrecht, A.L. Halpin, M. Karlsson, J.K. Rasheed, A. Kallen); New Mexico Emerging Infections Program, Santa Fe, New Mexico, USA (E.B. Hancock, E.C. Phipps); University of New Mexico, Albuquerque, New Mexico, USA (E.B. Hancock, E.C. Phipps, K. Culbreath); Tennessee Department of Public Health, Nashville, Tennessee, USA (D. Muleta, J. Mounsey, M.A. Kainer); University of Rochester, Rochester, New York, USA (C. Concannon, G. Dumyati, D.J. Hardy); Atlanta Veterans Affairs Medical Center, Decatur, Georgia, USA (C. Bower); Atlanta Research and Education Foundation, Decatur (C. Bower); Georgia Emerging Infections Program, Atlanta (C. Bower, J. Jacob); Emory University School of Medicine, Atlanta (J. Jacob); Oregon Health Authority, Portland, Oregon, USA (P.M. Cassidy, Z. Beldavs); TriCore Reference Laboratories, Albuquerque (K. Culbreath); Tristar Centennial Medical Center, Nashville (W.E. Phillips, Jr.); Rochester Regional Health, Rochester (R.L. Vargas); Providence Health and Services, Renton, Washington, USA (M. Oethinger)

DOI: https://doi.org/10.3201/eid2507.181200

Pseudomonas aeruginosa is an opportunistic pathogen that causes an estimated 51,000 healthcare-associated infections (HAI) in the United States annually and was the third most common gram-negative cause of selected HAI reported to the National Healthcare Safety Network (NHSN) during 2011–2014 (*1*,*2*). Infections caused by *P. aeruginosa* are associated with substantial morbidity and mortality rates; a recent study of bloodstream infections showed that patients with a *Pseudomonas* bloodstream infection had a higher mortality rate than patients with infections caused by members of *Enterobacteriaceae* or other non–lactose fermenting gram-negative bacilli (*3*). Infections with *P. aeruginosa* are often challenging to treat because of its intrinsic nonsusceptibility to many commonly used antimicrobial drugs; ≈13% of isolates causing HAI are multidrug resistant (MDR) (*2*). For these reasons, carbapenems have become important antimicrobial drugs for clinical management of serious *P. aeruginosa* infections.

Carbapenem resistance among *Pseudomonas* spp. is a concern; in 2014, 19.1% of *P. aeruginosa* associated with selected HAI and reported to the NHSN were not susceptible to carbapenems (*4*). Although this proportion has remained stable since 2009 (*1*,*5*), some reports have suggested recent increases in the prevalence of carbapenem-resistant *P. aeruginosa* (CRPA) among certain subpopulations, including children (*6*).

Carbapenem resistance in *P. aeruginosa* is due primarily to chromosomal mutations that alter porins, modify efflux pump activity, and derepress intrinsic β-lactamases. However, carbapenemase genes, commonly carried on

mobile genetic elements, have the potential for rapid dissemination. A study of isolates from 14 countries in Europe showed that the prevalence of metallo-β-lactamase (MBL)–producing *P. aeruginosa* increased from 12.3% in 2010 to 30.6% in 2011 (*7*).

In the United States, carbapenemase-producing CRPA (CP-CRPA) was first reported in 2001 in an isolate producing the Verona Integron Mediated (VIM) MBL from a cancer patient in Texas (*8*). CP-CRPA expressing the *Klebsiella pneumoniae* carbapenemase (KPC) (*9*), the active-on-imipenem (IMP) MBL (*10*,*11*), and New Delhi MBL (NDM) have also been identified (*10*,*12*,*13*), and healthcare-associated outbreaks of VIM-producing CRPA have occurred (*14*). Recent data from a convenience sample of CRPA tested through the Antibiotic Resistance Laboratory Network found that 1.9% of isolates produced a carbapenemase (*15*).

To date, the epidemiology of CRPA in the United States has not been systematically evaluated. Through the Centers for Disease Control and Prevention (CDC) Emerging Infections Program, we piloted surveillance for CRPA in 5 US metropolitan areas with the objective of developing a laboratory-based surveillance system to describe the epidemiology of CRPA.

Methods

During July 1–October 31, 2015, we performed sentinel surveillance for CRPA in Georgia (2 laboratories serving 3 acute care hospitals and multiple outpatient clinics), New Mexico (1 commercial laboratory serving the 3 largest healthcare systems in the state, including the regional cystic fibrosis [CF] referral center), Oregon (1 laboratory serving 8 acute care hospitals, multiple long-term care facilities, and >100 outpatient and specialty clinics), and Tennessee (2 laboratories serving 17 inpatient or residential facilities, including short-stay and long-term acute care hospitals and long-term care facilities, and 70 outpatient clinics). We conducted population-based surveillance in Monroe County, NY, which is served by 3 clinical laboratories. Laboratories that serve CF care centers were not recruited in Georgia, Oregon, or Tennessee.

We defined a CRPA case as isolation of *P. aeruginosa* resistant to ≥1 carbapenems with antipseudomonal activity (doripenem, imipenem, or meropenem) from any specimen source except the nares, rectum or perirectal area, or feces, because these excluded sources often represent surveillance rather than clinical cultures. We defined carbapenem resistance as MIC ≥8 μg/mL or zone diameter ≤15 mm using the results from the local clinical laboratory's primary antimicrobial drug testing methodology. Cases were identified through monthly queries of the automated testing instruments of participating laboratories for CRPA isolates; laboratories performing testing using the Kirby-Bauer disk diffusion method were asked to query the Laboratory Information System. To determine the percentage of *P. aeruginosa* resistant to carbapenems, we divided the number of CRPA by the total number of unique *P. aeruginosa* isolates from participating laboratories during the surveillance period.

We classified a patient's first CRPA case in a 30-day period as incident. For incident cases, we completed a case report form documenting patient characteristics, healthcare exposures, and provider-determined diagnoses through review of available medical records. If a patient had CRPA isolated from >1 specimen source at the time of incident culture, we classified their case as a single incident case from multiple specimen sources. We classified cases as healthcare-associated if the incident culture was collected from inpatients after hospital day 3 or from patients with a history of hospitalization, surgery, or residence in a long-term care facility during the previous year; an indwelling device in the previous 7 days; or chronic dialysis at the time of assessment. If none of these risk factors were present, we classified cases as community-associated. Death within 30 days of incident culture collection was determined by searching vital records for death certificates matching patient name, date of birth, and sex.

We calculated a Charlson Comorbidity Index score using underlying conditions abstracted from the medical record (*16*,*17*). We classified cases as associated with urinary tract infection if the urine sample was positive for ≥10^5 CFU/mL *P. aeruginosa* and if signs and symptoms consistent with urinary tract infection (UTI) were documented. We classified cases from lower respiratory tract specimens or with physician-diagnosed pneumonia as associated with pneumonia if all 3 of the following criteria were met: radiologic evidence of pneumonia from the chest image obtained closest to the culture collection and ≤2 calendar days before or after the day of culture collection; documentation of fever, leukocytosis, leukopenia, or, in persons ≥70 years of age, altered mental status; and documentation of ≥2 of the following: changes in character of sputum or increased respiratory secretions; new onset or worsening cough, dyspnea, or tachypnea; rales or bronchial breath sounds; and worsening gas exchange. We defined isolates as MDR using the clinical laboratory antimicrobial susceptibility testing results and a consensus definition (*18*).

Each site was asked to submit to CDC a convenience sample of ≤40 CRPA isolates from incident cases; we excluded isolates from CF patients. At CDC, we tested isolates for antimicrobial susceptibility by reference broth microdilution and screened them for carbapenemase activity by CarbaNP according to Clinical and Laboratory Standards Institute guidelines (*19*). We used the carbapenem inactivation method (CIM) to test isolates that were positive by CarbaNP and screened for the presence of carbapenemase-encoding genes (bla_{KPC}, bla_{NDM}, and bla_{VIM}) by PCR. We performed whole-genome sequencing on all

isolates using HiSeq (Illumina, https://www.illumina.com; 2 × 100-bp paired-end chemistry). We screened sequences for the presence of antimicrobial resistance genes, including carbapenemase genes and plasmid-mediated colistin resistance genes (*mcr-1*, *mcr-1.2*, and *mcr-2*) using ResFinder (*20*) and used the *P. aeruginosa* multilocus sequence type (MLST) database (https://pubmlst.org/paeruginosa) to identify strain types. We performed long-read sequencing on isolates harboring carbapenemase genes using RSII (Pacific Biosciences, https://www.pacb.com) and submitted raw sequencing reads under BioProject ID PRJNA483044.

We analyzed data using SAS version 9.3 (SAS Institute Inc., https://www.sas.com). We calculated crude incidence as the number of cases per 100,000 population, on the basis of the 2015 US Census population for Monroe County. We determined 95% CIs using Poisson regression. We assessed differences in frequency using the χ^2 test or Fisher exact test for small cell sizes; we considered $p<0.05$ in a 2-tailed test statistically significant.

The human subjects advisors in the National Center for Emerging and Zoonotic Infectious Diseases at CDC reviewed this activity. The advisors determined that the activity constituted public health surveillance.

Results

During July–October 2015, we identified 384 (9.1%) CRPA cases among 4,243 *P. aeruginosa* isolates (Table 1). The percentage that were carbapenem resistant ranged from 4.6% (26/560) at the Oregon site to 12.0% (68/566) at the Georgia site. During this period, we determined 294 incident cases from 274 unique patients; 14 (5.1%) patients had 2 incident cases and 3 (1.1%) had 3 incident cases. Among the sentinel sites, New Mexico (n = 85) and Tennessee (n = 79) had the most incident cases. In Monroe County, where we conducted population-based surveillance, we identified 60 incident cases, resulting in an overall crude incidence per 100,000 population of 8.02 (95% CI 6.23–10.33) for the 4-month period.

The 274 unique patients had a median age of 66 years (range <1 year to >89 years), and 114 (41.6%) were female (Table 2). Among the 226 patients with race recorded in the medical record, 181 (80.1%) were white. We completed case report forms for 281 (95.6%) incident cases; of those, 263 (96.0%) patients had a case report form completed for ≥1 incident case. Patients had a median Charlson Comorbidity Index score of 2 (range 0–14); the most frequently reported underlying conditions were chronic obstructive pulmonary disease (n = 95; 36.3%) and diabetes (n = 90; 34.4%). Six (2.3%) patients had no underlying conditions.

Cultures were collected in an acute care setting for 158 (53.9%) incident cases; among these, 132 (83.5%) were from a short-stay acute-care hospital and 26 (16.5%) were from a long-term acute-care hospital. Cultures were collected outside of acute-care settings for 135 (46.1%) incident cases, of which most were collected in outpatient settings (n = 83; 61.5%), followed by emergency departments (n = 33; 24.4%) and long-term care facilities (n = 19; 14.1%). Normally sterile sites were the source for 21 (7.1%) incident cases; among 273 cases associated only with nonsterile sites, the most frequent sources were respiratory specimens (n = 120; 44.0%) and urine (n = 111; 40.7%) (Table 3). The provider-diagnosed infections most frequently associated with the incident culture were UTI (n = 85; 31.7%) and pneumonia (n = 78; 29.1%). Among 76 patients with provider-diagnosed UTI, 17 (22.4%) met our criteria for UTI, and 6 (8.1%) of 74 patients with provider-diagnosed pneumonia met our pneumonia criteria. No provider-diagnosed infection was recorded for 35 (13.1%) cases.

Among 280 incident cases with patient healthcare exposure history available, 257 (91.8%) were healthcare associated and 23 (8.2%) were community associated (Table 3). The most common healthcare risk factors were acute-care hospitalization in the year before collection of the incident culture, reported for 83.0% (205/247) of cases, and presence of an indwelling device in the 7 days before culture collection, reported for 194 (69.3%) cases. Of 155 cases that occurred in hospitalized patients and had information available, 101 (65.2%) had received intravenous or oral antimicrobial drugs in the 14 days before culture collection; of those, 83 (82.2%) had received ≥1 β-lactam antimicrobial drug.

Table 1. Carbapenem-resistant *Pseudomonas aeruginosa* isolates identified through Emerging Infections Program sites, United States, July–October 2015*

Site	No. carbapenem-resistant *P. aeruginosa* isolates	Total no. *P. aeruginosa* isolates	% Carbapenem resistant	No. incident cases†
Georgia	68	566	12.0	49
Tennessee	91	890	10.2	79
New Mexico	116	1,295	9.0	85
New York‡	83	932	8.9	60
Oregon	26	560	4.6	21
Total	384	4,243	9.1	294

*Isolates are *P. aeruginosa* isolated from any specimen source except nares, rectum, perirectal area, or feces; carbapenem-resistant *P. aeruginosa* are isolates resistant to ≥1 carbapenem with anti-pseudomonal activity (doripenem, imipenem, or meropenem).
†Incident case defined as first carbapenem-resistant *P. aeruginosa* case in a patient in a 30-day period.
‡Cases in New York were identified through population-based surveillance; all other sites performed sentinel surveillance.

Hospitalization at the time of culture collection or in the following 30 days occurred in 61.8% (173/280) of incident cases. Of 121 cases in hospitalized patients who were not in the ICU before their positive culture, 31 (25.6%) were admitted to the ICU on the day of culture collection or in the following 7 days. The patient died within 30 days of incident culture collection in 34 (12.1%) of 281 cases with information available. Univariate analysis did not show a difference in death rate among patients with incident cases from sterile sites (2/16, 12.5%) compared with those from nonsterile sites (31/258, 12.0%; p = 1.0).

Antimicrobial susceptibility testing at the local clinical laboratory showed that most isolates remained susceptible to ≥1 aminoglycoside (246/276; 89.1%); of note, nearly two thirds (181/275; 65.8%) were susceptible to ceftazidime or cefepime (Table 4). Isolates from 181 (67.5%) of 268 cases were MDR. For all antimicrobial drugs, the percentage susceptible was higher among isolates from community-associated cases than among isolates from healthcare-associated cases, even though the difference was not statistically significant for amikacin, ceftazidime, levofloxacin, or tobramycin (Table 4). Similarly, 167 (71.4%) of 234 healthcare-associated cases were MDR, compared with 4 (20.0%) of 20 community-associated cases (p<0.01).

Table 2. Carbapenem-resistant *Pseudomonas aeruginosa* patient demographics and clinical characteristics, United States, July–October 2015*

Characteristic	Value
Patient demographics	N = 274
Sex	
F	114 (41.6)
M	160 (58.4)
Median age, y (range)	66 (<1–98)
Age group, y	
0–18	9 (3.3)
19–49	57 (20.8)
50–64	67 (24.5)
65–79	91 (33.2)
≥80	50 (18.2)
White ethnicity	181/226 (80.1)
Underlying clinical conditions†	N = 262
None	6 (2.3)
Chronic obstructive pulmonary disease	95 (36.3)
Diabetes	90 (34.4)
Chronic renal insufficiency	59 (22.5)
Decubitus ulcer	55 (21.0)
Congestive heart failure	51 (19.5)
Neurologic problems	49 (18.7)
Urinary tract problems/abnormalities	49 (18.7)
Obesity	44 (16.8)
Stroke	40 (15.3)
Dementia	37 (14.1)
Hemiplegia/paraplegia	35 (13.4)
Chronic skin breakdown	29 (11.1)
Prolonged surgical wound	15 (51.7)
Burn	1 (3.4)
Other type of chronic skin breakdown	14 (48.3)
Peripheral vascular disease	27 (10.3)
Solid tumor (nonmetastatic)	27 (10.3)
Other underlying conditions‡	125 (47.7)
Median CCI score (range)	2 (0–14)

*Values are no. (%) patients except as indicated. For patients with >1 incident case, demographics and clinical characteristics reflect those reported at the time of first incident case. CCI, Charlson Comorbidity Index.
†Total number of patients with a completed case report form and information available for underlying conditions.
‡Underlying conditions reported for <10% of patients are included in the other underlying conditions category: current smoker (n = 23), myocardial infarct (n = 18), metastatic solid tumor (n = 13), alcohol abuse (n = 10), chronic liver disease (n = 10), transplant recipient (n = 9), connective tissue disease (n = 8), chronic bronchiectasis (n = 6), liver failure (n = 6), hematologic malignancy (n = 5), spina bifida (n = 5), cystic fibrosis (n = 4), inflammatory bowel disease/Crohn's disease (n = 3), peptic ulcer disease (n = 3), HIV (n = 1), and intravenous drug user (n = 1).

Of the 129 isolates from incident cases in Georgia (n = 23), New Mexico (n = 26), New York (n = 25), Oregon (n = 20), and Tennessee (n = 35) submitted to CDC for further characterization, 106 (82.2%) were carbapenem-resistant by reference broth microdilution antimicrobial susceptibility testing. Five (3.9%) isolates showed carbapenemase activity by CarbaNP; among these, $bla_{\text{VIM-2}}$ and $bla_{\text{IMP-18}}$ were each identified in 1 isolate. One other isolate had a putative carbapenemase gene that encodes a protein with 90% amino acid identity to Hamburg MBL (HMB) 1, recently identified in a *P. aeruginosa* isolate in Germany (*21*); we called this homologue HMB-2 (Table 5). Of note, $bla_{\text{VIM-2}}$ and $bla_{\text{HMB-2}}$ were located on the chromosome; $bla_{\text{VIM-2}}$ was part of a Class 1 integron with 5 resistance gene cassettes, and $bla_{\text{HMB-2}}$ was located on a unique transposon similar to Tn3. The $bla_{\text{IMP-18}}$ gene was localized to a Class 1 integron on a plasmid with an unknown plasmid replicon type and lacking sequence similarity to plasmid sequences in publicly available databases. The patients with CRPA expressing VIM-2 or IMP-18 had documented prior healthcare exposure in the United States and no known international travel; the patient with HMB-2 was a traveler from Mexico. Two isolates that exhibited weak carbapenemase activity by CarbaNP but were negative for carbapenemase production by CIM did not harbor genes encoding any known or putative carbapenemases based on PCR and whole-genome sequencing analyses.

One of the 129 isolates was determined to be resistant to colistin by reference broth microdilution, but we identified no *mcr* genes; the isolate was susceptible to piperacillin/tazobactam, ceftazidime, cefepime, and aztreonam. Thirty-five isolates that were resistant to ceftazidime or harbored a carbapenemase gene were tested for susceptibility to ceftazidime/avibactam and ceftolozane/tazobactam; 24 (68.6%) were susceptible to ceftazidime/avibactam and 28 (80.0%) to ceftolozane/tazobactam (*19*). Among 128 sequenced isolates, 16 isolates did not match a sequence type (ST) in the database, and 45 unique STs were identified. The most common were ST235 (n = 13; 11.8%); ST298 (n = 10; 9.1%); and ST27, ST111, and ST395 (n = 6 for each; 5.5%). ST235, which has been associated with plasmid-mediated carbapenemases and rapid expansion, was the only ST identified in ≥1 isolate from each surveillance site.

All ST235 isolates were healthcare associated, and 11/13 (84.6%) were MDR.

Discussion

During this 4-month surveillance pilot, we identified 384 cases of CRPA across 9 clinical labs performing surveillance; 294 were incident cases. Although 9% of *P. aeruginosa* isolates from these sites were carbapenem-resistant, only 2% of CRPA isolates tested had an identified carbapenemase, suggesting that these resistance mechanisms are rare in *P. aeruginosa* at the surveillance sites. Nearly one third of CRPA were not MDR, and more than half appeared susceptible to ceftazidime or cefepime, according to current Clinical and Laboratory Standards Institute breakpoints. Although nearly half of cases were from specimens collected outside of acute-care settings (outpatient clinics, emergency departments, and long-term care facilities), 92% of patients had healthcare exposures before their positive culture, and 98% had ≥1 underlying condition, indicating that CRPA remains primarily a healthcare-associated pathogen affecting persons with serious medical issues.

CRPA is an opportunistic pathogen and was identified primarily in persons with underlying conditions, such as chronic obstructive pulmonary disease and diabetes. Although CRPA is frequently associated with CF, we identified a low number of CF patients, likely because the surveillance pilot was not optimally designed to capture cases from CF patients at sentinel sites. Community-associated cases were rare, comprising only 8% of identified CRPA cases, and some of these community-associated cases might represent misclassification due to limited documentation of previous medical encounters.

The overall crude mortality rate was substantial at 12% but lower than in a single-center study of CRPA bloodstream

Table 3. Culture source, provider-reported infection type, and prior healthcare risk factors in incident carbapenem-resistant *Pseudomonas aeruginosa* cases, United States, July–October 2015

Characteristic	No. (%) cases
Culture source	N = 294
Any sterile site*	21 (7.1)
Blood	10 (47.6)
Internal abscess	7 (33.3)
Pericardial fluid	1 (4.8)
Peritoneal fluid	1 (4.8)
Joint/synovial fluid	1 (4.8)
Other normally sterile sites	1 (4.8)
Nonsterile site†	273 (92.9)
Respiratory‡	120 (44.0)
Urine	111 (40.7)
Wound	35 (12.8)
Other nonsterile sites	9 (3.3)
Infection type§	N = 268
Urinary tract infection	85 (31.7)
Pneumonia	78 (29.1)
Septic shock	20 (7.5)
Bacteremia	20 (7.5)
Internal abscess	11 (4.5)
Other infection types¶	57 (21.3)
>1 Infection type	38 (14.2)
No infection	35 (13.1)
Risk factors	
Any healthcare exposure, n = 280#	257 (91.8)
Indwelling device placed <7 d before culture, n = 280	194 (69.3)
Surgery in prior year, n = 280	111 (39.6)
Long-term care facility resident in prior year, n = 280	84 (30.0)
Culture collected after hospital day 3, n = 280	64 (22.9)
Long-term acute care hospitalization in prior year, n = 280	40 (14.3)
Current chronic dialysis, n = 280	29 (10.4)
Hospitalization in prior year, n = 247	205 (83.0)
Antimicrobial drug ≤14 d before culture collection, n = 155**	101 (65.2)

*Includes 5 cases with CRPA from both sterile and nonsterile sites collected at time of incident culture (1 blood and tracheal aspirate; 1 blood and catheter tip; 1 blood and urine; 1 internal abscess and sputum; 1 pericardial fluid and wound).
†Total number of nonsterile sites is >273 because 2 case-patients had CRPA in multiple nonsterile sites at time of incident culture (1 sputum and tracheal aspirate; 1 urine and tracheal aspirate).
‡Includes sputum (n = 70), tracheal aspirate (n = 38), and bronchoalveolar lavage (n = 12).
§Total number of incident cases with completed case report form, excluding 13 cases with unknown infections.
¶Other infection types include the following: cellulitis (n = 9), decubitus/pressure ulcer (n = 7), chronic ulcer/wound (n = 6), bronchitis (n = 6), osteomyelitis (n = 6), surgical incision infection (n = 4), infection, not specified (n = 3), upper respiratory tract infection (n = 3), pyelonephritis (n = 2), cystic fibrosis exacerbation (n = 2), surgical site infection (n = 2), catheter site infection (n = 1), peritonitis (n = 1), skin abscess (n = 1), empyema (n = 1), septic arthritis (n = 1), wound (n = 1), other infection (n = 1).
#Total number of incident cases with completed case report form, excluding 1 case with unknown healthcare risk factors.
**Total number of incident cases with completed case report form and their culture collected in a short-stay or long-term acute care hospital.

Table 4. Antimicrobial susceptibility of carbapenem-resistant *Pseudomonas aeruginosa* isolates from incident cases based on testing at local clinical laboratory, by epidemiologic classification, United States, July–October 2015*

Antimicrobial agent	No. susceptible/total no. tested (%)	Epidemiologic classification, no. susceptible/ total no. tested (%)*		p value
		Healthcare-associated	Community-associated	
Cephalosporins				
Any cephalosporin	181/275 (65.8)	153/241 (63.5)	18/21 (85.7)	0.04
Ceftazidime	101/151 (66.9)	89/136 (65.4)	6/6 (100)	0.18†
Cefepime	146/273 (53.5)	120/239 (50.2)	17/21 (81.0)	0.01
Aminoglycosides				
Any aminoglycoside	246/276 (89.1)	212/241 (88.0)	21/21 (100)	0.14†
Amikacin	203/237 (85.7)	179/213 (84.0)	17/17 (100)	0.08†
Gentamicin	162/268 (60.5)	134/234 (57.3)	17/20 (85.0)	0.02
Tobramycin	118/180 (65.6)	104/164 (63.4)	7/7 (100)	0.10†
Fluoroquinolones				
Any fluoroquinolone	96/274 (35.0)	74/240 (30.8)	16/20 (80.0)	<0.01
Ciprofloxacin	93/266 (35.0)	71/233 (30.5)	16/19 (84.2)	<0.01
Levofloxacin	29/142 (20.4)	23/128 (18.0)	3/5 (60.0)	0.05†
Other antimicrobials				
Aztreonam	63/168 (37.5)	46/146 (31.5)	12/15 (80.0)	<0.01
Piperacillin/tazobactam	134/266 (50.4)	106/231 (45.9)	19/21 (90.5)	<0.01
Resistance	No. multidrug-resistant/total no. tested (%)			
Multidrug-resistant isolates‡	181/268 (67.5)	167/234 (71.4)	4/20 (20.0)	<0.01

*Antimicrobial susceptibilities by epidemiologic classification are restricted to the 281 incident cases with a completed case report form. Healthcare-associated is defined as a case with ≥1 healthcare risk factor reported; community-associated is defined as a case with no healthcare risk factors reported.
†By Fisher exact test.
‡Defined as an isolate resistant to 1 carbapenem (doripenem, imipenem, meropenem) and nonsusceptible to ≥1 antimicrobial drug in ≥2 of the following classes: cephalosporin (ceftazidime, cefepime), aminoglycoside (amikacin, gentamicin, tobramycin), fluoroquinolone (ciprofloxacin, levofloxacin), β-lactamase inhibitor combination (piperacillin-tazobactam), and monobactam (aztreonam).

infections, in which 30-day mortality rate was 30% (*22*). The difference could be due to the inclusion of nonsterile sites in our surveillance case definition, which might not represent true infections; however, mortality rates did not differ between patients with isolates from sterile sites and patients with only non–sterile site isolates. Although the crude mortality rate was similar to the rate observed in population-based surveillance for CRE (9.0%) and lower than for carbapenem-resistant *Acinetobacter baumannii* (17.9%) (*23*,*24*), these comparisons do not account for differences in culture sources and could reflect biases at the sentinel laboratories included in this pilot. Population-based surveillance in the same catchment areas is needed to further explore differences in mortality rates associated with carbapenem-resistant gram-negative rods.

A known or putative carbapenemase was only identified in 2.3% of isolates tested. The IMP-18– and VIM-2–producing isolates were both from the New Mexico sentinel site, where they accounted for 7.7% of isolates tested. *P. aeruginosa* with IMP-18 was previously identified in a motorcycle accident victim from the southwestern United States (*10*); the only additional reports of *P. aeruginosa* with IMP-18 have been 1 case in a patient in Mexico (*25*) and in multiple clinical isolates from hospitals in Puerto Rico (*11*). *P. aeruginosa* expressing VIM-2 has been associated with sporadic cases in the United States and with outbreaks among acutely ill patients at healthcare facilities in Illinois (*14*) and Florida (CDC, unpub. data). The patients with *P. aeruginosa* expressing VIM-2 or IMP-18 in this surveillance both had documented prior healthcare exposure in the United States but not elsewhere, raising concern for undetected transmission of CP-CRPA in US healthcare facilities. $bla_{\text{HMB-2}}$ is identical to a gene found in a *P. aeruginosa* isolate from a military medical facility in

Table 5. Characteristics of carbapenemase-producing isolates of carbapenem-resistant *Pseudomonas aeruginosa* from incident cases, United States, July–October 2015

Isolate no.	Site	Carbapenemase	Carbapenemase gene location	ST	Antimicrobial resistance pattern*
1	New Mexico	IMP-18	Plasmid	ST179	AMK, CAZ, CAZ/AVI, CIP, DOR, FEP, GEN, IMI, LEV, MER, CEF/TAZ, TOB
2	New Mexico	VIM-2	Chromosomal	ST308	CAZ,† CAZ/AVI, CIP, DOR, GEN, IMI, LEV, MER, CEF/TAZ, TOB
3	Tennessee	HMB-2	Chromosomal	ST235	AMK, CAZ, CAZ/AVI, CIP, DOR, FEP, GEN, LEV, MER, PIP/TAZ, CEF/TAZ, TOB

*Resistance pattern based on reference broth microdilution testing using 2019 Clinical and Laboratory Standards Institute interpretative criteria. AMK, amikacin; CAZ, ceftazidime; CAZ/AVI, ceftazidime/avibactam; CEF/TAZ, ceftolozane-tazobactam; CIP, ciprofloxacin; DOR, doripenem; FEP, cefepime; GEN, gentamicin; HMB, Hamburg metallo-β-lactamase; IMI, imipenem; IMP, active-on-imipenem; LEV, levofloxacin; MER, meropenem; PIP/TAZ, piperacillin/tazobactam; ST, sequence type; TOB, tobramycin; VIM, Verona integron mediated.
†Intermediate antimicrobial susceptibility.

San Antonio (GenBank accession no. KYO71936.1); identification of this novel carbapenemase gene highlights the importance of combining phenotypic testing for carbapenemase activity with whole-genome sequencing analysis to identify new resistance mechanisms.

Although we found drug susceptibilities similar to those reported in previous studies of CRPA in the United States (*26*), it is nevertheless notable that only 68% of CRPA identified through this surveillance were MDR, including 71% of healthcare-associated cases. Moreover, 89% of isolates remained susceptible to ≥1 aminoglycoside, whereas two thirds were susceptible to ceftazidime or cefepime. This high proportion of non-MDR isolates, in combination with the low proportion of carbapenemase producers, suggests that most carbapenem resistance is conferred by other mechanisms, such as mutation of the porin D gene *oprD*; changes in efflux pump expression; or inducible or mutational changes in expression of intrinsic, chromosomally encoded β-lactamases, such as AmpC.

This evaluation is subject to several limitations. First, our data are from a 4-month pilot study of 5 sites, 4 of which conducted sentinel surveillance. Although sentinel sites were primarily laboratories with diverse catchments, some settings, such as long-term care and outpatient clinics, might be underrepresented. At 3 sites, laboratories serving CF centers were not enrolled, which may have inadvertently skewed our catchment away from large academic medical centers that often care for the most acutely ill patients. For these reasons, the results cannot be generalized to the metropolitan areas in which the sentinel laboratories were located. Second, some of the cases identified probably represent colonization and not true infection. However, pneumonia, skin and soft tissue infections, and UTIs are common syndromes caused by CRPA, and therefore it was important that we include isolates from sources that could represent these infections. To provide context about the clinical relevance of isolates from these sites, we abstracted information from the medical records to determine whether infection was diagnosed by a physician; we also applied surveillance definitions for pneumonia and UTIs. However, both of these methods are imperfect for differentiating true infection from colonization. UTI and pneumonia definitions were designed to be specific but might have limited sensitivity. Finally, we were able to collect and test only 44% of isolates from incident cases. Because carbapenemase-producing *P. aeruginosa* appear to be rare in the United States, the small sample limited our ability to precisely characterize carbapenemase-production among CRPA.

This surveillance pilot provides a snapshot of the epidemiology of CRPA at 5 US sites and shows a large burden of CRPA that occurs primarily among persons with underlying conditions and recent exposure to healthcare. The CRPA we identified displayed varied antimicrobial susceptibilities, ranging from extensively drug-resistant to resistant only to carbapenems, and whole-genome sequence analysis indicates that the CRPA population at these sites is diverse. Carbapenemase production was rare in the isolates tested, and because of the high burden of CRPA, a more specific phenotypic definition might be needed for more targeted identification of carbapenemase-producing isolates. Based on findings from this pilot study, the Emerging Infections Program began population-based surveillance at 8 US sites in August 2016 to better describe the epidemiology of CRPA and monitor changes in the incidence of CRPA and carbapenemase-producing strains.

Acknowledgments

We thank Wendy Bamberg, Sarah Janelle, Lucy Wilson, Elisabeth Vaeth, Ruth Lynfield, and Paula Vagnone for their scientific input in the development of this surveillance pilot; Shirley Zhang for assistance with database development; and the participating clinical laboratories for their ongoing support of Emerging Infections Program surveillance.

The Healthcare-Associated Infections Community Interface (HAIC) MuGSI program is supported through CDC cooperative agreement CDC-RFA-CK17-1701.

About the Author

Dr. Walters is an epidemiologist in the Division of Healthcare Quality Promotion, National Center for Emerging and Zoonotic Infectious Diseases, CDC. Her interests include surveillance and prevention of antimicrobial drug–resistant bacteria, especially carbapenem-resistant gram-negative bacilli.

References

1. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol. 2016;37:1288–301. http://dx.doi.org/10.1017/ice.2016.174
2. US Centers for Disease Control and Prevention. Antibiotic resistance threats in the United States, 2013. 2013 [cited 2019 Apr 19]. https://www.cdc.gov/drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf
3. Thaden JT, Park LP, Maskarinec SA, Ruffin F, Fowler VG Jr, van Duin D. Results from a 13-year prospective cohort study show increased mortality associated with bloodstream infections caused by *Pseudomonas aeruginosa* compared to other bacteria. Antimicrob Agents Chemother. 2017;61:e02671–16. http://dx.doi.org/10.1128/AAC.02671-16
4. US Centers for Disease Control and Prevention. Antibiotic resistance patient safety atlas [cited 2017 Jun 22]. https://gis.cdc.gov/grasp/PSA/MapView.html
5. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to

the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009–2010. Infect Control Hosp Epidemiol. 2013;34:1–14. http://dx.doi.org/10.1086/668770
6. Logan LK, Gandra S, Mandal S, Klein EY, Levinson J, Weinstein RA, et al. Multidrug- and carbapenem-resistant *Pseudomonas aeruginosa* in children, United States, 1999–2012. J Pediatric Infect Dis Soc. 2017;6:352–9. https://doi.org/10.1093/jpids/piw064
7. Castanheira M, Deshpande LM, Costello A, Davies TA, Jones RN. Epidemiology and carbapenem resistance mechanisms of carbapenem-non-susceptible *Pseudomonas aeruginosa* collected during 2009–11 in 14 European and Mediterranean countries. J Antimicrob Chemother. 2014;69:1804–14. http://dx.doi.org/10.1093/jac/dku048
8. Toleman MA, Rolston K, Jones RN, Walsh TR. blaVIM-7, an evolutionarily distinct metallo-β-lactamase gene in a *Pseudomonas aeruginosa* isolate from the United States. Antimicrob Agents Chemother. 2004;48:329–32. http://dx.doi.org/10.1128/AAC.48.1.329-332.2004
9. Poirel L, Nordmann P, Lagrutta E, Cleary T, Munoz-Price LS. Emergence of KPC-producing *Pseudomonas aeruginosa* in the United States. Antimicrob Agents Chemother. 2010;54:3072. http://dx.doi.org/10.1128/AAC.00513-10
10. Hanson ND, Hossain A, Buck L, Moland ES, Thomson KS. First occurrence of a *Pseudomonas aeruginosa* isolate in the United States producing an IMP metallo-β-lactamase, IMP-18. Antimicrob Agents Chemother. 2006;50:2272–3. http://dx.doi.org/10.1128/AAC.01440-05
11. Wolter DJ, Khalaf N, Robledo IE, Vázquez GJ, Santé MI, Aquino EE, et al. Surveillance of carbapenem-resistant *Pseudomonas aeruginosa* isolates from Puerto Rican medical center hospitals: dissemination of KPC and IMP-18 β-lactamases. Antimicrob Agents Chemother. 2009;53:1660–4. http://dx.doi.org/10.1128/AAC.01172-08
12. Delaware Health and Social Services, Division of Public Health. Health alert: first confirmed US case of NDM-producing carbapenem-resistant *Pseudomonas aeruginosa*. May 5, 2014 [cited 2017 Jun 22]. http://dhss.delaware.gov/dph/php/alerts/dhan324.html.
13. Lonsway DR, Travis TC, Kongphet-Tran T, Anderson KF, Limbago BM, Rasheed JK. Evaluation of phenotypic screening methods to detect metallo-β-lactamase production in *Pseudomonas aeruginosa*. Presented at: American Society for Microbiology 115th General Meeting; May 30–June 2, 2015; New Orleans, LA, USA.
14. Lolans K, Queenan AM, Bush K, Sahud A, Quinn JP. First nosocomial outbreak of *Pseudomonas aeruginosa* producing an integron-borne metallo-β-lactamase (VIM-2) in the United States. Antimicrob Agents Chemother. 2005;49:3538–40. http://dx.doi.org/10.1128/AAC.49.8.3538-3540.2005
15. Woodworth KR, Walters MS, Weiner LM, Edwards J, Brown AC, Huang JY, et al. Vital signs: containment of novel multidrug-resistant organisms and resistance mechanisms—United States, 2006–2017. MMWR Morb Mortal Wkly Rep. 2018;67:396–401. http://dx.doi.org/10.15585/mmwr.mm6713e1
16. McGregor JC, Kim PW, Perencevich EN, Bradham DD, Furuno JP, Kaye KS, et al. Utility of the Chronic Disease Score and Charlson Comorbidity Index as comorbidity measures for use in epidemiologic studies of antibiotic-resistant organisms. Am J Epidemiol. 2005;161:483–93. http://dx.doi.org/10.1093/aje/kwi068
17. McGregor JC, Perencevich EN, Furuno JP, Langenberg P, Flannery K, Zhu J, et al. Comorbidity risk-adjustment measures were developed and validated for studies of antibiotic-resistant infections. J Clin Epidemiol. 2006;59:1266–73. http://dx.doi.org/10.1016/j.jclinepi.2006.01.016
18. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18:268–81. http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x
19. Clinical Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement (M100–S29). Wayne (PA): The Institute; 2019.
20. Zankari E. Comparison of the web tools ARG-ANNOT and ResFinder for detection of resistance genes in bacteria. Antimicrob Agents Chemother. 2014;58:4986. http://dx.doi.org/10.1128/AAC.02620–14
21. Pfennigwerth N, Lange F, Belmar Campos C, Hentschke M, Gatermann SG, Kaase M. Genetic and biochemical characterization of HMB-1, a novel subclass B1 metallo-β-lactamase found in a *Pseudomonas aeruginosa* clinical isolate. J Antimicrob Chemother. 2017;72:1068–73.
22. Buehrle DJ, Shields RK, Clarke LG, Potoski BA, Clancy CJ, Nguyen MH. Carbapenem-resistant *Pseudomonas aeruginosa* bacteremia: risk factors for mortality and microbiologic treatment failure. Antimicrob Agents Chemother. 2017;61:e01243–16. http://dx.doi.org/10.1128/AAC.021643–16
23. Guh AY, Bulens SN, Mu Y, Jacob JT, Reno J, Scott J, et al. Epidemiology of carbapenem-resistant enterobacteriaceae in 7 US communities, 2012–2013. JAMA. 2015;314:1479–87. http://dx.doi.org/10.1001/jama.2015.12480
24. Bulens SN, Yi SH, Walters MS, Jacob JT, Bower C, Reno J, et al. Carbapenem-nonsusceptible *Acinetobacter baumannii*, 8 US metropolitan areas, 2012–2015. Emerg Infect Dis. 2018;24:727–34. http://dx.doi.org/10.3201/eid2404.171461
25. Garza-Ramos U, Tinoco P, Silva-Sanchez J, Morfin-Otero R, Rodriguez-Noriega E, Leon-Garnica G, et al. Metallo-β-lactamase IMP-18 is located in a class 1 integron (In96) in a clinical isolate of *Pseudomonas aeruginosa* from Mexico. Int J Antimicrob Agents. 2008;31:78–80. http://dx.doi.org/10.1016/j.ijantimicag.2007.08.003
26. Buehrle DJ, Shields RK, Chen L, Hao B, Press EG, Alkrouk A, et al. Evaluation of the in vitro activity of ceftazidime-avibactam and ceftolozane-tazobactam against meropenem-resistant *Pseudomonas aeruginosa* isolates. Antimicrob Agents Chemother. 2016;60:3227–31. http://dx.doi.org/10.1128/AAC.02969–15

Address for correspondence: Maroya Spalding Walters, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop H16-3, Atlanta, GA 30329-4027, USA; email: MSWalters@cdc.gov

Added Value of Comprehensive Program to Provide Universal Access to Care for Sputum Smear–Negative Drug-Resistant Tuberculosis, China

Fei Huang, Susan van den Hof, Yan Qu, You Li, Hui Zhang, Lixia Wang, Miaomiao Sun, Wei Lu, Shuangyi Hou, Tianhua Zhang, Shitong Huan, Daniel P. Chin, Frank Cobelens

The increase in drug-resistant tuberculosis in China calls for scaling up rapid diagnosis. We evaluated introduction of rapid resistance testing by line-probe assay for all patients with a diagnosis of pulmonary tuberculosis in 2 prefectures in middle and eastern China. We analyzed sputum samples for smear-positive patients and cultures for smear-negative patients. We used a before–after comparison of baseline and intervention periods (12 months each) and analyzed data for 5,222 baseline period patients and 4,364 intervention period patients. The number of patients with rifampin resistance increased from 30 in the baseline period to 97 in the intervention period for smear-positive patients and from 0 to 13 for smear-negative patients, reflecting a low proportion of positive cultures (410/2,844, 14.4%). Expanding rapid testing for drug resistance for smear-positive patients resulted in a 3-fold increase in patients with diagnoses of rifampin-resistant tuberculosis. However, testing smear-negative patients had limited added value because of a low culture-positive rate.

Drug-resistant tuberculosis (TB) poses a major threat to TB control and elimination (*1*). China, where an estimated 73,000 patients showed development of rifampin-resistant TB (which requires longer and more toxic second-line treatment) during 2017, contains 13% of new cases of rifampin-resistant TB worldwide (*2*). However, of the 778,390 TB patients reported in 2017, only 14% were tested for drug resistance. Only 13,069 patients were reported to have rifampin-resistant TB, leaving >80% of cases undetected.

Recognizing the threat of rifampin-resistant TB, the Chinese Ministry of Health and the Bill & Melinda Gates Foundation have collaborated since 2009 to develop an improved TB control program to expand access to diagnosis, quality treatment, and affordable treatment for rifampin-resistant TB (*3*). In the first phase of the program during 2009–2011, pilot studies were conducted in 4 cities. In each city, 1 hospital was designated for diagnosis and treatment of rifampin-resistant TB and equipped with the Genechip line-probe assay (LPA; CapitaBio, http://www.capitalbiotech.com) for rapid molecular testing for isoniazid and rifampin resistance for all patients given a diagnosis of smear-positive pulmonary TB, rather than only those for whom rifampin-resistant TB was suspected (presumptive rifampin-resistant TB). In addition, collaborative mechanisms between the hospital, the local Center for Disease Control (CDC), and community health centers were set up to avoid loss of patients, specimens, and information as patients moved among these facilities. These pilot studies showed a 10-fold increased number of diagnoses of rifampin-resistant TB, a decrease in time from resistance testing to initiation of second-line treatment (by 90%), and an increased retention in treatment by 6 months, from 8% to 80% (*3*).

The Genechip LPA and the World Health Organization–endorsed GenoType MTBDRplus LPA (Bruker-Hain Lifesciences, https://www.hain-lifescience.de) were approved only for smear-positive sputum samples and culture isolates at the time of this study. A systematic review (3,451 samples in 4 datasets) of LPA performance for detecting TB

Author affiliations: Chinese Center for Disease Control and Prevention, Beijing, China (F. Huang, Y. Qu, Y. Li, H. Zhang, L. Wang); The Royal Netherlands Tuberculosis Foundation, The Hague, the Netherlands (S. van den Hof); Academic Medical Center, Amsterdam, the Netherlands (S. van den Hof, F. Cobelens); PATH, Beijing (M. Sun); Jiangsu Provincial Center for Disease Control and Prevention, Jiangsu, China (W. Lu); Hubei Provincial Center for Disease Control and Prevention, Hubei, China (S. Hou); Shaanxi Provincial Institute for Tuberculosis Control and Prevention, Shaanxi, China (T. Zhang); Bill & Melinda Gates Foundation, Beijing (S. Huan); Bill & Melinda Gates Foundation, Seattle, Washington, USA (D.P. Chin)

DOI: https://doi.org/10.3201/eid2507.181417

showed a pooled sensitivity of 94% on smear-positive samples but only 44% on smear-negative samples (*4*). Because sputum cultures were not routinely performed in China, rapid resistance testing in the first phase of the program was limited to smear-positive TB patients.

In China, most (68% in 2017) reported patients with pulmonary TB are smear negative and given treatment without bacteriological confirmation and drug resistance testing. Therefore, China introduced a policy to scale up mycobacterial culture and rapid resistance testing. Its National TB Control Plan for 2016–2020, issued in February 2017, set targets of bacteriological confirmation for ≥50% of all reported patients with pulmonary TB and drug resistance screening for ≥95% of all patients with pulmonary TB at high risk for rifampin-resistant TB (*5*). In 2017, only 32% of TB cases in China were bacteriologically confirmed, 14% of pulmonary TB cases were tested for drug resistance, and 18% of estimated rifampin-resistant TB cases were diagnosed. The second phase of the program during 2012–2015 piloted a policy of adding sputum culture and LPA-based resistance testing of culture isolates to the diagnostic algorithm for smear-negative patients with pulmonary TB.

An alternative to culture and LPA testing might have been Xpert MTB/RIF (http://www.cepheid.com), an automated within-cartridge molecular assay, which tests for *Mycobacterium tuberculosis* and rifampin resistance and has been recommended by the World Health Organization as the primary diagnostic test for pulmonary TB where it can be afforded (*6*,*7*). Xpert can be used directly for smear-positive and smear-negative samples, and showed a pooled sensitivity similar to that for solid media culture (89%) when used as an initial test, but a lower sensitivity (67%) when used as an add-on test for smear-negative samples (*6*,*8*). Moreover, the sensitivity and specificity of Xpert MTB/RIF for detecting rifampin resistance are high and similar to that for LPAs (*9*), although it does not detect isoniazid resistance (*10*). However, because Xpert MTB/RIF has not yet been approved in China for case detection, it was not included as an alternative test in the study. Also, because the price for end-users is high (*11*), its scale-up as a first-line test in the TB and rifampin-resistant TB algorithm was considered less affordable than scaling up culture and LPAs.

In this study, we established the added value of expanding the diagnostic algorithm for diagnosis and treating bacteriologically confirmed pulmonary TB and rifampin-resistant TB. Specifically, we quantified the additional diagnostic yield, the number of case-patients needed to test to find 1 case of rifampin-resistant TB, the time to initiation of second-line treatment, and the number of patients lost in the diagnosis and treatment cascade for the culture-based algorithm.

Methods

Study Design and Population

The pilot studies combined innovative methods and tools with proven effectiveness, as evaluated in the first phase of the program, with health sector changes. These changes are being implemented by the Chinese National Health and Family Planning Commission. In addition to sputum culture and LPA-based resistance testing of culture isolates for smear-negative pulmonary TB, this second program phase introduced several other components. To reduce financial barriers to access to treatment, efforts were made to use health insurance reimbursement and other forms of social protection payment to reduce out-of-pocket costs for treatment of TB to 30% and for treatment of rifampin-resistant TB to 10%. As TB diagnosis and treatment tasks were being shifted from local CDCs to designated hospitals, a case-based payment mechanism was designed, which aimed for cost containment and standardized good clinical practice in care for patients with TB or rifampin-resistant TB (*12*,*13*).

We quantified the effect of implementation of the second phase of the program on the diagnosis of bacteriologically confirmed smear-negative pulmonary TB and rifampin-resistant TB in a before–after design. The program was designed to select 1 prefecture, respectively, from the eastern and middle regions of China as pilot study sites. Zhenjiang City (Jiangsu Province) and Yichang City (Hubei Province) were also selected based on good performance of features of the National TB Program in China (i.e., funding, laboratory capacity, and case detection). Prefectures consisted of the main municipality (city) and surrounding counties. The total population size in this pilot study was 6.9 million in 15 counties. Details of these prefectures have been described elsewhere (*14*). We assessed changes in numbers of patients given a diagnosis and compared a 1-year intervention period after implementation of the program (April 2014–March 2015) to a baseline period (January–December 2012). Both pilot study sites introduced all health sector changes.

Diagnosis and Treatment

During the baseline period, presumptive TB patients (i.e., patients who had TB symptoms) underwent a chest radiograph and smear examination by Ziehl–Neelsen staining according to usual clinical routine. Clinical management was based on judgment of the clinician and national guidelines: clinical smear-negative TB diagnosis requires TB symptoms, chest radiograph abnormalities indicative of TB, and 3 negative sputum smear examinations. Both prefectures had experience with diagnosis and treatment of multidrug-resistant TB (MDR

TB), which was defined as resistance to rifampin and isoniazid, through an earlier project supported by the Global Fund to Fight AIDS, Tuberculosis, and Malaria (GFATM) that included drug-resistance screening of patients at high risk for MDR TB by using sputum culture and phenotypic drug susceptibility testing (DST). However, after the GFATM project ended, the degree of implementation varied among sites.

During the intervention period, patients with presumptive pulmonary TB had a chest radiograph and smear examination. In addition, culture (1 sputum specimen in Löwenstein-Jensen medium) was used in county-level laboratories for smear-negative patients. Smear-positive samples and culture isolates were tested in city-level laboratories with Genechip or GenoType MTBDRplus assays. The Genechip test, a domestically developed diagnostic test, has a sensitivity of 87.6% and a specificity of 98.0% for rifampin resistance in this setting and a sensitivity of 80.3% and a specificity of 95.8% for isoniazid resistance (*15*). A systematic review of the GenoType assay showed a pooled sensitivity of 94.6% and a specificity of 98.2% for rifampin resistance, and a sensitivity of 83.4% and a specificity of 99.6% for isoniazid resistance (*9*). We compiled additional details of the diagnostic algorithm (Table 1).

Data Collection and Analysis

We extracted data for the baseline and the intervention periods from the routine electronic recording and reporting system for notification and management of TB patients (TBIMS) (*16*). This system contains data about demographic characteristics of patients, laboratory test results, TB diagnosis, treatment provided, and treatment outcomes. Patient data were entered at the clinic where the patient was registered for treatment. The system is maintained by the national-level CDC, which also provides supervision and data quality checks. Data collection and data capture format was similar for the 2 periods except for results of molecular tests, which were captured only for the intervention period. TBIMS data for diagnosis and treatment for patients with TB or rifampin-resistant TB were exported and merged by using unique identification numbers.

Pulmonary TB was defined as a diagnosis of pulmonary TB in the TBIMS. Bacteriologic confirmation was defined as TB confirmed by smear examination or culture. Rifampin resistance was determined by LPA or phenotypic DST on Löwenstein-Jensen medium.

We performed analyses by using Stata version 13 (https://www.stata.com). We compared numbers and proportions of patients given a diagnosis of any type of pulmonary TB, bacteriologically confirmed pulmonary TB, and rifampin-resistant TB between the baseline and intervention periods. We calculated reporting rates by dividing the number of observed cases by the population size, available by age and sex on district level, in 2012 for the baseline period and in 2014 for the intervention period. We used MDR TB patients in the baseline period as the comparison group for rifampin-resistant TB patients in the intervention period because rifampin-resistant TB patients and MDR TB patients were treated and managed the same way in the intervention period. Patients with TB resistant to isoniazid but not rifampin received the standard first-line treatment.

We tested distributions for categorical data by using 2-sided Fisher exact tests and for numerical data by using the nonparametric Wilcoxon rank test. We used logistic regression to determine patient characteristics associated with culture positivity for smear-negative pulmonary TB patients given a diagnosis during the intervention period, and characteristics associated with rifampin resistance for all bacteriologically confirmed pulmonary TB patients during the intervention period. Potential characteristics available and included in univariable and multivariable regression were age, sex, treatment history, disease severity, and migrant status. All statistical testing used 0.05 as the significance level.

Results

A total of 4,553 pulmonary TB patients were reported during the intervention period and 5,269 during the baseline period. These findings reflected a 15% decrease in the annual reporting rate for all cases of pulmonary TB from 72.6 cases/100,000 persons to 61.7 cases/100,000 persons, which was consistent across the 2 prefectures (Yichang 9.2%, Zhenjiang 21.8%). After excluding 256 patients who

Table 1. Diagnostic algorithm used during baseline and intervention periods in study of added value of comprehensive program to provide universal access to care for sputum smear–negative drug-resistant TB, China*

Level	Baseline	Intervention
County	Light microscopy (Ziehl–Neelsen staining), smear examination, and chest radiograph for all presumptive TB patients	LED fluorescence microscopy (auramine staining), smear examination, and chest radiograph for all presumptive TB patients; culture for all patients with a diagnosis of smear-negative TB
City	Culture and phenotypic DST only for patients at high risk for MDR TB; culture for smear-positive patients in Zhenjiang	Line-probe assay (Genechip; CapitaBio, http://www.capitalbiotech.com) used in Yichang; GenoType (Hain Lifesciences, https://www.hain-lifescience.de/en/company/contact.html) used in Zhenjiang for all bacteriologically confirmed pulmonary TB

*DST, drug susceptibility testing; LED, light-emitted diode; MDR TB, multidrug-resistant tuberculosis; TB, tuberculosis.

had only pleural TB, we included 5,222 (99.1%) patients for the baseline period and 4,364 (95.8%) for the intervention period: overall, 1,808 (34.6%) smear-positive patients and 3,414 (65.4%) smear-negative patients during the baseline period, compared with 1,509 (34.6%) smear-positive patients and 2,805 (65.4%) smear-negative patients during the intervention period (Figure).

Culture Confirmation

During the baseline period 0.5% (12/2,258) of smear-negative patients were culture-confirmed in Yichang and 1.6% (19/1,156) in Zhenjiang. These proportions increased to 17.4% (358/2,055) in Yichang and 6.5% (52/800) in Zhenjiang, or 14.4% overall (95% CI 13.0%–15.7%), during the intervention period ($p<0.001$ for both prefectures) (Table 2). In Yichang, 5% of results were indeterminate, compared with 12% in Zhenjiang. The analysis of determinants of culture positivity among smear-negative patients showed strong effect modification by prefecture. Therefore, analysis was performed separately for each prefecture. In Yichang, the proportion culture-positive was independently associated with a history of previous TB treatment (adjusted odds ratio [aOR] 7.7, 95% CI 4.2–14.1), more severe disease (aOR 2.1, 95% CI 1.5–2.9), and being an internal migrant (aOR 3.1, 95% CI 2.2–4.1). In Zhenjiang, none of these determinants showed a significant association with culture positivity (Table 3).

Drug Resistance Testing

The proportion of smear-positive patients tested for drug resistance increased from 42.3% (899/2,126) to 83.0% (988/1,191; $p<0.001$) overall between the baseline and intervention periods, from 3.1% to 90.9% ($p<0.001$) for Yichang, and from 74.5% to 92.5% ($p<0.001$) for Zhenjiang (Table 2). In contrast, for smear-negative patients, the proportion tested for drug resistance increased between the baseline and intervention periods from 0.7% (23/3,414) to only 14.2% (405/2,855) ($p<0.01$); overall, from <0.1% (1/2,258) to 17.1% (352/2,055) ($p<0.001$) in Yichang and from 1.9% (22/1,156) to 6.6% (53/800) ($p<0.001$) in Zhenjiang.

Drug Resistance Results

For smear-positive patients during the intervention period, rifampin resistance was detected in 7.1% (67/949; 95% CI 5.5–8.9) in Yichang and 5.4% (30/560; 95% CI 3.6–7.6) in Zhenjiang (Table 2). Overall, the number of smear-positive patients with rifampin resistance more than tripled from 30 during the baseline period to 97 during the intervention period.

For smear-negative patients during the intervention period, a drug resistance result was available for 16.1% of smear-negative patients in Yichang and for 5.9% of smear-negative patients in Zhenjiang. Rifampin resistance was detected in 13 patients with a smear-negative, culture-positive result: 3.5% of those tested with results, and 0.5% of all smear-negative patients. Two additional smear-negative patients who were culture negative showed rifampin resistance by LPA directly on sputum; thus, the physicians ordered the LPA directly on sputum, contrary to the guideline, to have the result as quickly as possible, because the patients had been considered at high risk for MDR TB.

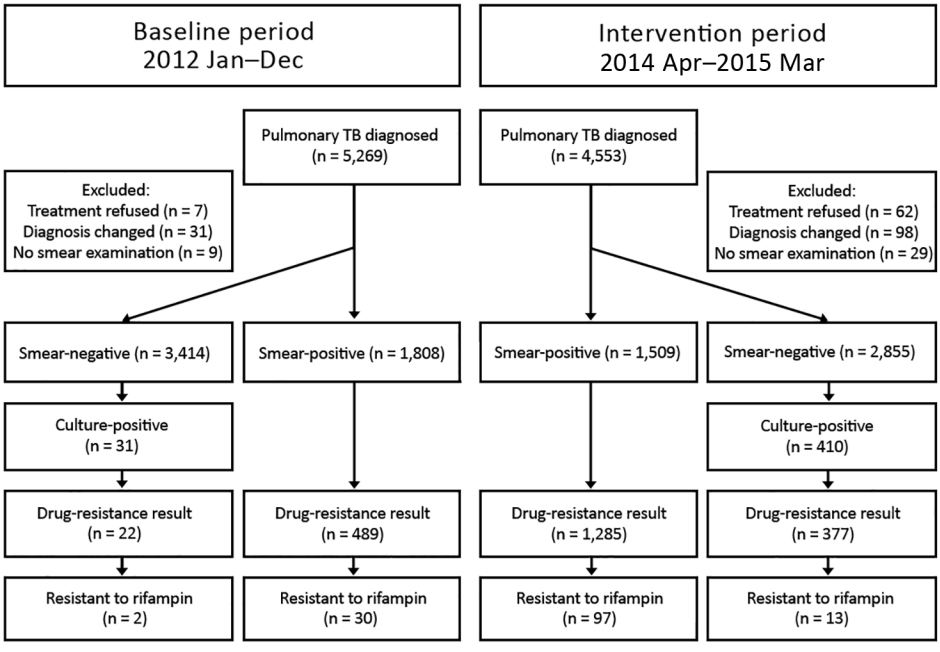


Figure. Flow diagram of TB patients given diagnoses at pilot prefectures in a baseline and intervention study of added value of a comprehensive program to provide universal access to care for sputum smear–negative drug-resistant TB, China. TB, tuberculosis.

Table 2. Drug resistance testing and results by prefecture and smear status in study of comprehensive program to provide universal access to care for sputum smear–negative drug-resistant tuberculosis, China*

Characteristic	Yichang, no. (%)		Zhenjiang, no. (%)	
	Baseline	Intervention	Baseline	Intervention
Smear-positive patients				
Patients given a diagnosis	1,177 (100)	949 (100)	631 (100)	560 (100)
Patients tested for drug resistance	36 (3.1)	863 (90.9)	470 (74.5)	518 (92.5)
Rapid resistance result available	36 (3.1)	828 (87.2)	453 (71.8)	457 (81.6)
Drug resistance test result				
No resistance to rifampin and isoniazid	24 (2.0)	715 (75.3)	405 (64.2)	389 (69.5)
Resistance to only isoniazid	3 (0.3)	46 (4.8)	26 (4.1)	38 (6.8)
Any rifampin resistance	9 (0.8)	67 (7.1)	21 (3.3)	30 (5.4)
MDR TB	6 (0.5)	30 (3.2)	14 (2.2)	18 (3.2)
Resistance to only rifampin	3 (0.3)	37 (3.9)	7 (1.1)	12 (2.1)
Smear-negative patients				
Patients given a diagnosis	2,258 (100)	2,055 (100)	1,156 (100)	800 (100)
Cultures performed	14 (0.6)	1,842 (89.6)	19 (1.6)	749 (93.6)
Cultures positive	12 (0.5)	358 (17.4)	19 (1.6)	52 (6.5)
Patients tested for drug resistance†	1 (<0.1)	352 (17.1)	22 (1.9)	53 (6.6)
Rapid resistance result available	1 (<0.1)	330 (16.1)	21 (1.8)	47 (5.9)
Drug resistance test result				
No resistance to rifampin and isoniazid	0	299 (14.5)	19 (1.6)	43 (5.4)
Resistance to only isoniazid	1 (<0.1)	19 (0.9)	0	3 (0.4)
Any rifampin resistance‡	0	12 (0.6)	2 (0.2)	1 (0.1)
MDR TB	0	10 (0.5)	2 (0.2)	1 (0.1)
Resistance to only rifampin	0	2 (0.1)	0	0

*Differences in numbers of patients tested and numbers with a test result reflect indeterminate test results. MDR TB, multidrug-resistant tuberculosis (i.e., resistant to rifampin and isoniazid); TB, tuberculosis.
†Including 10 smear-negative patients who had no culture results and 24 smear-negative culture-negative patients for whom a line-probe assay was performed directly for sputum.
‡Including 2 smear-negative patients for whom a line-probe assay was performed directly for sputum.

The proportion of patients that showed rifampin resistance among those tested was significantly higher for smear-positive patients (97/1,381, 7.0%) than for smear-negative patients (13/405, 3.2%) (p = 0.005). Overall, the addition of culture and rapid resistance testing during the intervention period yielded 10.9% (13/119; 95% CI 5.9%–18.0%) additional diagnoses of rifampin-resistant TB and no significant difference between prefectures (p = 0.180). For isoniazid-monoresistant TB, the additional increase was 20.8% (22/106; 95% CI 13.5%–29.7%). A total of 13/2,591 patients were determined to have rifampin-resistant TB, and 22/2,591 were determined to have isoniazid-monoresistant TB.

Second-Line Treatment

Of the 110 (including 13 smear-negative) rifampin-resistant patients with MDR TB given a diagnosis during the intervention period, 94 (85.5%) started second-line treatment, compared with 20 (60.6%) of 33 during the baseline period (p<0.001) (Table 4). The proportion that started treatment during the intervention period was 100% (13/13)

Table 3. Patient characteristics associated with culture positivity for smear-negative TB patients, Yichang and Zhenjiang prefectures, China*

Characteristic	Yichang				Zhenjiang			
	Culture, no. (%)		Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Culture, no. (%)		Unadjusted OR (95% CI)	Adjusted OR (95% CI)
	Negative	Positive			Negative	Positive		
Age, y								
0–39	401	102 (20.3)	Referent	Referent	166	12 (6.7)	Referent	Referent
40–59	565	129 (18.6)	0.9 (0.7–1.2)	0.9 (0.7–1.2)	215	14 (6.1)	0.9 (0.4–2.0)	1.0 (0.4–2.3)
≥60	507	127 (20.0)	1.0 (0.7–1.3)	1.0 (0.8–1.4)	316	26 (7.6)	1.1 (0.6–2.3)	1.3 (0.6–2.8)
Sex								
M	1,001	260 (20.6)	Referent	Referent	514	38 (6.9)	Referent	Referent
F	472	98 (17.2)	0.8 (0.6–1.0)	0.8 (0.6–1.0)	183	14 (7.1)	1.0 (0.6–2.0)	1.1 (0.6–2.0)
TB treatment history								
New	1,455	325 (18.3)	Referent	Referent	604	42 (6.5)	Referent	Referent
Retreatment	18	33 (64.7)	**8.2 (4.6–14.8)**	**7.7 (4.2–14.1)**	93	10 (9.7)	1.5 (0.8–3.2)	1.6 (0.8–3.3)
Disease severity								
Not severe	1,313	284 (17.8)	Referent	Referent	645	48 (6.9)	Referent	Referent
Severe	160	74 (31.6)	**2.1 (1.6–2.9)**	**2.1 (1.5–2.9)**	52	4 (7.1)	1.0 (0.4–3.0)	1.0 (0.3–2.9)
Citizenship								
Local	1,326	267 (16.8)	Referent	Referent	523	34 (6.1)	Referent	Referent
Migrant	147	91 (38.2)	**3.1 (2.3–4.1)**	**3.1 (2.2–4.1)**	174	18 (9.4)	1.6 (0.9–2.9)	1.8 (0.9–3.3)
Total	1,473	358 (19.6)	NA	NA	697	52 (6.9)	NA	NA

*Values in bold indicate a statistically significant association (p<0.05). OR, odds ratio; NA, not applicable; TB, tuberculosis.

Table 4. Smear results for patients who did and did not start SLD treatment and outcomes for baseline and intervention periods in study of added value of comprehensive program to provide universal access to care for sputum smear–negative drug-resistant TB, China*

Characteristic	Baseline, no. (%)		Intervention, no. (%)	
	Smear-positive	Smear-negative	Smear-positive	Smear-negative
Initiated SLD treatment	18	2	83	11
Cure	9 (50.0)	1 (50)	14 (16.9)	0
Treatment completion	1 (5.6)	1 (50)	27 (32.5)	6 (54.5)
Death	2 (11.1)	0	9 (10.8)	1 (9.1)
Treatment failure	3 (16.7)	0	5 (6.0)†	1 (9.1)
Lost to follow-up	0	0	6 (7.2)	0
Stop treatment because of side effects	0	0	8 (9.6)	0
Other	3 (16.7)	0	14 (16.9)	3 (27.3)
Did not initiate SLD treatment	13	0	14	2 (18.2)
No regimen change, continued first-line treatment	11 (84.6)‡	0	2 (14.3)	0
Death	1 (7.7)	0	4 (28.6)	0
Lost to follow-up	0	0	3 (21.4)	0
Treatment refusal because of nonfinancial reason	0	0	1 (7.1)	1 (50.0)
Other	1 (7.7)	0	4 (28.6)	1 (50.0)
Total	31	2	97	13

*SLD, second-line drug; TB, tuberculosis.
†Includes 10 patients with TB resistant to rifampin but not isoniazid, who were not eligible for SLD treatment during the baseline period.
‡Includes 2 patients given treatment for extensively drug-resistant tuberculosis.

for smear-negative patients and 83.5% (81/97) for smear-positive patients (p = 0.243).

For smear-positive patients, the median delay between presentation for diagnosis and initiation of second-line drug treatment decreased from 16 weeks during the baseline period to 16 days during the intervention period. For smear-negative patients, the median delay decreased from 21 weeks to 7 weeks. Among patients given second-line treatment, 12 (60.0%) of 20 patients during the baseline period and 47 (50.0%) of 94 patients during the intervention period had treatment success (cure or completion) as the outcome (p = 0.572). The proportion with treatment failure or death did not differ significantly between the intervention (16 patients, 17.0%) and baseline (5 patients, 25.0%) periods (p = 0.603). During the intervention period, 6 patients did not return for follow-up appointments and 8 stopped treatment because of side effects (total 14.9%), whereas neither finding was reported during the baseline period (p = 0.143). Three (9.1%) eligible patients during the baseline period and 16 (14.5%) patients during the intervention period were known not to have started second-line treatment (Table 4).

Discussion

In this pilot study, expanding drug resistance testing for smear-positive TB patients suspected of having only MDR TB to all smear-positive TB patients resulted in a 3-fold increase in the number of patients identified having rifampin-resistant TB and a 5-fold increase in the number of patients given second-line treatment. This distinct increase was observed despite a reduction in numbers of pulmonary TB patients given a diagnosis at the 2 pilot study sites, which is consistent with decreased TB reporting in China (*2*). Introduction of rapid testing for rifampin and isoniazid resistance by LPA clearly was a decisive factor. The addition of mycobacterial culture, which enabled line-probe testing for smear-negative patients (i.e., those with low bacterial load in their sputum), yielded only 11% more cases of rifampin-resistant TB. This finding is a low yield given that smear-negative diagnosis accounted for 62% of all pulmonary TB cases; 199 cultures were required for each case of smear-negative rifampin-resistant TB detected. However, we detected 13 additional rifampin-resistant TB cases that would have been missed otherwise.

For smear-positive patients, the intervention decreased median delays until initiation of second-line treatment more than for smear-negative patients, reflecting the need for a culture isolate on which to perform LPA. However, this difference did not affect the proportion starting second-line treatment or treatment outcomes.

For smear-positive and smear-negative patients, we detected a similar absolute number of isolates resistant to isoniazid only as we detected rifampin-resistant isolates. At the time of this study, no differential regimens had been recommended yet for isoniazid-resistant TB, which could have led to improved regimens for 5%–7% of all patients given a diagnosis of pulmonary TB.

Addition of culture resulted in a low (14%) bacterial confirmation rate for smear-negative patients. This finding could reflect a high rate of false-negative cultures. False-negative sputum cultures are known to occur as a result of long sample transit times, harsh decontamination, or other shortcomings in laboratory procedures (*17*). The difference for the intervention period in the proportion of positive cultures between Yichang (19.4%) and Zhenjiang (6.9%) suggests that there were performance differences for the respective culture laboratories. An alternative explanation

might be false-positive diagnosis of pulmonary TB based on minimal chest radiograph abnormalities and symptoms without any other test. This finding might imply overdiagnosis and overtreatment: a considerable proportion of these patients might not have had TB and therefore did not need anti-TB treatment. Conversely, the sensitivity of a single sputum culture on solid media, as performed in this pilot study, was not >90% (*6*), and studies have suggested that patients with typical chest radiograph abnormalities but repeatedly negative cultures are at high risk for development of culture-positive TB over the next 2 years (*18*). Irrespective of their cause, these low bacterial confirmation rates need to be taken into account when scaling up culture for resistance testing purposes in the current laboratory and clinical system in China.

Although the intervention of improved collaboration between hospitals and CDC, as well as improved health insurance, increased the number of patients receiving second-line treatment, a total of 14% of patients given a diagnosis of rifampin-resistant MDR TB did not start second-line treatment, half of them because they died or did not return for care before treatment could be initiated. In addition, treatment success of second-line treatment remained rather low (50%), and 25% of patients stopping treatment because of side effects or unknown reasons. This finding clearly calls for improvement of linkage into care and retention in treatment. In these pilot studies, patients with rifampin-resistant or MDR TB were given second-line drugs for 20–24 months; results for both categories might be improved by shortening second-line treatment to 9–12 months, as currently recommended for selected patients (*19*).

The new National TB Control Plan for China calls for capacity for culture, strain identification, phenotypic DST, and molecular resistance testing in all prefecture-level TB-designated hospitals, and for capacity for molecular resistance testing for 70%–80% of counties, districts, and cities throughout the country by 2020 (*5*). However, investments for scaling up this laboratory capacity will be substantial and need to include quality assurance, biosafety (for culture and DST), and prevention of cross-contamination (for PCR-based LPAs). Given the low additional yield of testing smear-negative pulmonary TB patients, scaling up use of Xpert, despite higher end-user prices, has a better cost–benefit ratio, and warrants formal analyses to establish cost-effectiveness and budget impacts by comparing various diagnostic algorithms and scale-up strategies (*20*).

Our study had limitations. First, the low bacterial confirmation rate for smear-negative patients might underestimate the effect of LPA for detection of rifampin-resistant TB, specifically in settings with higher bacterial confirmation rates. Second, both prefectures already had second-line treatment in place for patients given a diagnosis of MDR TB. Thus, these prefectures were not representative of the situation in most of China. This limitation implies that the increase in number of patients given a diagnosis of rifampin-resistant TB in our pilot study might have been less than in prefectures not supported through the GFATM, and the expected effect of new diagnostic interventions on case findings for rifampin-resistant TB might have been underestimated. Third, patients who did not come to the county CDC (the so-called TB dispensary) were not included. Patients with presumptive TB at general hospitals should be referred to the TB dispensary; however, not all patients will be referred. Also, there would have been TB patients who did not come to general hospitals or TB dispensaries. It is not expected that self-referral and referral by general hospitals and village healthcare workers would have changed greatly from baseline to intervention periods. Fourth, we included only 2 prefectures that had different LPAs implemented. Therefore, we could not fully appreciate possible time effects on indicators, such as decreasing case reporting rates because of underlying epidemiologic changes.

Expansion of drug-resistance testing by rapid molecular assays to all pulmonary TB patients resulted in a 3-fold increase in numbers of patients given a diagnosis of rifampin-resistant MDR TB and who received appropriate treatment. Even so, testing smear-negative pulmonary TB patients had limited added value because of the low proportion of patients who had cultures positive for *M. tuberculosis*. Given the availability of alternative methods, such as Xpert, cost–benefit and budget impact analyses are warranted to determine if use of culture should be continued as part of the diagnostic algorithm to identify laboratory-confirmed TB cases. Despite several health system improvements, the linkage of care and retention of second-line TB treatment remained suboptimal.

Acknowledgments

We thank government officials and experts at the national level and in the project provinces and cities for their commitment and dedication to implementation of the tuberculosis program.

The study was supported by the Chinese National Health Commission and the Bill & Melinda Gates Foundation Collaboration TB Program (grant no. 51914).

About the Author

Dr. Huang is an epidemiologist at the Chinese National Center for TB Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing, China. His primary research interest is tuberculosis control and treatment.

References

1. Dheda K, Barry CE III, Maartens G. Tuberculosis. Lancet. 2016; 387:1211–26. http://dx.doi.org/10.1016/S0140-6736(15)00151-8
2. World Health Organization. Global tuberculosis report, 2018 [cited 2019 Jan 15]. http://www.who.int/tb/publications/global_report/
3. Li R, Ruan Y, Sun Q, Wang X, Chen M, Zhang H, et al. Effect of a comprehensive programme to provide universal access to care for sputum-smear-positive multidrug-resistant tuberculosis in China: a before-and-after study. Lancet Glob Health. 2015;3:e217–28. http://dx.doi.org/10.1016/S2214-109X(15)70021-5
4. Nathavitharana RR, Cudahy PG, Schumacher SG, Steingart KR, Pai M, Denkinger CM. Accuracy of line probe assays for the diagnosis of pulmonary and multidrug-resistant tuberculosis: a systematic review and meta-analysis. Eur Respir J. 2017;49:1601075. http://dx.doi.org/10.1183/13993003.01075-2016
5. The State Council. The People's Republic of China. National tuberculosis control plan 2016–220, 2016 [cited 2019 Feb 22] http://www.gov.cn/zhengce/content/2017-02/16/content_5168491.htm
6. Boehme CC, Nicol MP, Nabeta P, Michael JS, Gotuzzo E, Tahirli R, et al. Feasibility, diagnostic accuracy, and effectiveness of decentralised use of the Xpert MTB/RIF test for diagnosis of tuberculosis and multidrug resistance: a multicentre implementation study. Lancet. 2011;377:1495–505. http://dx.doi.org/10.1016/S0140-6736(11)60438-8
7. World Health Organization. Xpert MTB/RIF assay for the diagnosis of pulmonary and extrapulmonary TB in adults and children [cited 2017 Oct 8]. http://www.who.int/tb/publications/xpert-mtb-rif-assay-diagnosis-policy-update/
8. Steingart KR, Schiller I, Horne DJ, Pai M, Boehme CC, Dendukuri N. Xpert® MTB/RIF assay for pulmonary tuberculosis and rifampicin resistance in adults. Cochrane Database Syst Rev. 2014;1:CD009593.
9. Drobniewski F, Cooke M, Jordan J, Casali N, Mugwagwa T, Broda A, et al. Systematic review, meta-analysis and economic modelling of molecular diagnostic tests for antibiotic resistance in tuberculosis. Health Technol Assess. 2015;19:1–188, vii–viii. http://dx.doi.org/10.3310/hta19340
10. World Health Organization. | Treatment of drug-resistant TB: resources [cited 2017 Jun 20]. http://www.who.int/tb/areas-of-work/drug-resistant-tb/treatment/resources/
11. Albert H, Nathavitharana RR, Isaacs C, Pai M, Denkinger CM, Boehme CC. Development, roll-out and impact of Xpert MTB/RIF for tuberculosis: what lessons have we learnt and how can we do better? Eur Respir J. 2016;48:516–25. http://dx.doi.org/10.1183/13993003.00543-2016
12. Long Q, Qu Y, Lucas H. Drug-resistant tuberculosis control in China: progress and challenges. Infect Dis Poverty. 2016;5:9. http://dx.doi.org/10.1186/s40249-016-0103-3
13. Tang S, Wang L, Wang H, Chin DP. Access to and affordability of healthcare for TB patients in China: issues and challenges. Infect Dis Poverty. 2016;5:10. http://dx.doi.org/10.1186/s40249-016-0096-y
14. Zhou C, Long Q, Chen J, Xiang L, Li Q, Tang S, et al. Factors that determine catastrophic expenditure for tuberculosis care: a patient survey in China. Infect Dis Poverty. 2016;5:6. http://dx.doi.org/10.1186/s40249-016-0100-6
15. Pang Y, Xia H, Zhang Z, Li J, Dong Y, Li Q, et al. Multicenter evaluation of genechip for detection of multidrug-resistant *Mycobacterium tuberculosis*. J Clin Microbiol. 2013;51:1707–13. http://dx.doi.org/10.1128/JCM.03436-12
16. Huang F, Cheng S, Du X, Chen W, Scano F, Falzon D, et al. Electronic recording and reporting system for tuberculosis in China: experience and opportunities. J Am Med Inform Assoc. 2014;21:938–41. http://dx.doi.org/10.1136/amiajnl-2013-002001
17. Schaaf HS. Tuberculosis: a comprehensive clinical reference. Edinburgh: Saunders; 2009.
18. A controlled trial of a 2-month, 3-month, and 12-month regimens of chemotherapy for sputum smear-negative pulmonary tuberculosis: the results at 30 months. Hong Kong Chest Service/Tuberculosis Research Centre, Madras/British Medical Research Council. Am Rev Respir Dis. 1981;124:138–42.
19. World Health Organization. WHO treatment guidelines for drug-resistant tuberculosis, 2016 update. October 2016 revision. Geneva; The Organization; 2016.
20. Bilinski A, Neumann P, Cohen J, Thorat T, McDaniel K, Salomon JA. When cost-effective interventions are unaffordable: Integrating cost-effectiveness and budget impact in priority setting for global health programs. PLoS Med. 2017;14:e1002397. http://dx.doi.org/10.1371/journal.pmed.1002397

Address for correspondence: Hui Zhang, National Center for Tuberculosis Prevention and Control, Chinese Center for Disease Control and Prevention, Beijing 102206, China; email: zhanghui@chinacdc.cn

Macrolide-Resistant *Mycoplasma genitalium* in Southeastern Region of the Netherlands, 2014–2017

Liesbeth Martens, Sharon Kuster, Wilco de Vos, Maikel Kersten, Hanneke Berkhout, Ferry Hagen

Mycoplasma genitalium infections of the urogenital tract are usually treated with azithromycin; however, for the past several years, rates of azithromycin treatment failure have increased. To document the occurrence and frequency of macrolide resistance–mediating mutations (MRMMs) in *M. genitalium* infections, we collected 894 *M. genitalium*–positive samples during April 2014–December 2017 and retrospectively tested them for MRMMs. We designated 67 samples collected within 6 weeks after a positive result as test-of-cure samples; of these, 60 were MRMM positive. Among the remaining 827 samples, the rate of MRMM positivity rose from 22.7% in 2014 and 22.3% in 2015 to 44.4% in 2016 but decreased to 39.7% in 2017. Because of these high rates of MRMMs in *M. genitalium* infections, we recommend that clinicians perform tests of cure after treatment and that researchers further explore the clinical consequences of this infection.

Since *Mycoplasma genitalium* was first isolated from men with nongonococcal urethritis in 1981, this bacterium has been recognized as a possible pathogen of the genitourinary tract (*1*). During the first decade after its discovery, its fastidious nature and slow growth rate complicated research into its clinical significance. Its association with nongonococcal urethritis in men was not established until the mid-1990s (*2*,*3*), when molecular testing in research settings had become available. Now *M. genitalium* is widely recognized as a frequent cause of male urethritis (*4*,*5*), a likely cause of cervicitis and pelvic inflammatory disease in women (*6*), and a possible cause of several other genitourinary syndromes (*4*–*7*).

The commonly used treatment for *M. genitalium* infections is azithromycin, either in a single dose of 1,000 mg or as a 5-day regimen (500 mg on the first day, followed by 250 mg on the subsequent 4 days). During the past decade, azithromycin treatment failure has been reported with increasing frequency (*8*). Clinical cure rates reported before 2008 were generally >80% (*9*–*11*) but more recently have dropped to as low as 54% (*12*,*13*). A single dose of azithromycin is the preferred treatment for nongonococcal urethritis in many countries, including Australia (*14*), the Netherlands (*15*), the United States (*16*), and the United Kingdom (*17*). Moreover, *Chlamydia trachomatis* infections are also commonly treated with a single dose of azithromycin, often without excluding co-infection with *M. genitalium* (*14*–*17*). However, it has been suggested that the single-dose regimen of azithromycin is actually facilitating the development of macrolide resistance (*18*,*19*).

In the Netherlands, routine testing for *M. genitalium* is not included in national sexually transmitted disease (STD) screening protocols (*15*,*20*,*21*). In a recent revision of the national protocol, screening for *M. genitalium* infections in men with nongonococcal urethritis is mentioned but not explicitly advised (*22*). The protocol states that the treatment of choice for symptomatic men and their partners is azithromycin for 5 days or moxifloxacin for 7–10 days. Follow-up is not mentioned (*22*). Most local hospital-based guidelines advise azithromycin in a 5-day regimen only; some advise a test of cure. Detection of macrolide resistance–mediating mutations (MRMMs) has, so far, not been included as part of the diagnostic work-up.

To document the occurrence and frequency of MRMMs, we conducted a retrospective study in the Netherlands during April 2014–December 2017. We tested samples that were positive for *M. genitalium* during the study period for MRMMs by using a molecular diagnostic approach.

Material and Methods

Sampling

The Laboratory of Medical Microbiology of the Canisius-Wilhelmina Hospital in Nijmegen, the Netherlands, is the primary diagnostic laboratory for the hospital itself, other care institutions, and general practitioners in the area. All samples referred to this laboratory for STD diagnostics

Author affiliations: Radboud University Medical Center, Nijmegen, the Netherlands (L. Martens); Canisius-Wilhelmina Hospital, Nijmegen (L. Martens, S. Kuster, W. de Vos, M. Kersten, H. Berkhout, F. Hagen); Westerdijk Fungal Biodiversity Institute, Utrecht, the Netherlands (F. Hagen)

DOI: https://doi.org/10.3201/eid2507.181556

starting in April 2014 were tested for *M. genitalium*, and samples that were positive were stored at –80°C. The study was approved by the Canisius-Wilhelmina Hospital Institution Review Board. Because patient identities were anonymous, written informed consent of participants was not required.

DNA Extraction

We collected *M. genitalium*–positive samples (determined by routine diagnostic in-house quantitative PCR [qPCR]) from the –80°C storage. After thawing the samples, we homogenized them by short vortexing and subsequently mixed a 200-μL sample with 20 μL internal control (phocine herpes virus [PhHV]) into a 96–deep well plate and extracted nucleic acids by using the MagNA Pure 96 automatic platform (Roche, http://www.roche.com). Nucleic acids eluates were directly subjected to molecular testing and stored at –20°C until further use.

Detection of STDs

We again subjected all samples to the same routine diagnostic in-house qPCR that simultaneously detects *Trichomonas vaginalis*, *M. genitalium*, and the internal extraction and amplification control PhHV. The qPCR mixture consisted of 2 μL primers and probes MgeFwd (5′-GAGAAATACCTTGATGGTCAGCAA-3′, 5 pmol/μL), MgeRvd (5′-GTTAATATCATATAAAGCTCTAC-CGTTGTTATC-3′, 5 pmol/μL), MgeProbe (5′-FAM-ACTTTGCAATCAGAAGGT-MGB-3′, 2 pmol/μL), TvaFwd (5′-CCTCAGTTCGCAAAGGC-3′, 5 pmol/μL), TvaRvd (5′-TTCAGCGACCATTCCCA-3′, 5 pmol/μL), TvaProbe (5′-HEX-CATTGACGCACTCATGACGAAC-GA-BHQ1-3′, 2 pmol/μL), PhHVFwd (5′-GGGCGAAT-CACAGATTGAATC-3′, 5 pmol/μL), PhHVRvd (5′-GCG-GTTCCAAACGTACCAA-3′, 5 pmol/μL), and PhHVProbe (5′-Cy5-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ2-3′, 2 pmol/μL) (*23*); 10 μL 2× Fast Advanced Master Mix (Applied Biosystems, https://www.thermofisher.com); and 8 μL DNA sample. The qPCR reaction was conducted in a LightCycler 480-II (Roche Diagnostics) with the following protocol: UNG (uracil-*N*-glycosylase) treatment for 2 min at 50°C, 10 min polymerase activation at 96°C, 45 cycles of denaturation for 5 s at 96°C and 12 s at 60°C (fluorescence measurement), and a final cooling step for 30 s at 40°C. We included a negative extraction control, a negative template control, and a positive template control.

Detection of *M. genitalium* and MRMMs

The TVMGres qPCR assay developed by NYtor (https://www.nytor) is a multiplex qPCR assay that detects *T. vaginalis* and *M. genitalium* and includes the internal control PhHV. The assay simultaneously detects the single-nucleotide polymorphisms A2058C, A2058G, A2058T, and A2059G in the 23S ribosomal RNA–encoding region of *M. genitalium,* which together account for >95% of the cases of azithromycin resistance (*24*). We added 5 μL of the nucleic acids extract to 15-μL reaction mix that contained qPCR master mix and the primer/probe mix from the TVMGres qPCR assay. We performed the qPCR reactions on a LightCycler 480-II instrument (Roche Diagnostics) using a reaction protocol consisting of a polymerase activation step of 3 min at 95°C, followed by 45 cycles of 15 s at 95°C and 1 min at 60°C. Detection was done by measuring the fluorescence signals of FAM (for *T. vaginalis*), CAL Fluor Orange 560 (for *M. genitalium* mutations), CAL Fluor Red 610 (for *M. genitalium* detection), and TYE665 (for internal control) (https://eu.idtdna.com for all dyes). We considered a sample to be valid if an amplification curve for any of the pathogen targets or the internal control was present.

We defined wild-type *M. genitalium* as that in a sample that was positive for *M. genitalium* without the presence of a positive MRMM signal. We defined resistant *M. genitalium* as that in a sample positive for both *M. genitalium* and MRMMs.

Statistical Analyses

We collected data in Microsoft Excel (Microsoft, https://www.microsoft.com) and used Microsoft Office Excel and SPSS (IBM, https://www.ibm.com) for analysis. We calculated p values in SPSS by using the χ^2 test and 2-sample *t*-test.

Results

We tested 28,408 samples from 20,537 patients for the presence of STD organisms. Most (n = 25,132; 88.5%) samples were provided by general practitioners, 3,087 (10.9%) by hospitals, and 189 (0.7%) from other and unknown locations. *M. genitalium* was detected in 1,146 (4.0%) samples from 879 patients (4.3%). For 7 patients, multiple samples that were collected on the same day were positive; for each of these patients, we included only 1 of these samples, which left 1,139 samples, 936 of which were available for further testing. The remaining 203 samples were either not stored or were of inadequate volume for further analysis. We found no statistically significant differences between available and unavailable samples (Table).

A total of 23 samples were negative for *M. genitalium* by the routine diagnostic in-house qPCR and the TVMGres qPCR assay. We excluded these samples from our study, together with 18 samples repeatedly showing incongruous results (i.e., in-house qPCR result not matching the TVMGres qPCR result). We also excluded another sample that repeatedly had an inhibited internal control in the TVMGres qPCR assay because of a concurrent *T. vaginalis* infection. We provide an overview of all included and excluded samples (Figure 1).

Table. Characteristics of samples available and unavailable for further testing in study of macrolide-resistant *Mycoplasma genitalium* in southeastern region of the Netherlands, 2014–2017*

Characteristic	Available samples, % (n = 936)	Unavailable samples, % (n = 203)	p value
Patient sex			
F	67.3	69.5	0.546
M	32.7	30.5	
Proportion sent in by general practitioner	92.2	90.2	0.343
Proportion test of cure	7.3	8.9	0.357

*Average ages: patients for whom samples were available, 29.8 ± 9.3 y; patients for whom samples were not available, 30.9 ± 9.5 y; p = 0.118.

Characteristics of the Study Population

Of the 28,408 samples that were tested for *M. genitalium* during the study period, 19,393 (68.3%) originated from female patients and 9,015 (31.7%) from male patients (2.2:1 ratio). The percentages of *M. genitalium* positivity were similar for male (4.1%) and female (4.0%) patients.

Average age at the time of testing was 32.0 years; female patients (average 31.3 years of age) were younger than male patients (average 33.6 years of age). The average age of *M. genitalium*–positive male patients (33.1 years) was slightly lower than that of *M. genitalium*–negative male patients (33.6 years; p = 0.36). *M. genitalium*–positive female patients were 2.8 years younger than *M. genitalium*–negative female patients (28.5 vs. 31.3 years; p<0.001).

Epidemiology

A total of 894 samples had positive results for *M. genitalium* when tested by the routine diagnostic in-house qPCR and by the TVMGres qPCR assay. Of these, 67 (7.5%) samples were collected within 6 weeks of a previous positive *M. genitalium* result. These retests were considered positive results for tests of cure, which were analyzed separately. Of the remaining 827 samples, 281 (34.0%) were *M. genitalium* MRMM–positive by qPCR. The frequency of MRMMs almost doubled from 22.7% (27/119) in 2014 and 22.3% (47/211) in 2015 to 44.4% (92/207) in 2016, then decreased to 39.7% (115/290) in 2017 (Figure 2).

Tests of Cure

Samples taken from a patient within 6 weeks of an *M. genitalium*–positive sample were defined as test-of-cure samples. During the study period, we collected 214 samples for test of cure, most (163; 76.2%) from the second half of 2016 on. Of the 214 samples, 86 (40.2%) were *M. genitalium* positive; of these, 67 (77.9%) were available for further testing. Only 7 (10.4%) samples were found to contain wild-type *M. genitalium* (Figures 3, 4).

For 57 positive test-of-cure samples, both the initial sample and the test(s)-of-cure samples were available. In

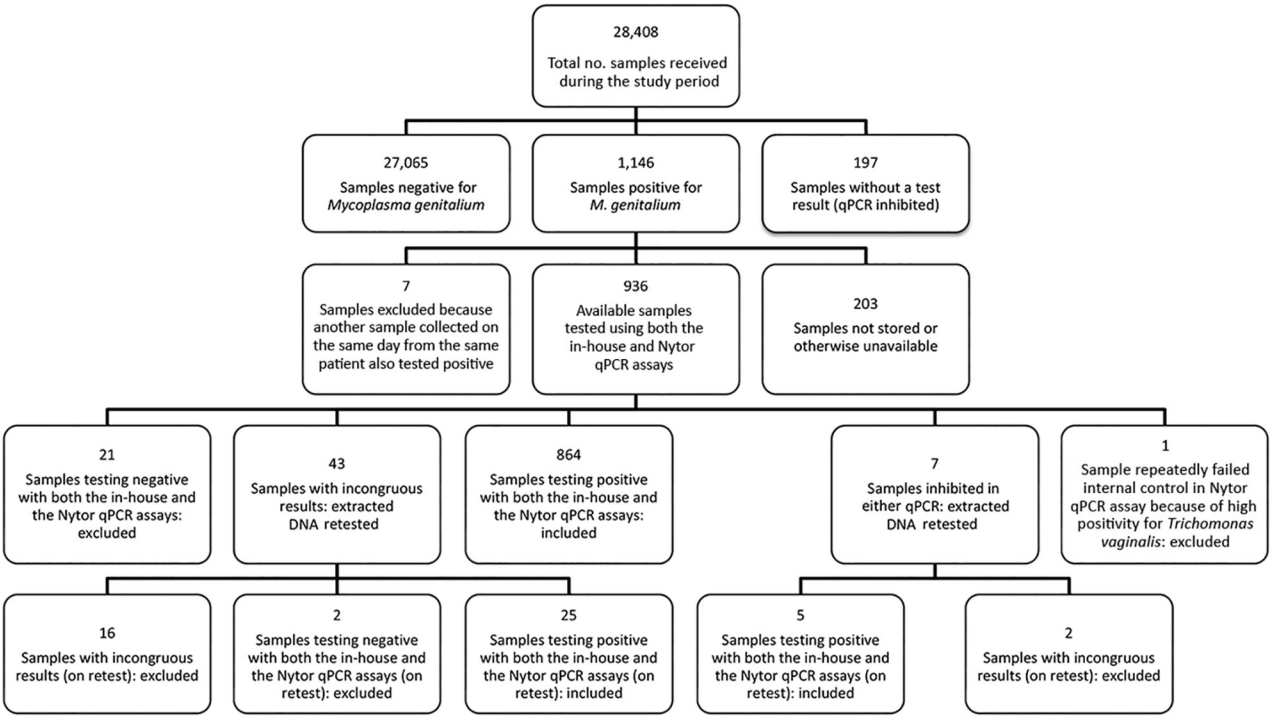


Figure 1. Characteristics of all samples received during study of macrolide-resistant *Mycoplasma genitalium* in southeastern region of the Netherlands, 2014–2017. qPCR, quantitative PCR.

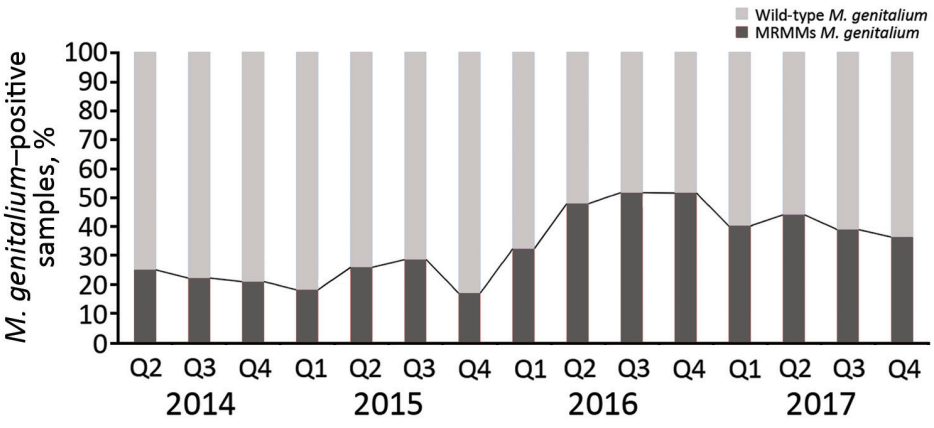


Figure 2. Proportion of *Mycoplasma genitalium*–positive samples containing MRMMs in study of macrolide-resistant *M. genitalium* in southeastern region of the Netherlands, 2014–2017. Percentages of *M. genitalium*–positive samples that are either wild-type or positive for MRMMs are based on quantitative PCR. MRMM, macrolide resistance–mediating mutation; Q, quarter.

most instances, both samples were positive for MRMMs (45/57; 78.9%).

Seven patients who were originally infected with a wild-type strain had resistant *M. genitalium* in the test-of-cure sample. For 1 patient, the initial sample was positive for MRMMs but the test-of-cure sample, collected 41 days later, contained wild-type *M. genitalium*. For the remaining 4 patients, both the initial sample and the test-of-cure sample contained wild-type *M. genitalium*.

Discussion

This retrospective epidemiologic study shows that the frequency of MRMMs in *M. genitalium* in the southeastern region of the Netherlands almost doubled from 2014 and 2015 to 2016. After 2016, the frequency of MRMMs gradually decreased through the last quarter of 2017.

High MRMM frequency in *M. genitalium* has been described before (*25*–*36*) and is thought to be a consequence of using the single-dose azithromycin treatment regimen for *C. trachomatis* infections without excluding the presence of *M. genitalium* nongonococcal urethritis in men, *M. genitalium* infections, or both (*18*,*19*). To our knowledge, the decreasing number of MRMMs observed in *M. genitalium* from our study population in 2017 compared with 2016 has not been described before; however, compared with 2014 and 2015, the MRMM prevalence trend is increasing. During the study period, local protocols for the treatment of the aforementioned conditions were unchanged; however, starting on October 3, 2016, the advice to perform a test of cure 3 weeks after treatment was added in print to every report of a positive *M. genitalium* test result. In the preceding months, the advice was often given on an individual basis. Thus, in the second half of 2016, the number of tests of cure increased sharply (Figure 3). This increased testing may help explain the decrease in MRMM frequency because the 40.2% with a test-of-cure result that was positive for *M. genitalium* would logically have been subsequently treated with a different treatment regimen. However, even in 2017, the year in which most tests of cure were conducted, only about one third of the positive *M. genitalium* samples were followed up by a test of cure. Regardless whether the decreased MRMM frequency resulted from the increased number of tests of cure, the high rate of MRMMs in itself calls for a test of cure after treatment with azithromycin.

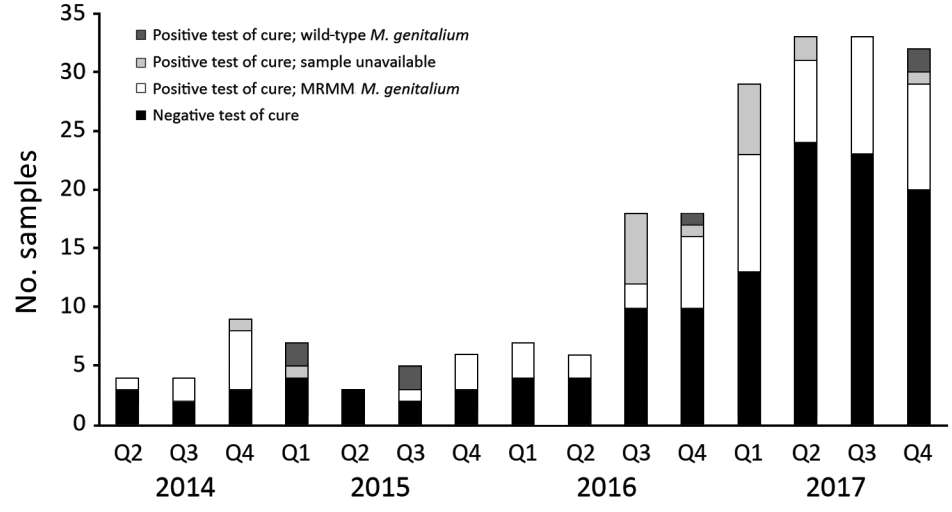


Figure 3. Characteristics of test-of-cure samples in study of macrolide-resistant *Mycoplasma genitalium* in southeastern region of the Netherlands, 2014–2017. Positive results indicate presence of *M. genitalium*; negative results indicate no *M. genitalium*. Q, quarter.

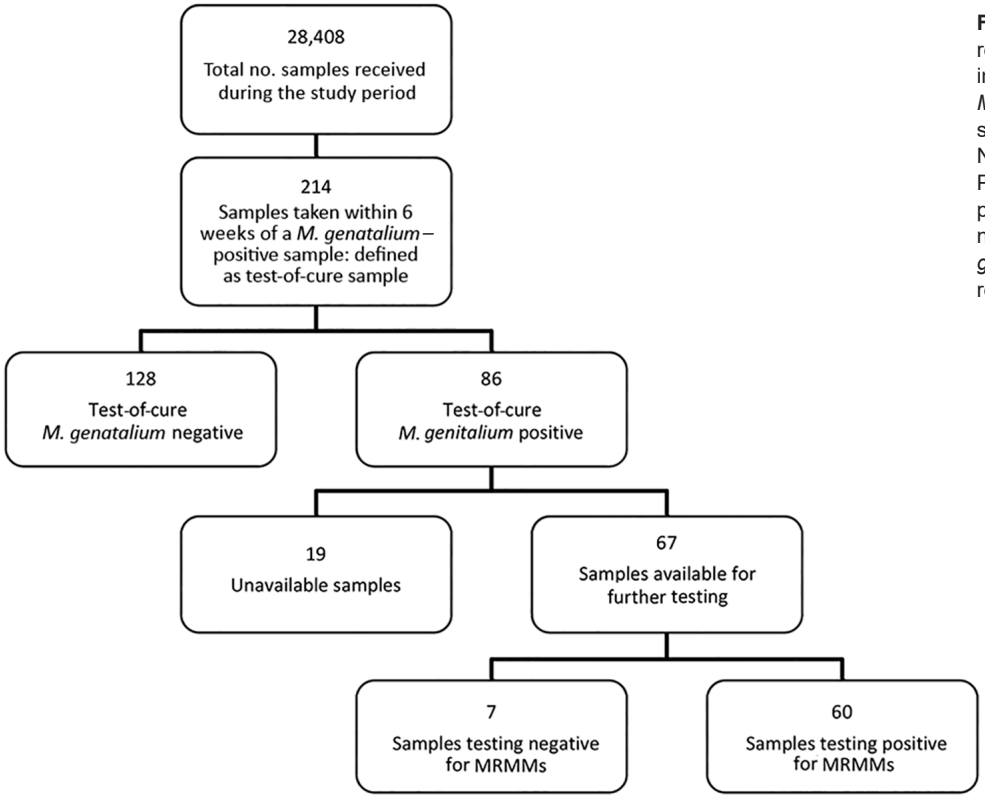


Figure 4. Identification and test results for test-of-cure samples in study of macrolide-resistant *Mycoplasma genitalium* in southeastern region of the Netherlands, 2014–2017. Positive results indicate presence of *M. genitalium*; negative results indicate no *M. genitalium*. MRMM, macrolide resistance–mediating mutation.

Most positive test-of-cure samples (60/67; 89.6%) contained MRMMs. This finding was expected because given that azithromycin is the preferred treatment for *M. genitalium* according to local protocol, macrolide-susceptible strains would have resulted in a negative test-of-cure result. Of note, for 7 patients who were originally infected with a wild type strain, test-of-cure results indicated a resistant *M. genitalium* strain. One patient with a resistant strain turned out to have a wild-type strain 6 weeks later; however, because it is unknown what treatment these patients received and whether they were at risk of acquiring a second *M. genitalium* infection, we cannot comment on the reason. The 4 patients with a persistent wild-type *M. genitalium* infection may have been reinfected, may have had a resistant *M. genitalium* infection that cannot be detected by the qPCR, may have been prescribed a nonmacrolide drug (e.g., doxycycline), or may have neglected to take the prescribed treatment.

A limitation of our study is the number of samples unavailable for further testing, which was 203 (17.8%) of 1,139. The reasons were generally either the sample not being stored or the volume of the stored sample being too low. In the third quarter of 2016, a disproportionately large number of samples (52/83; 62.7%) was lost because of a misunderstanding in storage protocol (i.e., which samples should and should not be stored). Unfortunately, this period coincided with a peak of MRMM frequency (17/31; 54.8%). However, in the fourth quarter of 2016, only 9/81 (11.1%) of samples were lost and MRMM frequency was the same (38/69; 55.1%). Statistically, we found no difference in characteristics of the populations for whom samples were available or unavailable (Table).

The question whether asymptomatic persons should be screened for *M. genitalium* can only be answered with more research into the clinical aspects and (long-term) complications of (co-)infection with *M. genitalium*. However, if a single dose of azithromycin indeed predisposes toward the development of MRMMs, *M. genitalium* infection should be excluded before *C. trachomatis* infections are treated with this regimen. In addition, treatment of nongonococcal urethritis should be reevaluated.

In conclusion, high rates (up to 44.4%) of MRMMs in *M. genitalium* were found among patients from the southeastern region of the Netherlands who were screened for STDs during 2014–2017. After a sharp initial increase, MRMM prevalence among *M. genitalium*–positive samples declined from the first quarter of 2017 on. This finding may or may not be a consequence of the increased number of tests of cure performed during the months immediately preceding the decline. However, regardless of the reason for the decline, we believe that the rates of MRMMs in *M. genitalium* call for a recommendation to perform a test of cure after treatment of *M. genitalium* infections.

Acknowledgments

We thank NYtor for providing the necessary number of TVMGres qPCR kits free of charge.

About the Author

Dr. Martens is a resident physician in clinical microbiology at the Canisius-Wilhelmina Hospital and Radboud University Medical Centre in Nijmegen, the Netherlands. Her research interests include public health and sexual health.

References

1. Tully JG, Taylor-Robinson D, Cole RM, Rose DL. A newly discovered *Mycoplasma* in the human urogenital tract. Lancet. 1981;1:1288–91. http://dx.doi.org/10.1016/S0140-6736(81)92461-2
2. Horner PJ, Gilroy CB, Thomas BJ, Naidoo RO, Taylor-Robinson D. Association of *Mycoplasma genitalium* with acute non-gonococcal urethritis. Lancet. 1993;342:582–5. http://dx.doi.org/10.1016/0140-6736(93)91411-E
3. Jensen JS, Orsum R, Dohn B, Uldum S, Worm AM, Lind K. *Mycoplasma genitalium*: a cause of male urethritis? Genitourin Med. 1993;69:265–9.
4. Horner PJ, Martin DH. *Mycoplasma genitalium* infection in men. J Infect Dis. 2017;216(suppl_2):S396–405. http://dx.doi.org/10.1093/infdis/jix145
5. Bradshaw CS, Jensen JS, Tabrizi SN, Read TR, Garland SM, Hopkins CA, et al. Azithromycin failure in *Mycoplasma genitalium* urethritis. Emerg Infect Dis. 2006;12:1149–52. http://dx.doi.org/10.3201/eid1207.051558
6. Wiesenfeld HC, Manhart LE. *Mycoplasma genitalium* in women: current knowledge and research priorities for this recently emerged pathogen. J Infect Dis. 2017;216(suppl_2):S389–95. http://dx.doi.org/10.1093/infdis/jix198
7. Taylor-Robinson D, Jensen JS. *Mycoplasma genitalium*: from chrysalis to multicolored butterfly. Clin Microbiol Rev. 2011;24:498–514. http://dx.doi.org/10.1128/CMR.00006-11
8. Manhart LE, Jensen JS, Bradshaw CS, Golden MR, Martin DH. Efficacy of antimicrobial therapy for *Mycoplasma genitalium* infections. Clin Infect Dis. 2015;61(Suppl 8):S802–17. http://dx.doi.org/10.1093/cid/civ785
9. Gambini D, Decleva I, Lupica L, Ghislanzoni M, Cusini M, Alessi E. *Mycoplasma genitalium* in males with nongonococcal urethritis: prevalence and clinical efficacy of eradication. Sex Transm Dis. 2000;27:226–9. http://dx.doi.org/10.1097/00007435-200004000-00008
10. Wikström A, Jensen JS. *Mycoplasma genitalium*: a common cause of persistent urethritis among men treated with doxycycline. Sex Transm Infect. 2006;82:276–9. http://dx.doi.org/10.1136/sti.2005.018598
11. Stamm WE, Batteiger BE, McCormack WM, Totten PA, Sternlicht A, Kivel NM; Rifalazil Study Group. A randomized, double-blind study comparing single-dose rifalazil with single-dose azithromycin for the empirical treatment of nongonococcal urethritis in men. Sex Transm Dis. 2007;34:545–52.
12. Manhart LE, Gillespie CW, Lowens MS, Khosropour CM, Colombara DV, Golden MR, et al. Standard treatment regimens for nongonococcal urethritis have similar but declining cure rates: a randomized controlled trial. Clin Infect Dis. 2013;56:934–42. http://dx.doi.org/10.1093/cid/cis1022
13. Anagrius C, Loré B, Jensen JS. Treatment of *Mycoplasma genitalium*. Observations from a Swedish STD clinic. PLoS One. 2013;8:e61481. http://dx.doi.org/10.1371/journal.pone.0061481
14. Australasian Sexual Health Alliance. Guideline on urethritis [cited 2018 Oct 5]. http://www.sti.guidelines.org.au/syndromes/urethritis-male#management
15. De Vries HJC, Van Doornum GJJ. Toelichting bij de nieuwe samenvattingskaart 2014/2015. Diagnostiek en behandeling van seksueel overdraagbare aandoeningen. Tijdschrift voor Infectieziekten. 2014;9:9–11.
16. Centers for Disease Control and Prevention. Sexually transmitted diseases treatment guidelines [cited 2018 Oct 5]. https://www.cdc.gov/std/tg2015/default.htm
17. British Association for Sexual Health and HIV. Guideline on NGU [cited 2018 Oct 1]. https://www.bashh.org/guidelines
18. Jensen JS, Bradshaw CS, Tabrizi SN, Fairley CK, Hamasuna R. Azithromycin treatment failure in *Mycoplasma genitalium*-positive patients with nongonococcal urethritis is associated with induced macrolide resistance. Clin Infect Dis. 2008;47:1546–53. http://dx.doi.org/10.1086/593188
19. Horner P, Ingle SM, Garrett F, Blee K, Kong F, Muir P, et al. Which azithromycin regimen should be used for treating *Mycoplasma genitalium*? A meta-analysis. Sex Transm Infect. 2018;94:14–20. http://dx.doi.org/10.1136/sextrans-2016-053060
20. Netherlands Federation of General Physicians. Guideline on sexually transmitted infections [in Dutch] [cited 2018 Oct 5]. https://www.nhg.org/standaarden/volledig/nhg-standaard-het-soa-consult
21. National Institute for Public Health and the Environment. Guideline on sexually transmitted infections in government-funded STI-clinics [in Dutch] [cited 2018 Oct 5]. https://lci.rivm.nl/draaiboeken/consult-seksuele-gezondheid
22. Netherlands Association of Dermatology and Venereology. Multidisciplinary guideline on diagnostics and treatment of sexually transmitted infections [in Dutch] [cited 2018 Oct 5]. http://www.nvdv.nl/wp-content/uploads/2014/08/Definitieve-multidisciplinaire-richtlijn-soa-herziening-2018.pdf
23. Jensen JS, Björnelius E, Dohn B, Lidbrink P. Use of TaqMan 5′ nuclease real-time PCR for quantitative detection of *Mycoplasma genitalium* DNA in males with and without urethritis who were attendees at a sexually transmitted disease clinic. J Clin Microbiol. 2004;42:683–92. http://dx.doi.org/10.1128/JCM.42.2.683-692.2004
24. Twin J, Jensen JS, Bradshaw CS, Garland SM, Fairley CK, Min LY, et al. Transmission and selection of macrolide resistant *Mycoplasma genitalium* infections detected by rapid high resolution melt analysis. PLoS One. 2012;7:e35593. http://dx.doi.org/10.1371/journal.pone.0035593
25. Anderson T, Coughlan E, Werno A. *Mycoplasma genitalium* macrolide and fluoroquinolone resistance detection and clinical implications in a selected cohort in New Zealand. J Clin Microbiol. 2017;55:3242–8. http://dx.doi.org/10.1128/JCM.01087-17
26. Barberá MJ, Fernández-Huerta M, Jensen JS, Caballero E, Andreu A. *Mycoplasma genitalium* macrolide and fluoroquinolone resistance: prevalence and risk factors among a 2013–2014 cohort of patients in Barcelona, Spain. Sex Transm Dis. 2017;44:457–62. http://dx.doi.org/10.1097/OLQ.0000000000000631
27. Braam JF, Slotboom B, Van Marm S, Severs TT, Van Maarseveen NM, Van Zwet T, et al. High prevalence of the A2058T macrolide resistance-associated mutation in *Mycoplasma genitalium* strains from the Netherlands. J Antimicrob Chemother. 2017;72:1529–30. http://dx.doi.org/10.1093/jac/dkw584
28. Couldwell DL, Jalocon D, Power M, Jeoffreys NJ, Chen SCA, Lewis DA. *Mycoplasma genitalium*: high prevalence of resistance to macrolides and frequent anorectal infection in men who have sex with men in western Sydney. Sex Transm Infect. 2018;94:406–10. http://dx.doi.org/10.1136/sextrans-2017-053480
29. Gossé M, Lysvand H, Pukstad B, Nordbø SA. A novel SimpleProbe PCR assay for detection of mutations in the 23S rRNA gene associated with macrolide resistance in *Mycoplasma*

genitalium in clinical samples. J Clin Microbiol. 2016;54:2563–7. http://dx.doi.org/10.1128/JCM.01233-16
30. Gratrix J, Plitt S, Turnbull L, Smyczek P, Brandley J, Scarrott R, et al. Prevalence and antibiotic resistance of *Mycoplasma genitalium* among STI clinic attendees in western Canada: a cross-sectional analysis. BMJ Open. 2017;7:e016300. http://dx.doi.org/10.1136/bmjopen-2017-016300
31. Nijhuis RH, Severs TT, Van der Vegt DS, Van Zwet AA, Kusters JG. High levels of macrolide resistance-associated mutations in *Mycoplasma genitalium* warrant antibiotic susceptibility-guided treatment. J Antimicrob Chemother. 2015; 70:2515–8. http://dx.doi.org/10.1093/jac/dkv136
32. Read TRH, Fairley CK, Tabrizi SN, Bissessor M, Vodstrcil L, Chow EPF, et al. Azithromycin 1.5 g over 5 days compared to 1 g single dose in urethral *Mycoplasma genitalium*: impact on treatment outcome and resistance. Clin Infect Dis. 2017;64:250–6. http://dx.doi.org/10.1093/cid/ciw719
33. Salado-Rasmussen K, Jensen JS. *Mycoplasma genitalium* testing pattern and macrolide resistance: a Danish nationwide retrospective survey. Clin Infect Dis. 2014;59:24–30. http://dx.doi.org/10.1093/cid/ciu217
34. Tabrizi SN, Su J, Bradshaw CS, Fairley CK, Walker S, Tan LY, et al. Prospective evaluation of ResistancePlus MG, a new multiplex quantitative PCR assay for detection of *Mycoplasma genitalium* and macrolide resistance. J Clin Microbiol. 2017; 55:1915–9. http://dx.doi.org/10.1128/JCM.02312-16
35. Tagg KA, Jeoffreys NJ, Couldwell DL, Donald JA, Gilbert GL. Fluoroquinolone and macrolide resistance-associated mutations in *Mycoplasma genitalium.* J Clin Microbiol. 2013;51:2245–9. http://dx.doi.org/10.1128/JCM.00495-13
36. Trembizki E, Buckley C, Bletchly C, Nimmo GR, Whiley DM. High levels of macrolide-resistant *Mycoplasma genitalium* in Queensland, Australia. J Med Microbiol. 2017;66:1451–3. http://dx.doi.org/10.1099/jmm.0.000584

Address for correspondence: Ferry Hagen, Canisius-Wilhelmina Hospital, Department of Medical Microbiology and Infectious Diseases, Weg door Jonkerbos 100, NL-6532SZ Nijmegen, the Netherlands; email: f.hagen@gmail.com

Essential Role of Interferon Response in Containing Human Pathogenic Bourbon Virus

Jonas Fuchs, Tobias Straub, Maximilian Seidl, Georg Kochs

Bourbon virus (BRBV) is a recently discovered tick-transmitted viral pathogen that is prevalent in the Midwest and southern United States. Since 2014, zoonotic BRBV infections have been verified in several human cases of severe febrile illness, occasionally with fatal outcomes, indicating a possible public health threat. We analyzed the pathology of BRBV infection in mice and found a high sensitivity of the virus to the host interferon system. Infected standard laboratory mice did not show clinical signs or virus replication. However, in mice carrying defects in the type I and type II interferon system, the virus grew to high titers and caused severe pathology. In cell culture, BRBV was blocked by antiviral agents like ribavirin and favipiravir (T705). Our data suggest that persons having severe BRBV infection might have a deficiency in their innate immunity and could benefit from an already approved antiviral treatment.

In the past 4 years, several reports from the Midwest and southern United States have described the detection of a new viral pathogen, called Bourbon virus (BRBV), associated with severe febrile illness (*1*–*4*). The isolation of BRBV from patients with a history of tick bites was unexpected, because BRBV belongs to tick-transmitted viruses of the genus Thogotoviruses, which are largely unknown in the United States. However, recent tick surveillance campaigns confirmed the prevalence of BRBV in the affected region (*5*,*6*).

Thogotoviruses are influenza virus–like arboviruses with a segmented RNA genome; they are frequently isolated in Africa, the Middle East, Asia, and southern Europe and are usually associated with diseases in livestock (*7*). BRBV is genetically most similar to Dhori virus (DHOV) from India (*8*). Although serologic surveys suggest the occurrence of zoonotic transmission of Thogotoviruses, few human cases have been well-documented (*9*,*10*). However, in laboratory mice, Thogotoviruses show an aggressive systemic infection affecting mainly the liver, lungs, and spleen, leading to a fatal acute hepatitis. This severe disease progression is accompanied by a massive induction of interferon (IFN) α without an apparent protective effect (*11*,*12*).

We conducted our study with the aim to evaluate the virulence and pathogenesis of BRBV in vivo. Furthermore, we assessed the antiviral effect of the host IFN system on BRBV replication.

Materials and Methods

Biosafety and Animal Ethics

Because of the unknown health risk associated with the human BRBV isolate, all work with infectious virus was performed under Biosafety Level 3 conditions. The animals were handled in accordance with guidelines of the Federation for Laboratory Animal Science Association and the national animal welfare body of Germany. Animal experiments were performed in compliance with animal protection laws in Germany and approved by the local animal welfare committee (Regierungspraesidium Freiburg, permit no. G-15/127).

Cells and Viruses

We cultivated human lung epithelial A549 cells (ATCC CCL-185), human hepatoma Huh7 cells (*13*), human cervix carcinoma HeLa cells (ATCC CCL-2), African green monkey kidney Vero cells (ATCC CCL-81), and transformed human dermal fibroblast cultures (*14*) in Dulbecco modified eagle medium supplemented with 10% fetal calf serum at 37°C and 5% CO_2. We treated Huh7 cells with recombinant human IFN-α2a (PBL Assay Science, https://www.pblassaysci.com) or recombinant human IFN-γ (R&D Systems, https://www.rndsystems.com) 16 h before and 2 h after infection. To test antivirals, we treated Huh7 cells with ribavirin (Sigma-Aldrich, https://www.sigmaaldrich.com) or favipiravir/T-705 (BioVision, https://www.biovision.com) 2 h after infection. To evaluate the cytotoxicity of these substances, we treated uninfected cells for 48 h with the maximum doses as

Author affiliation: University of Freiburg Medical Center, Faculty of Medicine, University of Freiburg, Freiburg, Germany

DOI: https://doi.org/10.3201/eid2507.181062

described. Afterward, we assessed the viability of the cells by measuring the activity of lactate dehydrogenase in the cell culture supernatant (Pierce LDH Cytotoxicity Assay Kit; ThermoFisher, https://www.thermofisher.com). To establish a positive control, we treated cells with the lysis buffer provided by the manufacturer.

Viruses and Infection

BRBV (strain NR-50132/ATCC VR-1842) (*1*) was kindly provided by Amy J. Lambert of the Centers for Disease Control and Prevention (Fort Collins, CO, USA). We produced virus stocks of BRBV, DHOV (strain India/1313/61) (*15*), and Thogoto virus (THOV) (strain SiAr126) (*16*) on Vero cells. To perform growth kinetics, we infected the cells with BRBV at a multiplicity of infection (MOI) of 0.001 in Dulbecco modified eagle medium with 1% fetal calf serum and 20 mmol/LM HEPES. We harvested supernatants at the indicated time points. We stored virus-containing supernatants at –80°C, and we determined viral titers by using plaque assay on Vero cells.

Animal Infections

We purchased wild-type C57BL/6 mice from Janvier Labs (https://www.janvier-labs.com). We bred mice with defects in the IFN pathway (*17*) in house. We conducted all experiments by using sex- and age-matched animals (7–9 week-old mice). We infected animals intraperitoneally with BRBV or DHOV diluted in 100 μL PBS with 0.3% bovine serum albumin. Depending on the experimental setup, we measured weight, survival, and clinical score daily for each animal (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1062-App1.pdf). We euthanized the animals by using cervical dislocation at the indicated time points. To determine survival of the animals after infection, we euthanized animals if the weight loss was >25% or the mice showed severe clinical signs. We harvested organs (liver, lung, spleen, and kidney) at day 4 postinfection and homogenized them by using FastPrep Homogenizer (MP Biomedicals, https://www.mpbio.com) in PBS. After centrifugation of the supernatants at 5,000 × *g* for 10 min at 4°C, we analyzed them by using plaque assay on Vero cells. We collected whole blood from animals anesthetized with ketamine/xylazine by using heart puncture before cervical dislocation. We prepared serum samples by using incubation at 37°C for 10 min and centrifugation at 5,000 × *g* for 10 min. We used serum samples directly to determine alanine transaminases by using an alanine color endpoint assay (MaxDiscovery; Bio Scientific, http://www.biooscientific.com), or we stored the samples at –20°C. We generated postinfectious serum directed against BRBV by challenging C57BL/6 mice with 10^5 PFU/animal. Fourteen days after infection, we harvested the serum. Because of the lethality of DHOV, we used $Mx1^{+/+}$ mice for the infection and production of specific antiserum directed against DHOV, as described previously (*18*).

We performed antibody treatment of the animals by intraperitoneal injection. To deplete IFN-γ, we injected 0.5 mg of IFN-γ monoclonal antibody (mAb) (XMG1.2; Biolegend, https://www.biolegend.com) at 1 day preinfection and 2 days postinfection. We achieved blockage of the type I IFN receptor (IFNAR) by treating the mice with 1 mg of anti-IFNAR-1 mAb (MAR1–5A3; BioXCell, https://bxcell.com) at 1 day preinfection and 1 day postinfection. To deplete natural killer (NK) cells, we treated $IFNAR^{-/-}$ mice infected with 100 PFU of BRBV with 0.25 mg of NK1.1 mAb (PK136, BioXcell) at 3 days preinfection and 1 day postinfection. At 4 days postinfection, we harvested organs and used fluorescence-activated cell sorting analysis to determine virus titers and confirm the depletion of $NK1.1^+$ cells.

We administered 20 mg or 40 mg of ribavirin (5 mg/mL in 0.9% NaCl; Sigma-Aldrich, https://www.sigmaaldrich.com) per kilogram bodyweight each day intraperitoneally, starting immediately postinfection. We mock-treated control animals with 0.9% NaCl only.

For histologic analysis, we harvested the organs, which we washed once in PBS and then fixed in 4% formaldehyde in PBS overnight. For cryoprotection, we incubated organs once in 15% sucrose (in H_2O) at 4°C for 4 h and afterward in 30% sucrose at 4°C overnight. After embedding in OCT medium (Tissue-Tek; Sakura, https://www.sakuraus.com), we performed 5-μm cryosections and stained them with hematoxylin and eosin.

Western Blot Analysis and Antibodies

We infected Vero cells with the indicated viruses (MOI 0.25) for 24 h and then lysed them in Passive Lysis Buffer (Promega, https://www.promega.com). We denaturated proteins in Lämmli buffer and incubated them at 95°C for 5 min. We separated the protein lysates by using 12% SDS-polyacrylamid gel electrophoresis and transferred them onto a PVDF membrane (Millipore Sigma, http://www.emdmillipore.com). We detected viral proteins by using polyclonal mouse antisera. We used β-actin–specific rabbit antiserum (Sigma-Aldrich) as an internal control. We detected primary antibodies by using fluorescent-labeled anti-mouse secondary antibodies (LI-COR, https://www.licor.com).

To test the antiserum for virus neutralization, we prepared serial dilutions of the polyclonal mouse serum in PBS and incubated them with a fixed amount of 100 PFU of BRBV for 1 h at room temperature. To establish a control, we incubated virus with PBS or an unspecific mouse serum. We transferred the virus–serum mixture onto Vero cells and performed a plaque assay. We normalized the PFU of the antibody-treated viruses to the control virus.

Real-Time Reverse Transcription PCR

RNA was extracted (NucleoSpin RNA kit; Macherey-Nagel, https://www.mn-net.com) from infected cells and subjected to cDNA synthesis (QuantiTect Reverse Transcription Kit; QIAGEN, https://www.qiagen.com). We performed real-time reverse transcription PCR (RT-PCR) by using 10 ng cDNA in a SYBR Green assay (QuantiTect PCR Kit, QIAGEN) with primers specific for human IFN-β (Hs_IFNB1_1, QIAGEN) and human γ-actin (Hs_ACTG1_1, QIAGEN). We normalized cycle threshold values to actin (ΔCT) and plotted them relative to the ΔCT values of the mock-treated control ($2^{-\Delta\Delta CT}$). We detected viral transcripts of BRBV and DHOV by using panspecific Thogotovirus primers (FW: TTCAATGAATGYTTGGACCCAGATGC [segment 2, nucleotides 940–965]; RW: TTGWACATYCCCATGAACAT [segment 2, nucleotides 1,210–1,229]) in a conventional RT-PCR; we detected the products by using an ethidium bromide–stained agarose gel.

Statistical Analyses

We analyzed all data with Prism 7 software (GraphPad, https://www.graphpad.com). We performed statistical analysis of viral titers on log-transformed values by using a 1-way analysis of variance with a Tukey multiple comparison test (for ≥3 groups) or a 2-sided *t*-test (for 2 groups). We plotted viral titers either on a log scale (geometric mean) or log transformed on a linear scale (mean ± SD). For weight loss, we calculated SEM.

Results

BRBV Sensitivity to Type I and Type II IFN

We studied the virulence of BRBV by infection of C57BL/6 (B6) mice with high challenge doses of BRBV that did not result in disease (Figure 1, panel A), as reported previously (*8*). The lack of pathogenicity is surprising because related Thogotoviruses regularly cause generalized

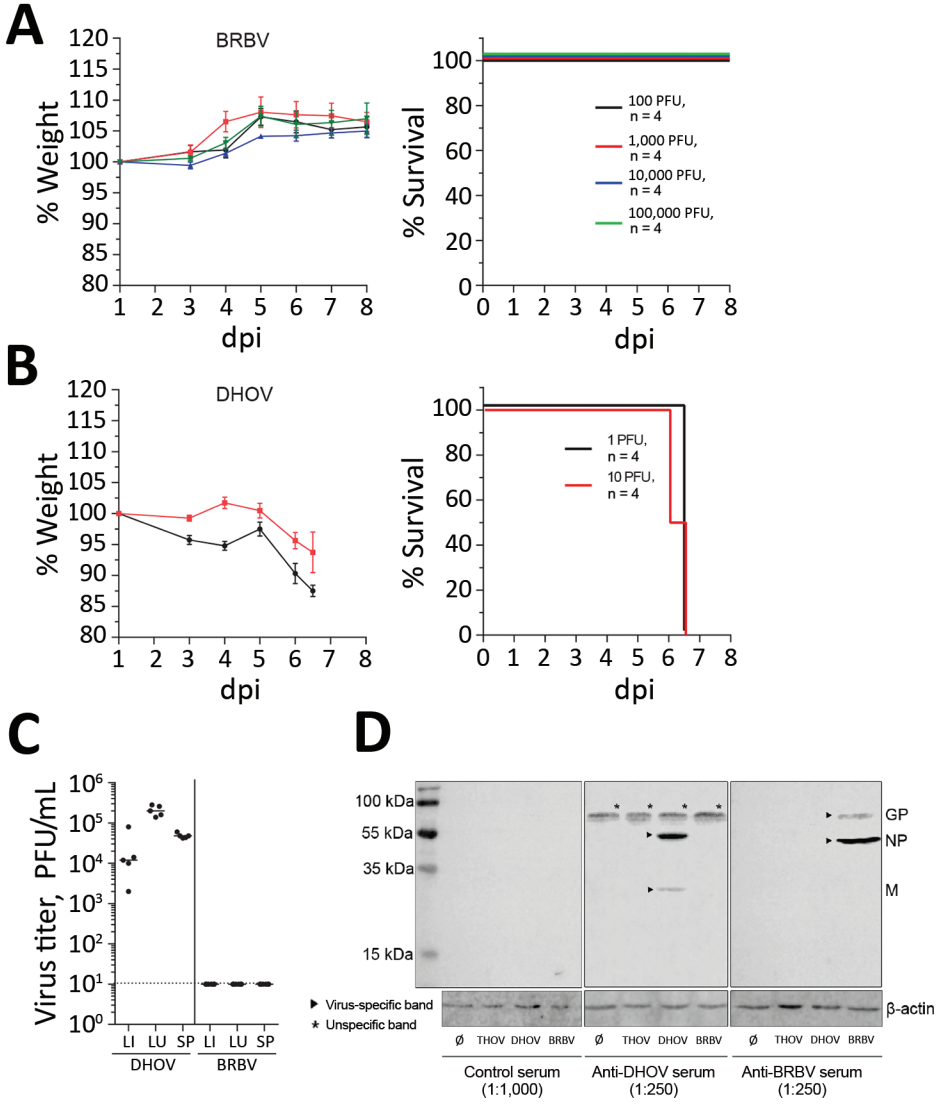


Figure 1. Results of virulence testing of BRBV in immunocompetent mice. A, B) B6 mice (n = 4) infected intraperitoneally with the indicated doses of BRBV (A) or DHOV (B) were monitored for weight (mean ±SEM) and survival. Animals were euthanized if they lost >25% body weight or showed severe illness. C) For virus growth, B6 mice (n = 5) were infected intraperitoneally with 100 PFU of DHOV and 1,000 PFU of BRBV. After 4 days, liver, lung, and spleen were harvested and a plaque assay performed. Geometric means are shown; dotted line indicates detection limit. D) Serum samples from BRBV- or DHOV-infected mice were pooled at 14 dpi. Vero cells were mock-treated or infected (multiplicity of infection 0.25) with BRBV, DHOV, and THOV and Western blot analysis of lysates from infected cells performed with antiserum at the indicated dilutions. Molecular weight and identity of the viral antigens are indicated. BRBV, Bourbon virus; DHOV, Dhori virus; dpi, days postinfection; GP, glycoprotein; LI, liver; LU, lung; M, matrix protein; NP, nucleoprotein; SP, spleen; THOV, Thogotovirus; ∅, mock-treated (control).

severe infections in IFN-competent laboratory mice (*11*). Infection of B6 mice with the closely related DHOV led to severe illness and early death (Figure 1, panel B). Virus replication was detectable in liver, lung, and spleen of DHOV-infected but not BRBV-infected animals (Figure 1, panel C). Successful BRBV infection was confirmed by seroconversion (Figure 1, panel D) and generation of neutralizing antibodies (Appendix Figure).

BRBV replicated to high titers in cells that are defective in IFN responses such as simian Vero or human Huh7 cells (*13*,*19*), as previously reported (*8*) (Figure 2, panel A). In IFN-responsive human A549 and HeLa cells, virus growth was reduced by 2 logs (Figure 2, panel A). Real-time RT-PCR analysis showed that BRBV and DHOV strongly induced IFN-β expression in A549 cells, whereas Huh7 cells were unresponsive, as reported previously (*13*). Both cell lines were efficiently infected, as demonstrated by viral PB1 gene expression (Figure 2, panel B). To confirm the role of type I IFN in the suppression of BRBV, we infected A549 cells that were stably overexpressing the IFN-antagonistic Npro protein of bovine viral diarrhea virus or the V protein of simian virus 5. Npro inhibits IFN synthesis by targeting transcription factor IRF3 (*20*), and the V protein blocks IFN signaling by targeting STAT1 for degradation (*21*). Both cultures showed enhanced BRBV replication compared with the parental A549 control cells (Figure 2, panel C), indicating that BRBV-induced IFN elicited an antiviral state that suppressed virus propagation. Enhanced growth of BRBV was also observed in transformed dermal fibroblast cultures obtained from an IFN-nonresponsive person with a genetic defect in STAT2 (*14*) (Figure 2, panel D). BRBV clearly is highly sensitive to the antiviral state induced by IFN.

We obtained similar results in vivo using IFN-nonresponsive animals. Mice lacking IFN-α/β receptor expression ($IFNAR^{-/-}$) cannot respond to type I IFN, whereas

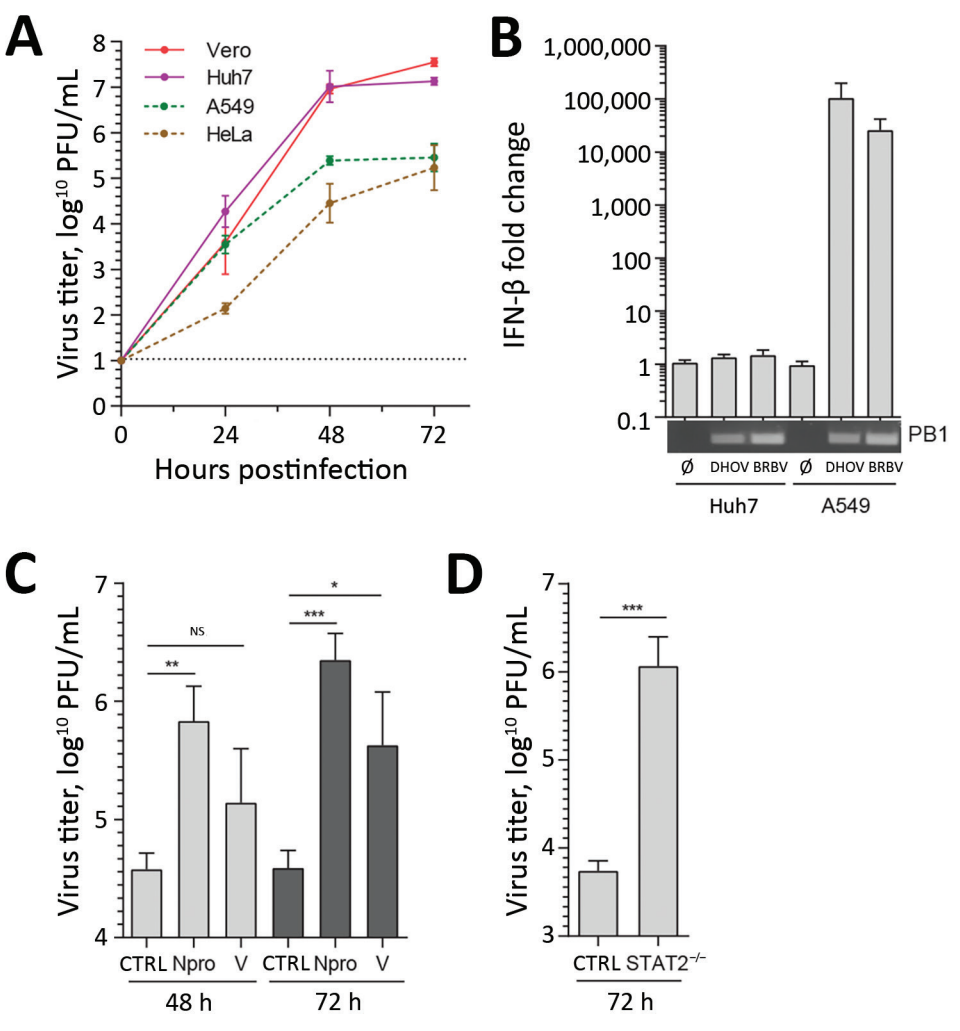


Figure 2. Results of sensitivity testing of BRBV to type I IFN–induced antiviral state in cell culture. A) Vero, Huh7, A549, and HeLa cells were infected with BRBV (multiplicity of infection [MOI] 0.001). At the indicated time points, the supernatants were harvested and viral titers determined. B) Huh7 or A549 was infected (MOI 0.25) with BRBV or DHOV for 16 h. Whole RNA was extracted and IFN-β and actin transcripts detected by real-time reverse transcription PCR. Changes in IFN-β transcripts were calculated in comparison to mock-treated cells. A conventional reverse transcription PCR assay with panspecific primers for viral segment 2 (PB1) was performed to control the infection. C, D) Defects in IFN induction or signaling enhancing BRBV propagation. Parental A549 and A549 cells stably expressing Npro of bovine viral diarrhea virus or the V protein of simian virus 5 (C), as well as (D) human skin fibroblast control cultures or with a defect in STAT2 infected with BRBV (MOI 0.001). Culture supernatants were collected and viral titers determined. Shown are the arithmetic means (±SD) of log-transformed values of 3 independent experiments. Statistical analyses were performed with a 1-way analysis of variance (Tukey multiple comparison test) (C) or a 2-tailed *t*-test (D). BRBV, Bourbon virus; CTRL, control; DHOV, Dhori virus; IFN, interferon; NS, nonsignificant; ∅, mock-treated (control). ***p<0.001; **p<0.01; *p<0.05.

mice devoid of both IFN-α/β and IFN-λ receptor expression (IFNAR$^{-/-}$ IL28R$^{-/-}$ double knockout mice) are nonresponsive to type I and type III IFNs (*17*). Although both types of mice supported BRBV growth in liver, lung, spleen, and kidney (Figure 3, panel A), virus growth was not detectable in single IL28R$^{-/-}$ mice, indicating that type III IFN plays a minor role. Unexpectedly, STAT1$^{-/-}$ mice were even more susceptible than the IFN receptor–deficient animals, and BRBV grew to higher titers (Figure 3, panel A). We reasoned that IFN-γ (type II IFN) might have a role in controlling virus replication because, like IFN-α/β, IFN-γ signaling relies on STAT1 for signal transduction and antiviral activity. We therefore treated B6 mice with monoclonal antibodies directed either against IFN-γ or against IFNAR. Blocking IFN-γ had no effect, whereas blocking IFNAR led to detectable BRBV replication in most organs tested except the liver (Figure 3, panel B).Treatment with a mixture of both antibodies massively increased virus growth in all organs, including the liver (Figure 3, panel B), indicating that type I and type II IFNs worked synergistically. The synergistic effect was also reflected in the severity of disease.

Treatment with IFNAR but not IFN-γ antibodies led to a transient drop in body weight with rapid recovery (Figure 3, panel C). In contrast, bodyweight loss in mice treated with both antibodies was dramatic (Figure 3, panel C), and the animals had to be euthanized 4 days after infection (Figure 3, panel D). The same effect was observed in IFNAR$^{-/-}$ IFNLR$^{-/-}$ double knockout mice treated with IFN-γ antibody (Figure 3, panels E–G). IFN-α/β clearly was the dominant antiviral factor controlling BRBV, but its antiviral effect was potentiated by IFN-γ, which is known to act on myeloid cells (*22*), the main target cells of Thogotoviruses (*23*). NK cells are known as the major producers of IFN-γ early during acute infections (*24*). However, depletion of NK cells by treatment of IFNAR$^{-/-}$ mice with NK1.1 antibody could not elevate virus replication (data not shown), leaving the identity of the IFN-γ–producing cell compartment unclear.

We investigated the clinical and pathologic changes in type I (IFNAR$^{-/-}$) and type I and II (STAT1$^{-/-}$) IFN–nonresponsive mice after BRBV infection (Figure 4). Wild-type mice remained asymptomatic throughout the course of infection and showed no gross abnormalities. STAT1$^{-/-}$ mice showed the most severe clinical score and became moribund, whereas IFNAR$^{-/-}$ mice were less severely affected and survived (Figure 4, panel A). Serum alanine aminotransferase levels were elevated as a sign of liver damage (Figure 4, panel B). Histologic liver sections showed massive hepatocellular destructions, including pathologic lesions and infiltrating inflammatory cells (Figure 4, panel C), consistent with acute degenerative hepatitis. Furthermore, the spleens of STAT1$^{-/-}$ animals showed massive necrosis and destruction of the normal architecture (Figure 4, panel C). We also detected infiltrations of granulocytes in lungs and kidneys of the infected STAT1$^{-/-}$ animals, indicating mild inflammation without apparent pathologic changes (data not shown).

The pathologic and clinical signs observed in mice were in some aspects compatible with the clinical manifestations described for the severe human BRBV cases in 2014 and 2017 (*1*,*3*). The mice showed a high degree of liver damage and elevated liver enzymes, as did the human patients. At later stages, the human patients had acute respiratory complications. Accordingly, the infected STAT1$^{-/-}$ mice showed high virus replication and infiltration of lymphocytes in their lungs accompanied by labored breathing. However, the prominent maculopapular rash that was described in both human patients was not observed in the STAT1$^{-/-}$ mice.

Antiviral Treatment Effect of Blocking BRBV Replication and Diminishing Pathology

As stated recently by the Centers for Disease Control and Prevention, no antiviral treatment of BRBV disease is available (*25*). Therefore, we tested the protective effect of IFN-α and IFN-γ in Huh7 cells. Treatment with IFN-α led to a 100-fold reduction in viral titers, whereas treatment with IFN-γ was less efficient. Combination of both cytokines led to >1,000-fold reduced virus titers, consistent with a synergistic mode of action (Figure 5, panel A). Treatment with the guanosine analog (ribavirin) and the guanine analog (favipiravir [T705]), both known inhibitors of viral RNA–dependent RNA polymerases (*26*,*27*), resulted in an up to 1 million–fold titer reduction (Figure 5, panels B, C). To evaluate the effect of ribavirin in combination with increasing amounts of IFN-α, we used moderate concentrations that correspond to pharmacologically relevant serum concentrations of 4–8 μmol/L for ribavirin and 100 IU/mL for IFNα (*28*) and observed an up to 1,000-fold reduction of virus replication (Figure 5, panel D). We did not observe cytotoxicity of IFN-α, IFN-γ, ribavirin, or favipiravir (T705) (Figure 5, panel E).

Our mouse models might be suitable to test new treatment regimens to curtail BRBV infections. IFNAR$^{-/-}$ and STAT1$^{-/-}$ mice were infected with a high dose of BRBV and treated daily with ribavirin. The amount of ribavirin used in these experiments was similar to that used in published experiments on influenza virus infections in mice (*26*) and the range of ribavirin treatment of hepatitis C patients (*28*). In our study, treatment reduced BRBV replication in most organs and resulted in efficient titer reductions in liver, lung, and kidney (Figure 5, panel F). Treatment also resulted in reduced weight loss (Figure 5, panel G) and a significant delay of 5 days in the mean time until death

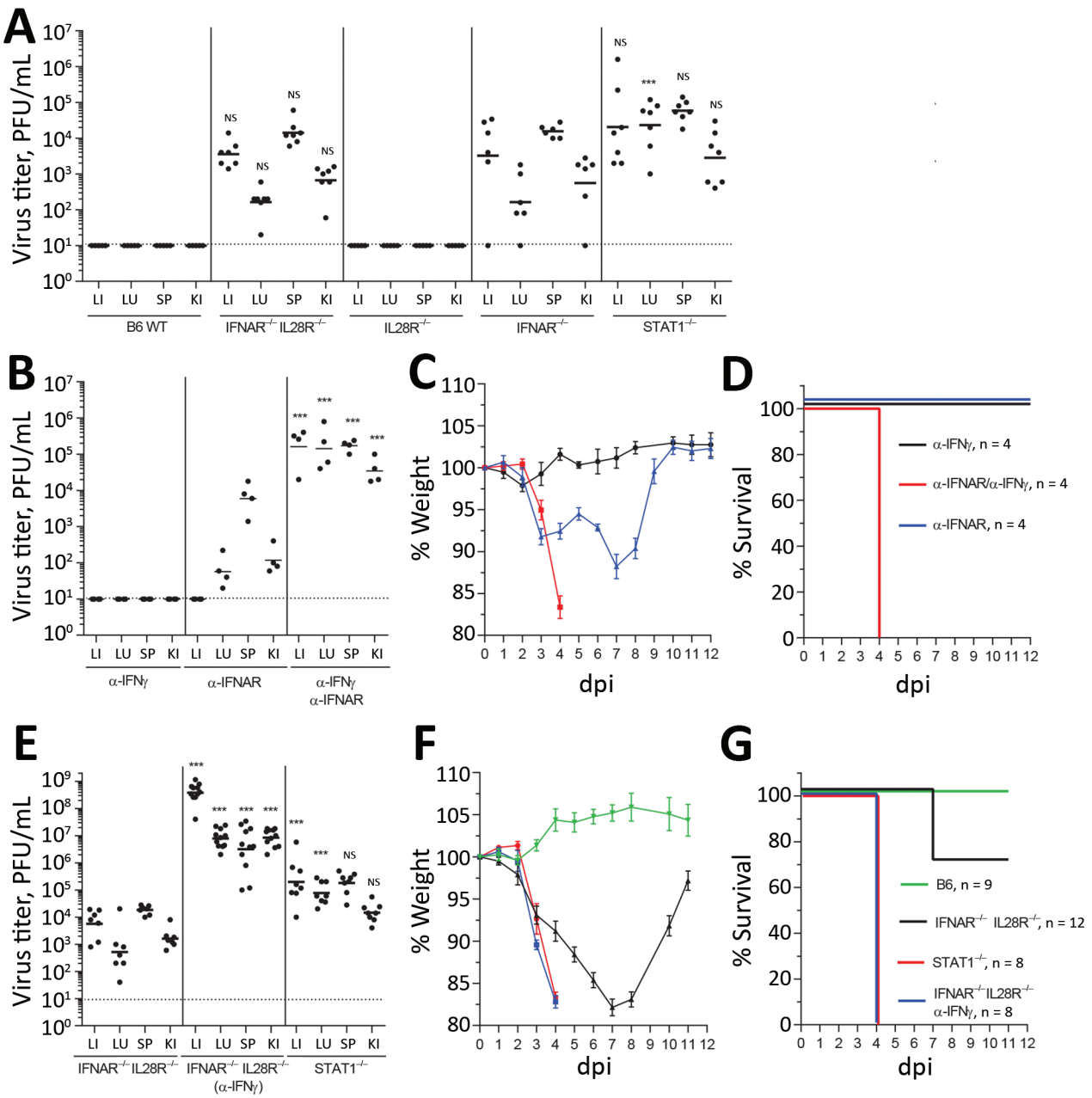


Figure 3. Immune response to BRBV in vivo. A) B6 WT (n = 5) mice or animals with a knockout in IFNAR ($IFNAR^{-/-}$) (n = 6), $IL28R^{-/-}$ (n = 6), $IFNAR^{-/-}$ $IL28R^{-/-}$ double knockout (n = 6), or $STAT1^{-/-}$ (n = 7) infected intraperitoneally with BRBV (1000 pfu). Liver, lung, spleen, and kidney were harvested at day 4 and viral titers determined. B) B6 WT animals (n = 4/group) treated with monoclonal antibodies directed against IFNAR-1 (0.5 mg/mouse 24 h before and 24 h after infection) or against IFN-γ (1 mg/mouse 24 h before and 48 h after infection) and infected with BRBV (100 PFU) for 4 d. C, D) B6 WT animals (4 per group) treated as in panel B and weight loss (mean ±SEM) and survival monitored. Animals were euthanized if they lost >25% body weight or showed severe clinical signs. E) $IFNAR^{-/-}$ $IL28R^{-/-}$ mice treated with α-IFNγ antibody (n = 11) or left untreated (n = 7) as described in panels B–D and $STAT1^{-/-}$ (n = 8) animals infected with BRBV (100 PFU). At day 4, postinfection viral titers were determined. F, G) The mice (E) together with additional B6 WT (n = 9) were monitored for weight loss (mean ±SEM) and survival as in panels C and D. In panels A, B, and E, geometric means are displayed and dotted lines indicate detection limits. Statistical analyses were performed on log-transformed values with a 1-way analysis of variance (Tukey multiple comparison test). Statistics are presented in comparison to the respective organs of $IFNAR^{-/-}$ (A), α-IFNAR (B), or $IFNAR^{-/-}$ $IL28R^{-/-}$ (E). BRBV, Bourbon virus; dpi, days postinfection; IFN, interferon; IFNAR, type I interferon receptor; KI, kidney; LI, liver; LU, lung; NS, nonsignificant; SP, spleen; WT, wild-type. ***p<0.001.

in the BRBV-infected STAT1$^{-/-}$ mice (Figure 5, panel H). Our results suggest that ribavirin, possibly in combination with pegylated IFN-α, might be an antiviral treatment option, as in the case of hepatitis C (*29*).

Discussion

Characterization of the human BRBV isolate demonstrated a surprisingly strong sensitivity to the type I and type II IFN system. Our results indicate that the few persons

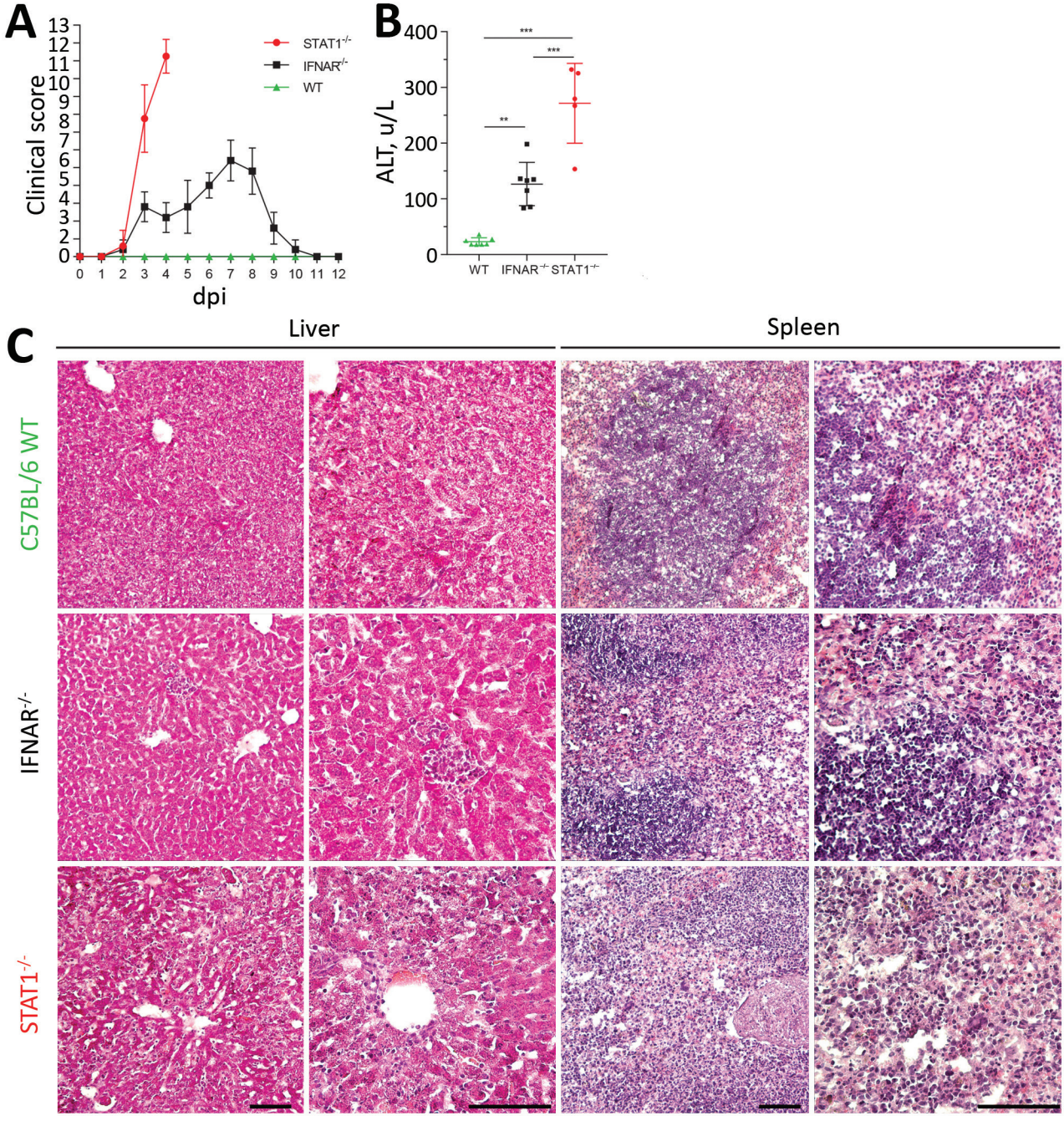


Figure 4. Pathology of BRBV-infected mice. A) B6 WT, IFNAR$^{-/-}$, and STAT1$^{-/-}$ animals (n = 5 for each group) infected intraperitoneally with 100 PFU of BRBV and monitored daily for weight and clinical signs (mean +SD); scoring system described in Appendix Table (https://wwwnc.cdc.gov/EID/article/25/7/18-1062-App1.pdf). B) Serum samples of infected B6 WT (n = 7), IFNAR$^{-/-}$ (n = 7), and STAT1$^{-/-}$ (n = 5) animals harvested at day 4 and analyzed for ALT (mean +SD). Statistical analysis was performed with a 1-way analysis of variance (Tukey multiple comparison test). C) Histologic results showing inflammatory organ damage at 4 dpi. Organs were fixed in 4% formaldehyde and embedded in OCT medium (Tissue-Tek; Sakura, https://www.sakuraus.com); cryosections were then stained with hematoxylin and eosin. Scale bars indicate 100 μm. ALT, alanine aminotransferase; BRBV, Bourbon virus; dpi, days postinfection; IFNAR, type I interferon receptor; WT, wild-type. ***p<0.001; **p<0.01.

to date who had a severe BRBV infection might have had an inborne or transient weakness in their innate antiviral immune response. Also, in case of an acute symptomatic infection with BRBV, our study provides a potential therapeutic option based on the long-approved treatment with ribavirin, possibly in combination with IFN-α.

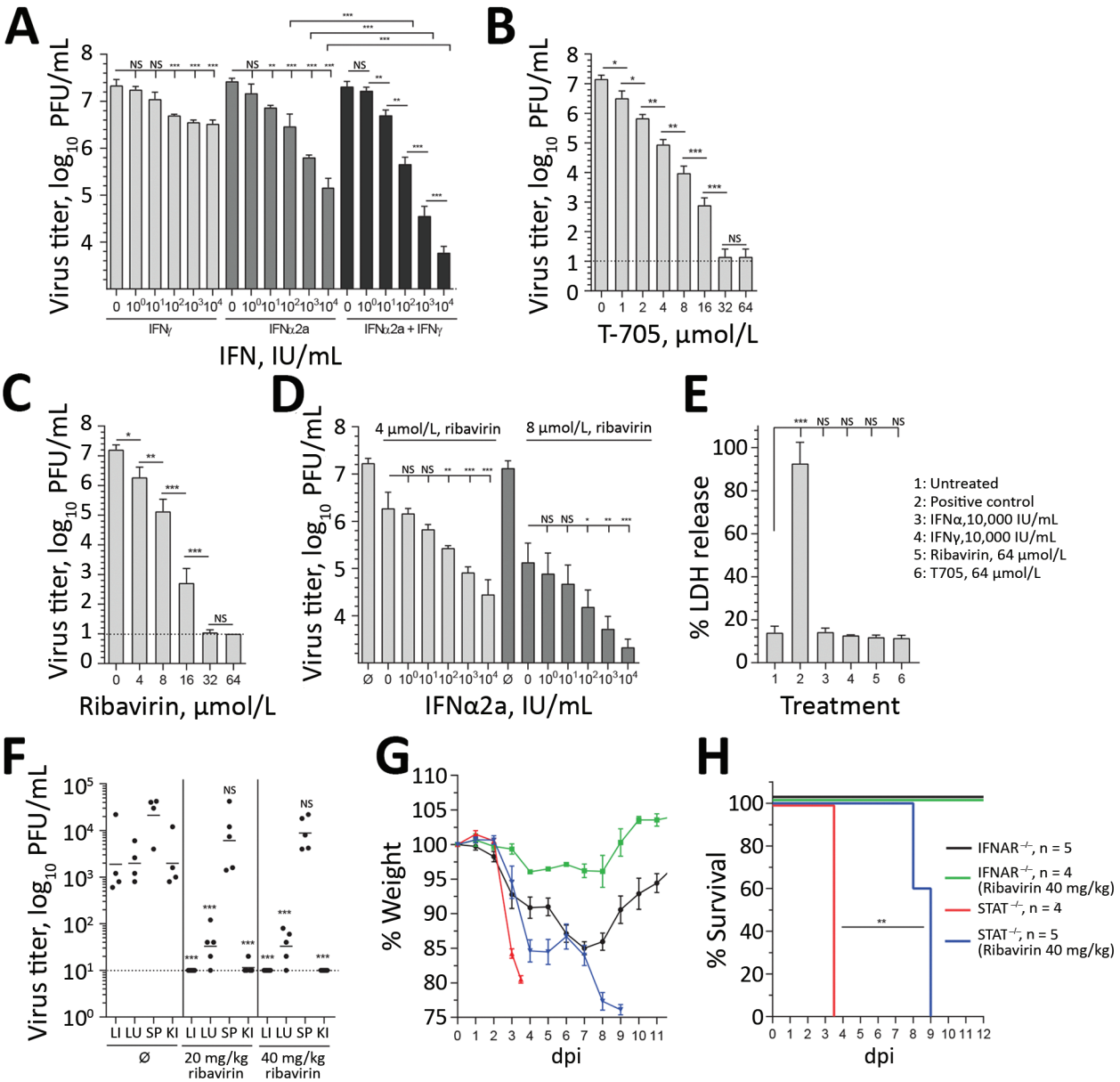


Figure 5. Antiviral treatment against BRBV. A–D) Huh7 cells infected with BRBV (multiplicity of infection 0.001) had viral titers determined at 48 hpi. Shown are the arithmetic means (±SD) of log-transformed values of 3 independent experiments. The cells were treated with increasing amounts of IFN-α2a, IFN-γ, or equal amounts of both IFNs 16 h prior and 2 hpi (A); increasing amounts of the antiviral drugs favipiravir (T705) and ribavirin 2 hpi (B, C); or a combination of ribavirin and IFN-α2a (D). E) To evaluate the cytotoxicity of these compounds, cells were treated with the indicated concentrations for 48 h, or as a positive control the cells were treated with lysis buffer. LDH activity in the supernatant was determined (normalized to positive control [n = 3, mean ±SD]). Statistical analyses were performed with a 1-way analysis of variance (Tukey multiple comparison test). F) IFNAR$^{-/-}$ animals (n = 5) treated by intraperitoneal injection with 0.9% NaCl (mock-treated) or 20 or 40 mg/kg/d ribavirin starting 4 hpi with 1,000 PFU of BRBV. At 4 dpi, viral titers were determined in liver, lung, spleen, and kidney. G, H) IFNAR$^{-/-}$ or STAT1$^{-/-}$ mice treated until day 7 dpi with ribavirin (40 mg/kg/d) as in panel F. Weight (mean ±SEM) and survival were monitored daily. The animals were euthanized if they lost >25% bodyweight or showed signs of severe illness. H) Statistical analysis for the survival curve performed with a log-rank (Mantel-Cox) test. BRBV, Bourbon virus; dpi, days postinfection; IFN, interferon; IFNAR, type I interferon receptor; hpi, hours postinfection; KI, kidney; LI, liver; LU, lung; LDH, lactate dehydrogenase; NS, nonsignificant; SP, spleen; ∅, mock-treated (control). ***p<0.001; **p<0.01; *p<0.05.

Future analysis of the seroprevalence to BRBV in humans is urgently needed to evaluate the zoonotic spread of the virus in the affected area.

Acknowledgments

We would like to thank our colleagues from the Centers for Disease Control and Prevention's Division of Vector-Borne Disease, National Center for Emerging and Zoonotic Infectious Diseases (Fort Collins, CO, USA), for providing us with a seed stock of Bourbon virus; Rick Randall (University of St. Andrews) for his constant support and for providing the transduced A549 cell lines; Sophie Hambleton (Newcastle University Medical School) for the STAT2-deficient fibroblast cultures; and Peter Staeheli for his support with the in vivo experiments. We also thank our colleagues Otto Haller, Peter Staeheli, Martin Schwemmle, and Laura Graf for their critical reviews of our manuscript.

The work was funded by Germany's National Platform on Zoonoses Research and Federal Ministry of Food and Agriculture through the Federal Office for Agriculture and Food (grant no. 2816HS008) and by the German Research Foundation (Deutsche Forschungsgemeinschaft) (grant no. KO 1579/12-1).

This work was conducted by J.F. in partial fulfillment of the requirements for a PhD degree from the Faculty of Biology of the University of Freiburg, Germany.

About the Author

Mr. Fuchs is a PhD student at the Institute of Virology at the Medical Center of the University of Freiburg, Germany. His research interests are vectorborne viral zoonoses and their interplay with the hosts' innate immune systems.

References

1. Kosoy OI, Lambert AJ, Hawkinson DJ, Pastula DM, Goldsmith CS, Hunt DC, et al. Novel Thogotovirus associated with febrile illness and death, United States, 2014. Emerg Infect Dis. 2015;21:760–4. http://dx.doi.org/10.3201/eid2105.150150
2. Charles M. Rare tick disease found in Payne County. Stillwater News Press [cited 2018 Apr 9]. https://www.stwnewspress.com/news/rare-tick-disease-found-in-payne-county/article_14ee1140-04f2-11e5-966e-6b24d85953ee.html
3. Miller M. Bourbon virus linked to death of park official. Missourian [cited 2018 Apr 9]. http://www.emissourian.com/local_news/county/bourbon-virus-linked-to-death-of-park-official–/article_057f0f0a-bddd-5f7a-aa0d-735187e4c379.html
4. Miller M. Bourbon virus—now listed as emerging illness. Missourian [cited 2018 Apr 9]. http://www.emissourian.com/local_news/county/bourbon-virus-now-listed-as-emerging-illness-new-case-in/article_5d67a0e6-bf55-5cfb-9e6c-9b73896fafad.html
5. Savage HM, Burkhalter KL, Godsey MS Jr, Panella NA, Ashley DC, Nicholson WL, et al. Bourbon virus in field-collected ticks, Missouri, USA. Emerg Infect Dis. 2017;23:2017–22. http://dx.doi.org/10.3201/eid2312.170532
6. Savage HM, Godsey MS Jr, Panella NA, Burkhalter KL, Manford J, Trevino-Garrison IC, et al. Surveillance for tick-borne viruses near the location of a fatal human case of Bourbon virus (family Orthomyxoviridae: genus Thogotovirus) in eastern Kansas, 2015. J Med Entomol. 2018;55:701–5. http://dx.doi.org/10.1093/jme/tjx251
7. McCauley JW, Hongo S, Kaverin NV, Kochs G, Lamb RA, Matrosovich MN, Perez DR, et al. Orthomyxoviridae. In: King AMQ, Lefkowitz E, Adams MJ, Carstens EB, eds. Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses. San Diego: Elsevier; 2012. p. 749–61.
8. Lambert AJ, Velez JO, Brault AC, Calvert AE, Bell-Sakyi L, Bosco-Lauth AM, et al. Molecular, serological and in vitro culture-based characterization of Bourbon virus, a newly described human pathogen of the genus Thogotovirus. J Clin Virol. 2015;73:127–32. http://dx.doi.org/10.1016/j.jcv.2015.10.021
9. Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, et al. Arthropod-borne viral infections of man in Nigeria, 1964–1970. Ann Trop Med Parasitol. 1975;69:49–64. http://dx.doi.org/10.1080/00034983.1975.11686983
10. Butenko AM, Leshchinskaia EV, Semashko IV, Donets MA, Mart'ianova LI. Dhori virus—a causative agent of human disease. 5 cases of laboratory infection [in Russian]. Vopr Virusol. 1987;32:724–9.
11. Mateo RI, Xiao SY, Lei H, DA Rosa AP, Tesh RB. Dhori virus (Orthomyxoviridae: Thogotovirus) infection in mice: a model of the pathogenesis of severe orthomyxovirus infection. Am J Trop Med Hyg. 2007;76:785–90. http://dx.doi.org/10.4269/ajtmh.2007.76.785
12. Li G, Wang N, Guzman H, Sbrana E, Yoshikawa T, Tseng CT, et al. Dhori virus (Orthomyxoviridae: Thogotovirus) infection of mice produces a disease and cytokine response pattern similar to that of highly virulent influenza A (H5N1) virus infection in humans. Am J Trop Med Hyg. 2008;78:675–80. http://dx.doi.org/10.4269/ajtmh.2008.78.675
13. Lanford RE, Guerra B, Lee H, Averett DR, Pfeiffer B, Chavez D, et al. Antiviral effect and virus-host interactions in response to alpha interferon, gamma interferon, poly(i)-poly(c), tumor necrosis factor alpha, and ribavirin in hepatitis C virus subgenomic replicons. J Virol. 2003;77:1092–104. http://dx.doi.org/10.1128/JVI.77.2.1092-1104.2003
14. Hambleton S, Goodbourn S, Young DF, Dickinson P, Mohamad SM, Valappil M, et al. STAT2 deficiency and susceptibility to viral illness in humans. Proc Natl Acad Sci U S A. 2013;110:3053–8. http://dx.doi.org/10.1073/pnas.1220098110
15. Clay WC, Fuller FJ. Nucleotide sequence of the tick-borne orthomyxo-like Dhori/India/1313/61 virus membrane protein gene. J Gen Virol. 1992;73:2609–16. http://dx.doi.org/10.1099/0022-1317-73-10-2609
16. Albanese M, Bruno-Smiraglia C, Di di Cuonzo G, Lavagnino A, Srihongse S. Isolation of Thogoto virus from *Rhipicephalus bursa* ticks in western Sicily. Acta Virol. 1972;16:267.
17. Mordstein M, Kochs G, Dumoutier L, Renauld JC, Paludan SR, Klucher K, et al. Interferon-lambda contributes to innate immunity of mice against influenza A virus but not against hepatotropic viruses. PLoS Pathog. 2008;4:e1000151. http://dx.doi.org/10.1371/journal.ppat.1000151
18. Thimme R, Frese M, Kochs G, Haller O. *Mx1* but not *MxA* confers resistance against tick-borne Dhori virus in mice. Virology. 1995;211:296–301. http://dx.doi.org/10.1006/viro.1995.1404
19. Mosca JD, Pitha PM. Transcriptional and posttranscriptional regulation of exogenous human beta interferon gene in simian cells defective in interferon synthesis. Mol Cell Biol. 1986;6:2279–83. http://dx.doi.org/10.1128/MCB.6.6.2279
20. Hilton L, Moganeradj K, Zhang G, Chen YH, Randall RE, McCauley JW, et al. The NPro product of bovine viral diarrhea virus inhibits DNA binding by interferon regulatory factor 3 and

targets it for proteasomal degradation. J Virol. 2006;80:11723–32. http://dx.doi.org/10.1128/JVI.01145-06
21. Didcock L, Young DF, Goodbourn S, Randall RE. The V protein of simian virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. J Virol. 1999;73:9928–33.
22. Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc Biol. 2004;75:163–89. http://dx.doi.org/10.1189/jlb.0603252
23. Kochs G, Anzaghe M, Kronhart S, Wagner V, Gogesch P, Scheu S, et al. in vivo conditions enable IFNAR-independent type I interferon production by peritoneal CD11b+ cells upon Thogoto virus infection. J Virol. 2016;90:9330–7. http://dx.doi.org/10.1128/JVI.00744-16
24. Biron CA, Brossay L. NK cells and NKT cells in innate defense against viral infections. Curr Opin Immunol. 2001;13:458–64. http://dx.doi.org/10.1016/S0952-7915(00)00241-7
25. Centers for Disease Control and Prevention. Bourbon virus [cited 2018 Apr 9]. https://www.cdc.gov/ncezid/dvbd/bourbon/index.html
26. Rowe T, Banner D, Farooqui A, Ng DC, Kelvin AA, Rubino S, et al. In vivo ribavirin activity against severe pandemic H1N1 influenza A/Mexico/4108/2009. J Gen Virol. 2010;91:2898–906. http://dx.doi.org/10.1099/vir.0.024323-0
27. Furuta Y, Gowen BB, Takahashi K, Shiraki K, Smee DF, Barnard DL. Favipiravir (T-705), a novel viral RNA polymerase inhibitor. Antiviral Res. 2013;100:446–54. http://dx.doi.org/10.1016/j.antiviral.2013.09.015
28. Tsubota A, Akuta N, Suzuki F, Suzuki Y, Someya T, Kobayashi M, et al. Viral dynamics and pharmacokinetics in combined interferon alfa-2b and ribavirin therapy for patients infected with hepatitis C virus of genotype 1b and high pretreatment viral load. Intervirology. 2002;45:33–42. http://dx.doi.org/10.1159/000050085
29. Feld JJ, Hoofnagle JH. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature. 2005;436:967–72. http://dx.doi.org/10.1038/nature04082

Address for correspondence: Georg Kochs, University of Freiburg, Institute of Virology, University Medical Center, Hermann-Herder-Strasse 11, 79104 Freiburg, Germany; email: georg.kochs@uniklinik-freiburg.de

Mitochondrial Junction Region as Genotyping Marker for *Cyclospora cayetanensis*

Fernanda S. Nascimento, John R. Barta, Julia Whale, Jessica N. Hofstetter, Shannon Casillas, Joel Barratt, Eldin Talundzic, Michael J. Arrowood, Yvonne Qvarnstrom

Cyclosporiasis is an infection caused by *Cyclospora cayetanensis*, which is acquired by consumption of contaminated fresh food or water. In the United States, cases of cyclosporiasis are often associated with foodborne outbreaks linked to imported fresh produce or travel to disease-endemic countries. Epidemiologic investigation has been the primary method for linking outbreak cases. A molecular typing marker that can identify genetically related samples would be helpful in tracking outbreaks. We evaluated the mitochondrial junction region as a potential genotyping marker. We tested stool samples from 134 laboratory-confirmed cases in the United States by using PCR and Sanger sequencing. All but 2 samples were successfully typed and divided into 14 sequence types. Typing results were identical among samples within each epidemiologically defined case cluster for 7 of 10 clusters. These findings suggest that this marker can distinguish between distinct case clusters and might be helpful during cyclosporiasis outbreak investigations.

Cyclospora cayetanensis is a coccidian parasite that causes human cyclosporiasis, an enteric infection associated with consumption of fecally contaminated fresh food or water harboring sporulated oocysts of this parasite. Cyclosporiasis most commonly occurs in tropical and subtropical regions (*1*). Cases in temperate regions are often associated with travel to countries where the disease is endemic or with foodborne outbreaks linked to various types of imported fresh produce (*2–4*). Cases in Canada and the United Kingdom have in recent years been increasingly associated with travel to the Riviera Maya and Cancun areas in Mexico (*5*,*6*).

In 2017, the Centers for Disease Control and Prevention was notified of 1,065 laboratory-confirmed cases of cyclosporiasis in the United States, of which ≥56% were domestically acquired (*7*). A case–control study identified green onions as being strongly associated with cyclosporiasis cases among 16 persons who dined at a Mediterranean-style restaurant chain in the Houston, Texas, area in 2017 (*8*). However, despite extensive epidemiologic investigation and trace-back efforts, the specific exposures associated with most of the cases in 2017 were not identified. The time lag between exposure to the contaminated source, the onset of clinical symptoms, and the epidemiologic investigation can be several weeks. Consequently, case-patients might be asked to recall relevant food exposure weeks to months before the interview and may not recall specific food exposures or identify ingredients included in certain dishes.

A validated molecular typing marker could help to improve our understanding of cyclosporiasis epidemiology and facilitate identification and investigation of disease clusters. Recent advances in next-generation sequencing have enabled whole-genome sequencing of the *C. cayetanensis* parasite (*9*,*10*), including its organellar genomes derived from the apicoplast (*11*,*12*) and mitochondrion (*12–14*). These advances facilitated development of a multilocus sequence typing (MLST) method based on 5 microsatellites. However, when this method was applied to stool samples, data were successfully obtained for all 5 loci for <60% of samples (*15*,*16*). In addition, the epidemiologic usefulness of the MLST method in outbreak investigations is currently unknown.

C. cayetanensis is a member of the phylum Apicomplexa. Its mitochondrial genome is ≈6.3 kb and is a linear molecule with ≥2 copies arranged in a concatemeric structure with a head-tail configuration (*12–14*). Comparison of the mitochondrial genomes of *C. cayetanensis* isolates from the United States and China showed only minor sequence variations (*12*). However, mitochondrial genomes from different isolates vary in length and seem to have a greater amount of variation in the junction area between the genome copies (*17*). The purpose of this study was to explore the sequence variation of this junction area of the mitochondrial genome and evaluate it as a potential typing marker for linking cyclosporiasis cases.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (F.S. Nascimento, J.N. Hofstetter, S. Casillas, J. Barratt, E. Talundzic, M.J. Arrowood, Y. Qvarnstrom); University of Guelph, Guelph, Ontario, Canada (J.R. Barta, J. Whale)

DOI: https://doi.org/10.3201/eid2507.141447

Table 1. *Cyclospora cayetanensis* mitochondrial junction types identified among 132 samples collected in different states, United States, 2013–2016*

Mitochondrial junction type	No. samples	Collection year (state)
Cmt154.A	50	2013 (TX); 2014 (MI, SC, TX); 2015 (GA, IL, TX, WI); 2016 (FL, GA, NE, TX)
Cmt154.B	34	2014–2016 (TX); 2016 (NE)
Cmt154.C	2	2013 (TX); 2015 (TX)
Cmt154.D	1	2015 (TX)
Cmt169.A	12	2013 (FL, TX); 2014 (MA, OH, PA)
Cmt169.B	7	2014–2016 (TX); 2015 (WI)
Cmt184.A	6	2013 (IA)
Cmt184.B	7	2014 (MA, MI, PA, TX); 2016 (FL)
Cmt184.C	5	2014 (ME); 2015 (TX)
Cmt184.D	3	2014 (MI, TX); 2016 (NE)
Cmt184.E	1	2013 (TX)
Cmt199.A	2	2014 (TX), 2016 (NE)
Cmt199.B	1	2014 (MA)
Cmt199.C	1	2016 (FL)

*Cmt, *Cyclospora* mitochondrial junction.

Methods

Sample Collection

Stool samples from 134 patients given a diagnosis of cyclosporiasis during 2013–2016 were sent to the Centers for Disease Control and Prevention from state public health laboratories in the United States for confirmatory diagnostic testing or as part of a research study. The samples had been collected in PCR-friendly stool preservatives (e.g., Zn-PVA) or transport medium (e.g., Cary-Blair) and were confirmed positive for *Cyclospora* sp. parasites by ultraviolet fluorescence microscopy (*18*). The samples were collected in the following states and years: Florida (n = 1), Iowa (n = 7), and Texas (n = 6), 2013; Maine (n = 4), Massachusetts (n = 5), Michigan (n = 6), Ohio (n = 1), Pennsylvania (n = 2), South Carolina (n = 3), and Texas (n = 24), 2014; Georgia (n = 1), Illinois (n = 1), Texas (n = 42) and Wisconsin (n = 6), 2015; and Florida (n = 4), Georgia (n = 1), Nebraska (n = 7), and Texas (n = 13), 2016.

Epidemiologic Investigations and Classification

We defined an outbreak as ≥2 epidemiologically linked cases (e.g., a cluster of cases in persons linked to a restaurant, grocery store, or social event). We defined a temporospatial cluster as cases that occurred in the same geographic area (e.g., in the same community or town) and had illness onset dates around the same time (e.g., within ≈15 days of each other). Epidemiologic evidence for linking cases with common exposures (e.g., restaurant, grocery store, or social events) is typically stronger than for temporospatial clusters. We defined an international travel–associated case as a case in a person who spent ≥1 day during their pertinent incubation period (i.e., 14 days before illness onset) outside the United States.

DNA Extraction and Molecular Detection

We washed 2 mL of each stool twice with phosphate-buffered saline, pH 7.4, and used 500 μL of the feces for DNA extraction by using the UNEX method, as described elsewhere (*19*). We amplified the mitochondrial junction region in a 25-μL PCR by using the NEBNext Q5 Hot Start HiFi PCR Master Mix (New England Biolabs, https://www.neb.com), 400 nmol/L of each of the forward (cyclo_mit-100F: TACCAAAGCATCCATCTACAGC) and reverse (cyclo_mit-54R: CCCAAGCAATCGGATCGTGTT) primers, and 1 μL of the DNA sample. The cycling conditions were 98°C for 2 min, followed by 35 cycles of 98°C for 15 s, 66°C for 15 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. PCR products of ≈200 bp were visualized by electrophoresis on a 1.5% agarose gel stained with ethidium bromide. We purified the PCR products by using the Monarch PCR and DNA Cleanup Kit (New England Biolabs) and sequenced them on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, https://www.thermofisher.com) in both directions by using the PCR primers and BigDye Terminator V3.1 chemistry (Applied Biosystems). We used the DyeEx 2.0 Spin Kit (QIAGEN, https://www.qiagen.com) to remove unincorporated dyes.

Data Analysis and Sequences

We aligned forward and reverse sequence reads by using the MAFFT version 7.222 (*20*) plug-in in Geneious R11 (*21*). The variant types of the mitochondrial junction are available in GenBank (accession nos. MH430075–88).

Ethics

We used stool samples in accordance with the Human Subjects Research Protocol (use of coded specimens for *Cyclospora* genomics research). This protocol was approved by the Human Research Protection Office in the Center for Global Health, Centers for Disease Control and Prevention (#2014–107).

Results

We amplified the mitochondrial junction region from 133 (99%) of 134 samples from patients with confirmed

diagnosis of cyclosporiasis; 1 sample from Iowa did not show any visible band after amplification. Sanger sequencing from 132 of these samples generated data of sufficient quality for analysis in both forward and reverse direction; 1 sample from Michigan did not produce readable sequences. The mitochondrial junction region of *C. cayetanensis* exhibited a high degree of variability between samples because of 3 variations of a 15-nt motif referred to as type I, TAGTATTATTTA**T**AA; type II, TAGTATTATTTT**T**AA; and type III, TAGTATTATTT**TA**AA (variant nucleotides are shown in bold) (Appendix Figure, https://wwwnc.cdc.gov/EID/article/25/7/18-1447-App1.pdf). These repeats were present in 2–5 copies in various combinations and resulted in different lengths and composition of the mitochondrial junction. On the basis of the number of repeats, we divided sequences into 4 main groups designated Cmt154, Cmt169, Cmt184, and Cmt199. Each main group could be further divided into 2–5 sequence types on the basis of the repeat motifs and 3 single-nucleotide polymorphisms (SNPs) present downstream of the repeat region. The sequence types were designated with an arbitrary letter following the group number (e.g., Cmt154.A, Cmt154.B). The combination of repeat motifs and SNPs resulted in 14 unique mitochondrial junction sequence (Cmt) types among the 132 samples analyzed (Table 1).

We determined the relationship between different Cmt sequences and their distribution among samples analyzed from epidemiologically linked or sporadic cases (Figure). This information includes all Cmt types publicly available in GenBank as of August 2018, including type Cmt214.A, which is the longest type described so far but was not encountered in this study. The Cmt types have 2–6 copies of

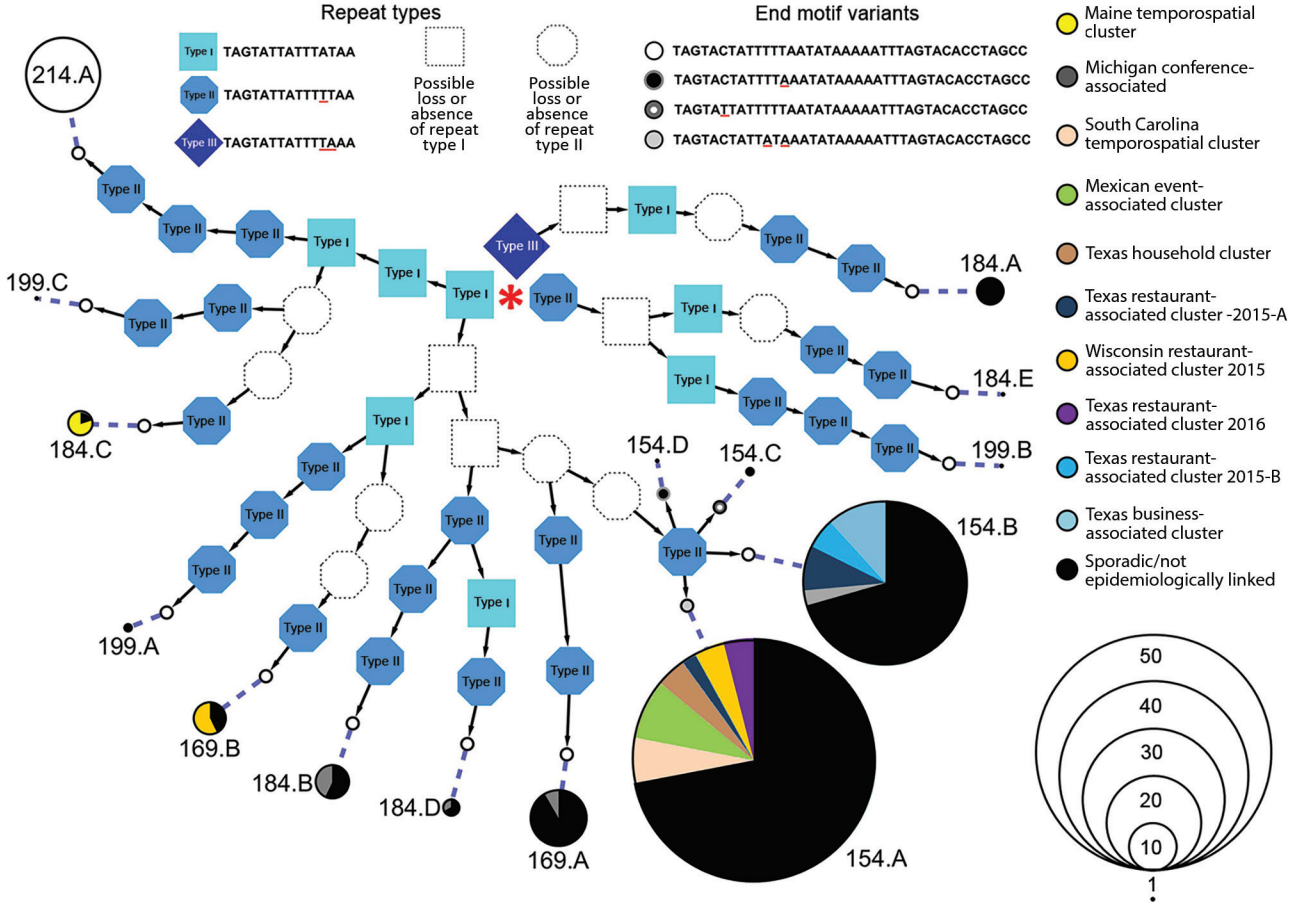


Figure. Relationships between detected *Cyclospora* mitochondrial junction (Cmt) types, United States. Fourteen unique Cmt types were detected. Cmt214.A (top left) was not detected in this study but was reported previously (GenBank accession no. MH430089.1); it represents the type with the largest number of 15-mer repeats (total 6) and is therefore included as reference for comparison. Three different 15-mer repeat sequences are known, and each Cmt type possesses 2–6 of these 15-mer repeats in various combinations. The sequence of each mt junction type can be elucidated from this figure starting with the first repeat, indicated by the red central asterisk, and then following the arrows to the end motif. A dashed line links the sequence to a pie chart that provides epidemiologic information. The size of the pie chart represents the number of times this particular Cmt type was detected. For instance, type 154.A was detected in 50 samples (as reflected by the scale) and represents the most common type. Red underlined letters indicate variable sites that exist in the end motif and 15-mer repeats.

the 3 different 15-mer repeats in various combinations. The predominant type, Cmt154.A, was found in 50 samples in this study, including 16 case-patients with a travel history to Mexico, 1 case-patient with a travel history to Costa Rica, and 14 case-patients linked to outbreaks/clusters in South Carolina (2014), Texas (2015–2016), and Wisconsin (2015). A total of 34 samples typed as Cmt154.B, including 11 samples from patients with a travel history to Mexico, 9 cases linked to several restaurant-associated outbreaks in Texas (2015), and 1 case linked to an event-associated outbreak in Michigan (2014). We also provide detailed typing and epidemiologic information for all 132 samples (Appendix Table).

A total of 37 of the analyzed samples were epidemiologically associated with 10 outbreaks or temporospatial case clusters (Table 2). Seven of these clusters had identical typing results among the samples within each cluster: 2 temporospatial clusters in South Carolina and Maine in 2014, an event in Mexico in 2015, a Texas household in 2015, and 3 restaurant outbreaks in Texas (2 in 2015 and 1 in 2016). Conversely, 2 restaurant-associated outbreaks in Wisconsin and Texas in 2015, and an event-associated outbreak in Michigan in 2014 had ≥2 types identified within each cluster.

Discussion

We investigated DNA sequence variations in the short junction segment of the mitochondrial genome in *C. cayetanensis* parasites. We distinguished 14 Cmt types among 132 samples collected in the United States during 2013–2016 on the basis of sequence length and the SNPs in this region. The variability of the mitochondrial junction region detected in our study adds to the current knowledge of the structure of the *C. cayetanensis* mitochondrial genome. A

Table 2. Distribution of *Cyclospora cayetanensis* mitochondrial junction types detected in epidemiologically linked samples, United States*

Collection state and year	Epidemiologic known link to case cluster/outbreak	Sample no.	International travel within 2 weeks before symptom onset	Cmt type
Maine 2014	Maine temporospatial cluster†	HCME548_14	No	Cmt184.C
		HCME550_14	No	Cmt184.C
		HCME552_14	No	Cmt184.C
		HCME298_14	No	Cmt184.C
Michigan 2014	Michigan event-associated cluster	HCMI030_14	Unknown	Cmt154.B
		HCMI040_14	No	Cmt184.D
		HCMI029_14	No	Cmt184.B
		HCMI039_14	Unknown	Cmt184.B
Pennsylvania 2014		HCPA556_14	No	Cmt184.B
		HCPA962_14	Unknown	Cmt169.A
South Carolina 2014	South Carolina temporospatial cluster†	HCSC052_14	No	Cmt154.A
		HCSC053_14	No	Cmt154.A
		HCSC054_14	No	Cmt154.A
Texas 2015	Mexican event-associated cluster	HCTX208_15	Mexico/Tulum	Cmt154.A
		HCTX219_15	Mexico/Tulum	Cmt154.A
		HCTX220_15	Mexico/Tulum	Cmt154.A
		HCTX547_15	Mexico/Tulum	Cmt154.A
	Texas household cluster‡	HCTX354_15	Mexico/Riviera Maya	Cmt154.A
		HCTX355_15	Mexico/Riviera Maya	Cmt154.A
	Texas restaurant-associated cluster 2015-A	HCTX353_15	No	Cmt154.A
		HCTX540_15	No	Cmt154.B
		HCTX551_15	No	Cmt154.B
		HCTX555_15	No	Cmt154.B
	Texas restaurant-associated cluster 2015-B	HCTX356_15	No	Cmt154.B
		HCTX357_15	No	Cmt154.B
	Texas local business-associated cluster	HCTX204_15	Mexico/Cozumel	Cmt154.B
		HCTX205_15	No	Cmt154.B
		HCTX206_15	No	Cmt154.B
		HCTX538_15	No	Cmt154.B
Wisconsin 2015	Wisconsin restaurant-associated cluster 2015	HCWI001_15	No	Cmt154.A
		HCWI003_15	No	Cmt154.A
		HCWI002_15	No	Cmt169.B
		HCWI004_15	No	Cmt169.B
		HCWI005_15	No	Cmt169.B
		HCWI006_15	No	Cmt169.B
Texas 2016	Texas restaurant-associated cluster 2016	HCTX471_16	No	Cmt154.A
		HCTX474_16	No	Cmt154.A

*Cmt, C*yclospora* mitochondrial junction.
†The terminology temporospatial cluster is used here for cases that were not linked to a particular establishment or event but were temporally and geographically clustered.
‡Case-patients were a married couple who traveled together to Riviera Maya, Mexico, during their incubation period. Because they did not spend the entire 14-d incubation period in Mexico, it is unclear whether they became infected in Texas or Mexico.

recently published strategy for assembly and comparison of mitochondrial genomes of *C. cayetanensis* reported a variable number of 15-mer repeats in the terminal region of the mitochondrial genome (*17*), a finding that we confirmed and expanded upon in our study. The sequence of type Cmt169.B, which was found in 6 samples in our study, is identical to the mitochondrial junction sequence found in a previously reported sample from Nepal (GenBank accession no. KP231180.1) (*14*). The most distinct mitochondrial genome reported so far is from an isolate from China (*12*), which, on the basis of the draft genome, has only 1 copy of the 15-mer repeat.

The copy number of the mitochondrial genome is still unknown for *C. cayetanensis*. Tang et al. (*12*) estimated 513 copies of the mitochondrial genome for each nuclear genome on the basis of the relative proportion of whole-genome sequencing reads mapped to each genome. However, this estimate seems high compared with the mitochondrial copy number in other apicomplexan parasites (e.g., 50 copies/nuclear genome in *Eimeria tenella* [*22*], 20 copies/nuclear genome in *Plasmodium falciparum* [*23*], and 150 copies/nuclear genome in *P. yoelli* [*24*]). Nevertheless, targeting a high copy number locus provides the greatest opportunity for successful amplification directly from clinical samples. We successfully amplified and sequenced the mitochondrial junction in 98.5% of the samples in this study. In contrast, an MLST method based on 5 microsatellite loci in the *C. cayetanensis* nuclear genome resulted in interpretable data from only 53%–59% of samples tested (*15*,*16*).

This study included ≥2 samples from 10 outbreaks associated with restaurants, specific events, or temporospatial case clusters. Samples from 7 of these clusters/outbreaks had identical typing results for all linked cases, and 3 clusters/outbreaks had linked cases that typed differently. Instances in which the same cluster showed >1 distinct type included an outbreak in Michigan (2014) in which 4 types were detected among 6 patients, an outbreak in Texas (2015) in which 1 patient had a type distinct from the other 3 patients, and an outbreak in Wisconsin (2015) in which 2 different types were detected among 6 patients. As suggested by Guo et al. (*15*), the presence of >1 type in a cluster might be indicative of produce contaminated with mixed populations of *C. cayetanensis*.

To date, epidemiologic investigations of cyclosporiasis cases and outbreaks have been limited by the lack of molecular typing methods that can reliably differentiate isolates of *C. cayetanensis.* Our study suggests that PCR amplification and DNA sequencing of a short region of the mitochondrial genome might provide useful typing information to aid such investigations. Performing amplicon deep sequencing of the Cmt region by using next-generation sequencing methods might also enable analysis of clinical or environmental samples containing multiple genotypes. Although further studies are required, including sampling from broader geographic areas, we propose that the mitochondrial junction region of *C. cayetanensis* shows promise as a molecular typing marker for this human pathogen.

Acknowledgments

We thank Subin Park and Erik Van Roey for assisting in preliminary bioinformatics analysis; Yaritbel Torres for help with sample processing; Rebecca L. Hall for providing epidemiologic assistance; and Cathy Snider, Chun Wang, Marie-Claire Rowlinson, Marek Pawlowicz, Jason Blanton, Tonia Parrott, and Meno Elcock for providing samples and contributing to the Advanced Molecular Detection and Response to Infectious Disease Outbreaks Initiative.

This study was supported by the Advanced Molecular Detection and Response to Infectious Disease Outbreaks Initiative of the Centers for Disease Control and Prevention.

About the Author

Dr. Nascimento is a postdoctoral research fellow in the Parasitic Diseases Branch, Division of Parasitic Diseases and Malaria, Center for Global Health, Centers for Disease Control and Prevention, Atlanta, GA. Her research interests include development and evaluation of molecular and immunological tools for investigation and diagnosis of parasitic diseases.

References

1. Ortega YR, Sanchez R. Update on *Cyclospora cayetanensis*, a food-borne and waterborne parasite. Clin Microbiol Rev. 2010;23:218–34. http://dx.doi.org/10.1128/CMR.00026-09
2. Hall RL, Jones JL, Hurd S, Smith G, Mahon BE, Herwaldt BL. Population-based active surveillance for *Cyclospora* infection—United States, Foodborne Diseases Active Surveillance Network (FoodNet), 1997–2009. Clin Infect Dis. 2012;54(Suppl 5):S411–7. http://dx.doi.org/10.1093/cid/cis049
3. Abanyie F, Harvey RR, Harris JR, Wiegand RE, Gaul L, Desvignes-Kendrick M, et al.; Multistate Cyclosporiasis Outbreak Investigation Team. 2013 multistate outbreaks of *Cyclospora cayetanensis* infections associated with fresh produce: focus on the Texas investigations. Epidemiol Infect. 2015;143:3451–8. http://dx.doi.org/10.1017/S0950268815000370
4. Herwaldt BL. *Cyclospora cayetanensis*: a review, focusing on the outbreaks of cyclosporiasis in the 1990s. Clin Infect Dis. 2000;31:1040–57. http://dx.doi.org/10.1086/314051
5. Nichols GL, Freedman J, Pollock KG, Rumble C, Chalmers RM, Chiodini P, et al. *Cyclospora* infection linked to travel to Mexico, June to September 2015. Euro Surveill. 2015;20:1–4. http://dx.doi.org/10.2807/1560-7917.ES.2015.20.43.30048
6. Marques DF, Alexander CL, Chalmers RM, Elson R, Freedman J, Hawkins G, et al. Cyclosporiasis in travellers returning to the United Kingdom from Mexico in summer 2017: lessons from the recent past to inform the future. Euro Surveill. 2017;22:1–4. http://dx.doi.org/10.2807/1560-7917.ES.2017.22.32.30592
7. Centers for Disease Control and Prevention. Cyclosporiasis outbreak investigations—United States, 2017 (updated June 10, 2017)

[cited 2018 Sep 6]. https://www.cdc.gov/parasites/cyclosporiasis/outbreaks/2017/index.html
8. Keaton AA, Hall NB, Chancey RJ, Heines V, Cantu V, Vakil V, et al. Notes from the field: Cyclosporiasis cases associated with dining at a Mediterranean-style restaurant chain—Texas, 2017. MMWR Morb Mortal Wkly Rep. 2018;67:609–10. http://dx.doi.org/10.15585/mmwr.mm6721a5
9. Qvarnstrom Y, Wei-Pridgeon Y, Li W, Nascimento FS, Bishop HS, Herwaldt BL, et al. Draft genome sequences from *Cyclospora cayetanensis* oocysts purified from a human stool sample. Genome Announc. 2015;3:1–2. http://dx.doi.org/10.1128/genomeA.01324-15
10. Liu S, Wang L, Zheng H, Xu Z, Roellig DM, Li N, et al. Comparative genomics reveals *Cyclospora cayetanensis* possesses coccidia-like metabolism and invasion components but unique surface antigens. BMC Genomics. 2016;17:316. http://dx.doi.org/10.1186/s12864-016-2632-3
11. Cinar HN, Qvarnstrom Y, Wei-Pridgeon Y, Li W, Nascimento FS, Arrowood MJ, et al. Comparative sequence analysis of *Cyclospora cayetanensis* apicoplast genomes originating from diverse geographical regions. Parasit Vectors. 2016;9:611. http://dx.doi.org/10.1186/s13071-016-1896-4
12. Tang K, Guo Y, Zhang L, Rowe LA, Roellig DM, Frace MA, et al. Genetic similarities between *Cyclospora cayetanensis* and cecum-infecting avian *Eimeria* spp. in apicoplast and mitochondrial genomes. Parasit Vectors. 2015;8:358. http://dx.doi.org/10.1186/s13071-015-0966-3
13. Ogedengbe ME, Qvarnstrom Y, da Silva AJ, Arrowood MJ, Barta JR. A linear mitochondrial genome of *Cyclospora cayetanensis* (Eimeriidae, Eucoccidiorida, Coccidiasina, Apicomplexa) suggests the ancestral start position within mitochondrial genomes of eimeriid coccidia. Int J Parasitol. 2015;45:361–5. http://dx.doi.org/10.1016/j.ijpara.2015.02.006
14. Cinar HN, Gopinath G, Jarvis K, Murphy HR. The complete mitochondrial genome of the foodborne parasitic pathogen *Cyclospora cayetanensis.* PLoS One. 2015;10:e0128645. http://dx.doi.org/10.1371/journal.pone.0128645
15. Guo Y, Roellig DM, Li N, Tang K, Frace M, Ortega Y, et al. Multilocus sequence typing tool for *Cyclospora cayetanensis.* Emerg Infect Dis. 2016;22:1464–7. http://dx.doi.org/10.3201/eid2208.150696
16. Li J, Chang Y, Shi KE, Wang R, Fu K, Li S, et al. Multilocus sequence typing and clonal population genetic structure of *Cyclospora cayetanensis* in humans. Parasitology. 2017;144:1890–7. http://dx.doi.org/10.1017/S0031182017001299
17. Gopinath GR, Cinar HN, Murphy HR, Durigan M, Almeria M, Tall BD, et al. A hybrid reference-guided de novo assembly approach for generating *Cyclospora* mitochondrion genomes. Gut Pathog. 2018;10:15. http://dx.doi.org/10.1186/s13099-018-0242-0
18. Berlin OG, Peter JB, Gagne C, Conteas CN, Ash LR. Autofluorescence and the detection of *Cyclospora* oocysts. Emerg Infect Dis. 1998;4:127–8. http://dx.doi.org/10.3201/eid0401.980121
19. Qvarnstrom Y, Benedict T, Marcet PL, Wiegand RE, Herwaldt BL, da Silva AJ. Molecular detection of *Cyclospora cayetanensis* in human stool specimens using UNEX-based DNA extraction and real-time PCR. Parasitology. 2018;145:865–70. http://dx.doi.org/10.1017/S0031182017001925
20. Katoh K, Misawa K, Kuma K, Miyata T. MAFFT: a novel method for rapid multiple sequence alignment based on fast Fourier transform. Nucleic Acids Res. 2002;30:3059–66. http://dx.doi.org/10.1093/nar/gkf436
21. Kearse M, Moir R, Wilson A, Stones-Havas S, Cheung M, Sturrock S, et al. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics. 2012;28:1647–9. http://dx.doi.org/10.1093/bioinformatics/bts199
22. Hikosaka K, Nakai Y, Watanabe Y, Tachibana S, Arisue N, Palacpac NM, et al. Concatenated mitochondrial DNA of the coccidian parasite *Eimeria tenella.* Mitochondrion. 2011;11:273–8. http://dx.doi.org/10.1016/j.mito.2010.10.003
23. Preiser PR, Wilson RJ, Moore PW, McCready S, Hajibagheri MA, Blight KJ, et al. Recombination associated with replication of malarial mitochondrial DNA. EMBO J. 1996;15:684–93. http://dx.doi.org/10.1002/j.1460-2075.1996.tb00401.x
24. Vaidya AB, Akella R, Suplick K. Sequences similar to genes for two mitochondrial proteins and portions of ribosomal RNA in tandemly arrayed 6-kilobase-pair DNA of a malarial parasite. Mol Biochem Parasitol. 1989;35:97–107. http://dx.doi.org/10.1016/0166-6851(89)90112-6

Address for correspondence: Yvonne Qvarnstrom, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop D64, Atlanta, GA 30329-4027, USA; email: bvp2@cdc.gov

Nationwide Stepwise Emergence and Evolution of Multidrug-Resistant *Campylobacter jejuni* Sequence Type 5136, United Kingdom

Bruno S. Lopes, Norval J.C. Strachan, Meenakshi Ramjee, Anne Thomson, Marion MacRae, Sophie Shaw, Ken J. Forbes

We examined whole-genome–sequenced *Campylobacter jejuni*/ and *C. coli* from 2012–2015 isolated from birds and human stool samples in North East Scotland for the presence of antimicrobial resistance genes. We found that sequence type (ST) 5136 (clonal complex 464) was the most prevalent multidrug-resistant strain of *C. jejuni* exclusively associated with poultry host reservoirs and recovered from human cases of campylobacteriosis. Tetracycline resistance in ST5136 isolates was due to a *tet*(O/32/O) mosaic gene, ampicillin resistance was conferred by G→T transversion in the –10 promoter region of $bla_{OXA-193}$, fluoroquinolone resistance was due to C257T change in *gyrA,* and aminoglycoside resistance was conferred by *aac*. Whole-genome analysis showed that the strain ST5136 evolved from ST464. The nationwide emergence of ST5136 was probably due to stepwise acquisition of antimicrobial resistance genes selected by high use of β-lactam, tetracycline, fluoroquinolone, and aminoglycoside classes of drugs in the poultry industry.

Campylobacter jejuni and *C. coli* are the most common causes of bacterial foodborne gastroenteritis in the industrialized world (*1*). In the United Kingdom alone, *Campylobacter* is implicated in >500,000 cases, 80,000 medical consultations, and 200 deaths and costs the economy an estimated £1 billion annually (*2,3*). According to World Health Organization estimates, *Campylobacter*-related sequelae affect ≈1% of the worldwide population (*4*). In the United Kingdom, human infection has been associated with retail chicken meat products (55%–75% attribution); cattle and sheep have a secondary role and wild birds, pigs, and dogs minor roles (*5*–*7*). Most human *Campylobacter* infections are mild and self-limiting and resolve within a few days, but severe or prolonged infections can occur, particularly in the young, elderly, and immunocompromised patients with AIDS or other vulnerable categories for which therapeutic intervention may be warranted (*8*).

For clinical therapy of campylobacteriosis, antimicrobial drugs, such as erythromycin, are usually prescribed, but ciprofloxacin is advised in moderately severe cases of nonconfirmed gastroenteritis and for travelers' diarrhea (*9*). In recent years, treatment with fluoroquinolones has been challenging because of an increasing prevalence of fluoroquinolone resistance in human *Campylobacter* isolates that led to the ban of the fluoroquinolone enrofloxacin for use in poultry in the United States in 2004 (*10*).

The Veterinary Medicines Directorate reports that in 2016 a total of 337 tons (17% decrease from 2015) of authorized veterinary antimicrobial drugs were sold in the United Kingdom. The sales of trimethoprim, sulphonamides, β-lactams, and aminoglycosides remained stable between 2012 and 2016, but notable reductions were observed for tetracycline (30%), macrolides (24%), and fluoroquinolones (29%) from 2015 to 2016 (*11*). Only enrofloxacin and difloxacin are licensed for poultry use; 0.5 metric tons of active ingredient were sold for use in poultry production in 2015 to the British Poultry Council, a national trade group that accounts for 90% of all broilers produced in the United Kingdom (*12*).

The main drivers for the acquisition of antimicrobial resistance (AMR) are selection pressure and the opportunity for horizontal gene transfer (*13*). Multidrug resistance has been defined as resistance to ≥3 classes of antimicrobial drugs and can occur by stepwise mutation or single plasmid acquisition of AMR genes (*14,15*). A report commissioned by the UK Food Standards Agency shows that drug-resistant *Campylobacter* species are becoming more

Author affiliation: University of Aberdeen, Foresterhill, Aberdeen, Scotland, UK

DOI: https://doi.org/10.3201/eid2507.181572

prevalent; evidence shows increasing levels of resistance in bacteria from poultry meat (*16*), which makes up around half of all meat by weight purchased in the United Kingdom (33 kg/person) (*17*).

The aim of this study was to establish the evolutionary course of events that led to the emergence of a multidrug-resistant (MDR) organism by considering the interplay between selection pressures and genetic changes. Specifically, we investigated whether antimicrobial resistance to β-lactams, tetracyclines, fluoroquinolones, and aminoglycosides in the ancestral lineages of *C. jejuni* ST5136 (Figure 1) led to the emergence of a strain that is the causative agent of human campylobacteriosis in 1 of 20 cases in the United Kingdom (*18*).

Materials and Methods

Bacterial Isolates and Genotyping

Campylobacter isolates for this study came from patients who had campylobacteriosis during 2012–2015 and from chicken and turkey retail meat samples. We incubated the meat samples at ambient temperature in enrichment broth for 1 h with occasional agitation and plated 100 μL of the broth on mCCDA plates (E & O Laboratories, http://www.eolabs.com). We then incubated the plates under microaerobic conditions at 37ºC for 48 h and extracted genomic DNA using the Wizard Genomic DNA Purification Kit (Promega, https://www.promega.com). Whole-genome sequencing was performed using HiSeq 2000 sequencer (Illumina, https://www.illumina.com) with 100 bped-end sequencing. We assembled FASTQ paired-end reads using Velvet (*19*) with a typical 50× coverage and assembled genome size of ≈1.6 Mbp. We uploaded the genomes to the Bacterial Isolate Genome Sequence Database (BIGSdb) and typed them using the 7-locus multilocus sequence typing (MLST) and whole-genome MLST (wgMLST) schemes (*20,21*).

Antimicrobial Resistance Gene Determinants

We assessed AMRs in silico for clonal complexes (CC) 464 (parent CC of ST5136), CC353, CC354, and CC574 (n = 494, North East Scotland; n = 68, England). We determined the tetracycline resistance (*CAMP1698*) and $bla_{\text{OXA-61}}$–like (*CAMP0265*) variants in accordance with BIGSdb (*20*). We assessed the flanking sequence of $bla_{\text{OXA-61}}$–like for the G→T transversion in the –10 promoter region by exporting 100 bp flanking allele sequences in XMFA/concatenated FASTA formats from *C. jejuni/coli* PubMLST isolate database (*22*). We also screened isolates for *gyrA* and 23S rRNA V domain mutations, $bla_{\text{OXA-184}}$–like and presence of *ermA*, *emrB*, *ermC*, and *ermF* using BIGSdb and Resfams (*23,24*). We identified putative aminoglycoside resistance genes encoding N-acetyltransferases (AAC), O-adenyltransferases (ANT), O-phosphotransferases (APH), and streptothricin acetyltransferase (SAT) using Resfams (*25*).

Genome Annotation and Phylogenetic Analyses

We chose 100 isolates with 1 *C. coli* (CC828) outgroup that were representative of the closely related clonal complexes CC464, CC353, CC354, and CC574 from BIGSdb. The group consisted of isolates from North East Scotland (n = 31), England (n = 68), and Estonia (n = 1). We annotated their genomes using RAST (*26*) and analyzed them by whole-genome multilocus sequence typing (wgMLST) using 1,643 Gundogdu loci (*27*). We constructed a phylogenetic tree in Bionumerics v. 7.6 (Applied Maths, http://www.applied-maths.com) using the topscore unweighted pair group method with arithmetic mean algorithm based on similarity of wgMLST loci.

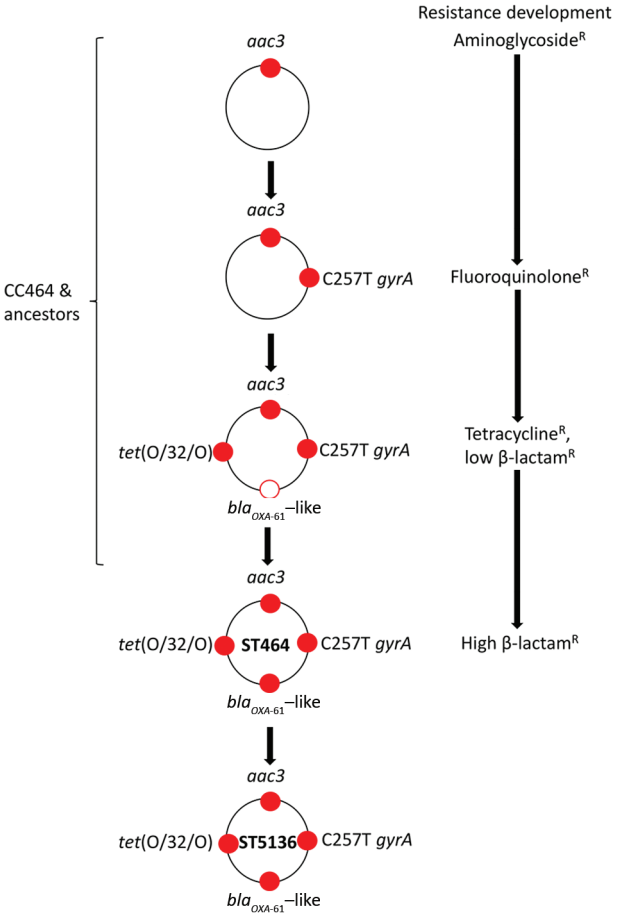


Figure 1. Stepwise sequential evolution of multidrug resistance in *Campylobacter jejuni* ST5136, Scotland. Red dots indicate resistance to antimicrobial drug as a result of genetic change or acquisition of resistance gene; white dot with red outline indicates acquisition of oxacillinase. Resistances are indicated as follows: *aac3,* aminoglycoside; C257T *gyrA,* fluoroquinolone; $bla_{\text{OXA-61}}$–like, β-lactam (variant in ST5136 is OXA-193); *tet*(O/32/O), tetracycline. CC, clonal complex; OXA, oxacillin; R, resistant; ST, sequence type.

We constructed maximum-likelihood trees for aminoglycoside resistance determinants in MEGA7 (*28*).

Antimicrobial Susceptibility Testing and Strain Stability

We determined antimicrobial susceptibility for 21 representative CC464 isolates with genotypic polymorphisms across the range of detected resistance markers. We used Mueller-Hinton agar with 5% defibrinated horse blood and 20 mg/L β-nicotinamide adenine dinucleotide for antimicrobial susceptibility testing using the disk diffusion method for ciprofloxacin (5 μg/disk), gentamicin (10 μg/disk), tetracycline (5 μg/disk), kanamycin (5 μg/disk), amikacin (30 μg/disk), tobramycin (10 μg/diskc), and streptomycin (30 μg/disk) (*29,30*). We determined MIC of ampicillin (AMP) by Etest (Oxoid, http://www.oxoid.com) and interpreted the MIC_{AMP} and disk susceptibility results using guidelines from the European Committee on Antimicrobial Susceptibility Testing and Clinical and Laboratory Standards Institute and interpreted them as described previously (*29*). We used the antimicrobial drug–susceptible isolate ARI3025 as a control. We subcultured the ST5136 isolate ARI4158 on antibiotic-free blood agar plate (ARI4158_20) 22 times to assess the stability of its antimicrobial resistance genes.

Statistical Analyses

We calculated 95% CIs by 10,000 bootstrap iterations to assess if there were significant differences in clonal complexes or individual strains with respect to resistance to different antimicrobial drug categories or AMR resistance alleles. If the CIs did not overlap (e.g., between 2 groups for a particular factor), then we inferred the comparison to be statistically significant ($p<0.05$).

Results

Genomic Analyses of Resistance in CC464, CC353, CC354, and CC574

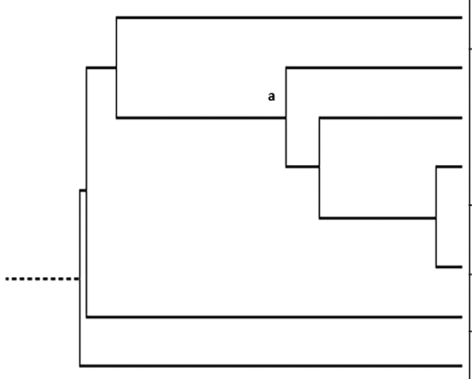
We compiled the metadata of 494 isolates from North East Scotland, including strain types and resistance genotypes (Appendix Table 1, https://wwwnc.cdc.gov/EID/article/25/7/18-1572-App1.xlsx). This list includes the antimicrobial resistance genotype of isolates in CC464 (n = 229), CC353 (n = 116), CC354 (n = 113), and CC574 (n = 36) from across the United Kingdom. Of these, 97% of CC464 isolates were MDR, followed by 95% of CC354 and 67% of CC574; CC353 (38%) showed lower levels of antimicrobial resistance. Multidrug resistance in CC464 was significantly higher ($p<0.05$) than that for the other clonal complexes. All ST5136 isolates had genes or mutations conferring resistance to tetracycline, ciprofloxacin (all isolates except 1), and ampicillin. We observed ≥1 aminoglycoside resistance gene (*aac3* variants in ST5136 isolates with 75% (n = 8) showing phenotypic resistance to kanamycin (Table 1; Figure 2).

We detected macrolide resistance in ARI2246 (ST2036, CC353), ARI2515 (ST3155, CC354), and ARI3316 (ST2438,

Table. Phenotypic evaluation of antimicrobial drug–resistant genotypes of *Campylobacter jejuni* ST5136 CC464 isolates, Scotland*

pubMLST ID	Isolate	Year	Source	*gyrA* C257T	CIP	OXA†	AMP	*tet* variant‡	TET	*aac3* variant	KAN	TOB
38459	ARI1530	2012	Human stool	T	R	A	S	*tet*(O)$_{80}$	R	G1	R	S
39121	ARI2250	2013	Human stool	T	R	A	S	A	S	G1, G4	R	S
41320	ARI3377	2014	Human stool	T	R	OXA-465	R	*tet*(O/32/O)$_7$	R	G1	R	S
40661	ARI3044	2013	Human stool	T	R	A	S	*tet*(O/M/O)$_{218}$	R	G1	R	S
38785	ARI1899	2012	Human stool	T	R	A	S	*tet*(O)$_{13}$	R	G1	R	R
38883	ARI2002_1	2012	Human stool	T	R	OXA-193 (G)	S	*tet*(O/32/O)$_7$	R	G1	R	S
39844	C0525	2013	Turkey	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1	R	S
42202	C1204	2014	Chicken	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	R
40371	ARI2986	2013	Human stool	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	I	S
48290	ARI4158	2015	Human stool	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	I	S
42443	C1522	2015	Chicken	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	S
58497	C0112	2010	Chicken	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1	R	S
58473	ARI0533	2010	Human stool	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1	R	S
41611	C0972	2014	Chicken	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G2	I	S
39810	C0469	2012	Turkey	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	S
58159	ARI3975	2015	Human stool	C	S	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	S
39411	ARI2623	2013	Human stool	C	S	OXA-193 (T)	R	A	S	G1	R	R
38522	ARI1599	2012	Human stool	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	R
38475	ARI1546	2012	Human stool	T	R	A	S	*tet*(O/32/O)$_7$	R	G1	R	R
48369	ARI3988	2015	Human stool	T	R	A	S	*tet*(O/32/O)$_7$	R	G1	R	S
42114	ARI3830	2015	Human stool	C	S	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	R	R
48290	ARI4158_20	2015	Human stool	T	R	OXA-193 (T)	R	*tet*(O/32/O)$_7$	R	G1, G4	I	S

*Result based on disk susceptibility testing, except for ampicillin, for which MIC was estimated. R indicates MIC >128mg/L. All isolates were sensitive to amikacin, gentamicin, and streptomycin. *aac3,* 3-N-aminoglycoside acetyltransferase gene groups (G1–G4); AMP, ampicillin; CC, clonal complex; CIP, ciprofloxacin; I, intermediate; ID, identification; KAN, kanamycin; R, resistant; S, sensitive; ST, sequence type as determined by multilocus sequence typing; TET, tetracycline; TOB, tobramycin.
†OXA-465 = bla$_{OXA-184}$-like gene; A indicates absence of oxacillinase gene; (G) or (T) indicates guanine or thymine mutation at −10 promoter of *bla*$_{OXA-193}$.
‡A indicates absence of tetracycline resistance gene.



Lineage, n	β-lactamase gene, %	β-lactam^R, %	*tet* gene	Tet^R, %	AG allele	AG^R, %	FQ^R allele	FQ^R, %	MDR, %
CC353, 116	OXA-193, 13	10	*tet*(O/32/O)$_7$,6	37	*aac*(3′)$_{22,17}$, 31	91	*gyrA*$_{65}$ 32	66	38
	Other, 3.5		Other, 31		Other, 60		Other, 34		
CC464[b], 39	OXA-193, 62	72	*tet*(O/32/O)$_7$, 69	85	*aac*(3′)$_{22,17}$, 51	100	*gyrA*$_{57}$ 26	95	90
	Other, 16		Other, 16		Other, 49		Other, 69		
ST5136, 190	OXA-193, 100	100	*tet*(O/32/O)$_7$, 99	100	*aac*(3′)$_{22,17}$, 42	100	*gyrA*$_{57}$ 99	99	100
			Other, 1		Other, 58		Other, 1		
CC574, 36	OXA-193, 78	58	*tet*(O)$_{10}$, 75	81	*aac*(3′)$_{22}$, 81	100	*gyrA*$_{68}$ 17	22	67
	Other, 6		Other, 6		Other, 19		Other, 5		
CC355, 113	OXA-460,83	70	*tet*(O)$_8$, 88	95	*aac*(3′)$_8$, 87	98	*gyrA*$_{20}$ 89	95	95
	Other, 17		Other, 7		Other, 12		Other, 6		

Figure 2. Whole-genome phylogenetic tree of *Campylobacter jejuni* CC464, CC353, CC574, and CC354 isolates, Scotland; a indicates CC464 root and b indicates CC464 isolates excluding ST5136. β-lactamase gene (*bla*$_{OXA-61}$–like) indicates presence of abundant allele and other OXA genes; β-lactam^R, resistant isolates (defined by -10 promoter mutation or presence of OXA-184–like gene). *tet* gene indicates presence of abundant tetracycline resistance allele and other alleles; Tet^R, tetracycline-resistant isolates. AG allele indicates presence of abundant aminoglycoside allele and other alleles in a group of strains associated at the CC level; other indicates any other *aac3* resistance allele or a combination of the abundant allele along with a second *aac3*; AG^R indicates aminoglycoside-resistant isolates. FQ allele indicates most abundant *gyrA* allele and other alleles that confer fluoroquinolone resistance; FQ^R indicates fluoroquinolone-resistant isolates. MDR is defined as resistance to ≥3 antimicrobial drugs. CC, clonal complex; MDR, multidrug resistant; OXA, oxacillin; R, resistant; ST, sequence type.

CC354), all of which had A2075G mutation in the 23S rRNA V domain, observed by erythromycin disk diffusion assay. We found no mutations at 2074 and observed no resistance due to *erm* (erythromycin ribosome methylation) genes in any of the isolates.

Fluoroquinolone resistance determined by the *gyrA* (*CAMP0950*) C257T mutation was significantly higher in CC464 (226/229, 99%), the parent clonal complex of ST5136; CC353 (77/116, 66%); and CC354 (107/113, 95%) isolates, compared with CC574 (8/36, 22%) ($p<0.05$) (Figure 2; Appendix Table 1). β-lactam resistance was conferred primarily by the *bla*$_{OXA-61}$–like (*CAMP0265*). *CAMP0265* allele 1 and 14 encoded the OXA-193 enzyme occurring in CC464, CC353, and CC574 isolates, whereas *CAMP0265* allele 38 (OXA-460) was significantly associated with CC354 group of isolates ($p<0.05$). ARI3377 (ST464) and ARI2874 (ST581) had *bla*$_{OXA-184}$–like and lacked any *bla*$_{OXA-61}$–like genes. CC353 isolates had relatively lower prevalence (19/113, 17%) of oxacillinase. Resistance to β-lactams as a result of a –10 promoter mutation upstream of the *bla*$_{OXA-61}$–like or the presence of *bla*$_{OXA-184}$–like was significantly greater in CC464 (217/229, 95%) and CC354 (79/113, 70%) compared with CC353 (11/116, 9%; $p<0.05$), whereas 21/36 (58%) of CC574 isolates were found to be resistant to β-lactams ($p>0.05$) (Appendix Table 1). Amino acid sequence identity revealed that OXA-193 (257 aa) and OXA-460 (253 aa) are 97.7% similar, which may be the result of environmental conditions or ecologic adaptations as listed previously (*31,32*).

Tetracycline resistance was significantly higher in CC464 (221/229, 97%), CC354 (108/113, 96%), and CC574 (29/36, 81%) than in CC353 (43/116, 37%; $p<0.05$) (Appendix Table 1). The mosaic *tet*(O/32/O)$_7$ was significantly associated with CC464, whereas the *tet*(O)$_8$ and *tet*(O)$_{10}$ were associated with CC354 and CC574 ($p<0.05$) (Figure 2). Of CC464 isolates, 93% (214/229) had the chromosomal *tet*(O/32/O)$_7$–like disrupting *dcuC* (*CAMP1639*). Two ST464 clinical isolates, ARI3458 and OXC6581, were positive for both *tet*(O/32/O)$_7$ and *tet*(O). In ARI3458, *tet*(O/32/O)$_7$ was chromosomal and *tet*(O) was plasmidborne, but the location could not be determined in OXC6581 (Figure 3; Appendix Table 2). We observed that *tet*(O/32/O) alleles 7 and 22 and *tet*(O)$_{10}$ occurred very rarely in CC353 isolates. We found the mosaic *tet*(O/32/O)$_7$–like determinants on either plasmid or chromosome in CC353, CC354, CC574, and other clonal complexes but at a much lower frequency (Appendix Table 2). The *CAMP1698*_10 variant occurred in OXC8770, ARI1655, and ARI3389 (ST3015, CC574) and had 98.7% similarity (631/639) to *Streptococcus* phage_phi-m46.1 *tet*(O) sequence FM864213.1.

The Resfams database identified aminoglycoside resistance determinants with lengths ranging from 96–263 aa. We did not assign allele numbers to determinants with polypeptide lengths of 96–169 aa and deemed them pseudogenes, whereas those with length >261 aa we identified as novel putative aminoglycoside resistance genes

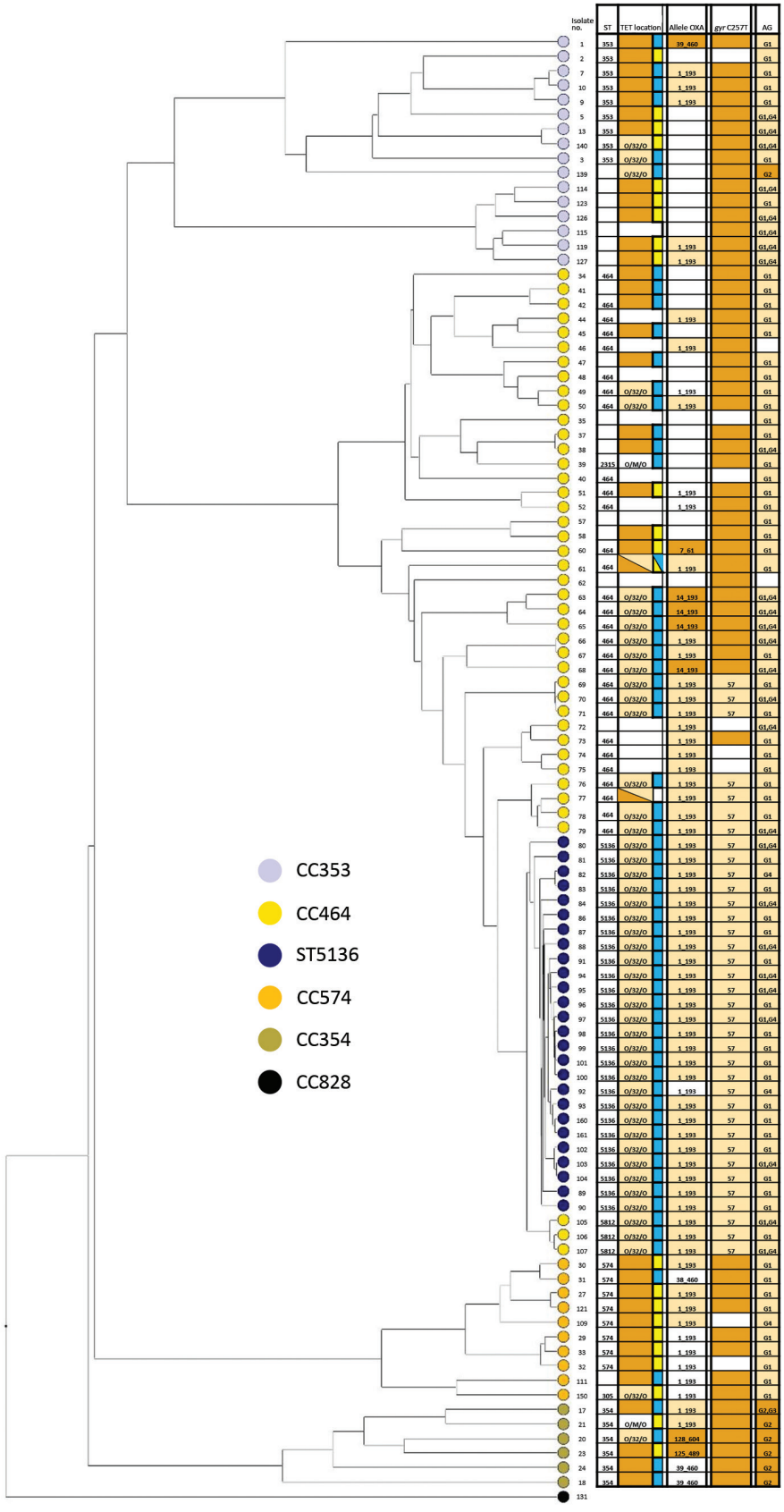


Figure 3. Whole-genome multilocus sequence typing of 100 selected isolates of *Campylobacter jejuni* from CC464, CC353, CC354, and CC574, Scotland. The tree was constructed using the UPGMA algorithm based on locus similarity. Isolate ID number indicated at branch end is linked to metadata in Appendix Table 2 (https://wwwnc.cdc.gov/EID/article/25/7/18-1572-App1.xlsx). Light orange boxes in the grid indicate variants associated with ST5136 isolates spread across the phylogeny; dark orange boxes denote resistance in individual isolates. Blue boxes represent tetracycline resistance in chromosome; yellow boxes, in plasmid. AG, aminoglycoside; CC, clonal complex; ID, identification; OXA, oxacillin; ST, sequence type; TET, tetracycline.

after BLAST query (Figure 4; Appendix Tables 1, 2). We identified a total of 17 *aac3* (groups G1–G4), 1 *aph* (O-phosphotransferase), and 1 *ant* aminoglycoside resistant gene. We grouped the *aac3* allelic variants as v7, v15, v17, v24–28, (G1); v8, v10, v29 (G2); v19 (G3); and v11, v22, v30–32 (G4) (Figure 4). We identified ≥1 *aac3* variant in all isolates and no *aac6′* enzymes. We identified streptothricin acetyltransferase (SAT_4), *aph* (v2), *aph3′-IIIa* (v1), and O-adenyltransferase (*aadE*/*ant6*, v2) in a single isolate of *C. jejuni* (ARI2517, ST2140/

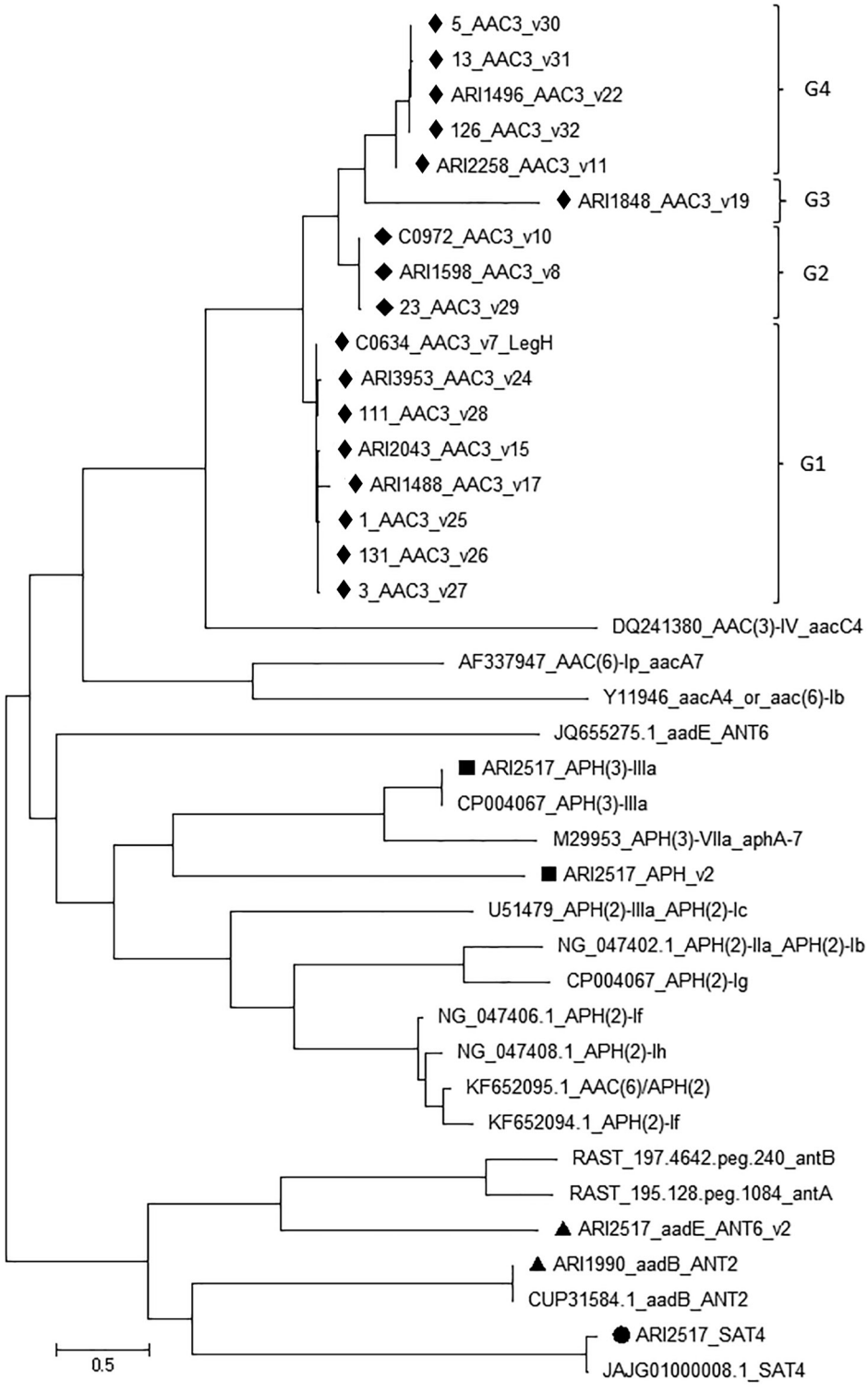


Figure 4. Maximum-likelihood tree of all aminoglycoside resistance determinants found in *Campylobacter jejuni* CC464, CC353, CC354, and CC574 isolates, Scotland. The tree was built using MEGA7 (https://www.megasoftware.net). The variants (v) listed in G1, G2, G3, G4 groups commonly occur in the 4 CCs. Scale bar indicates nucleotide substitutionsj per site. Aminoglycoside resistance gene variants identified in representative isolates of 4 CCs are shown by different symbols: black diamonds, *aac3*; black squares, *aph*; black triangles, *ant*; black circle, *sat.* CC, clonal complex.

CC574). We identified the *aadE*/*ant6*, v2 in ARI2168 (ST400/CC353) also, and *aadB* in ARI1990 (ST2116/CC353) (Appendix Table 1). All CC464 isolates were positive for ≥1 *aac3* gene (Figure 2; Appendix Table 1, 2). The *aac3* allele v17 was significantly more common in CC464 compared with v8 and v22 that occurred in other clonal complexes (Figure 2).

Antimicrobial Susceptibility Testing and Strain Stability

We performed susceptibility testing and corroborated 21 isolates of CC464 with genotypes predictive of resistant or sensitive phenotypes for different antimicrobial drugs (Table). Past studies show that disk diffusion correlated well to MIC determination and reliably distinguished between sensitive, intermediate, and resistant isolates of *Campylobacter* (*33*); hence, we used this method to assess and interpret genotypic data linked to isolate phenotype. Previous research has observed that the C257T *gyrA* genotype conferred ciprofloxacin resistance, G57T change in the -10 promoter of bla_{OXA-61}-like led to an increase in ampicillin resistance, and all tetracycline resistance genes conferred resistance to tetracycline (Table) (*22*,*34*,*35*). We observed resistance to kanamycin in 18/21 *aac3*-positive isolates harboring alleles v22 and v17, indicating that these variants may have the ability to hydrolyze kanamycin (Table); this finding warrants further investigation. Most of the isolates displayed a tobramycin-sensitive phenotype, whereas all were sensitive to amikacin, streptomycin, and gentamicin (Table). We subcultured isolate ARI4158 (ST5136) 22 times on antibiotic-free blood agar to assess the stability of its antimicrobial resistance genes, but we found it to be resistant like the parent isolate.

Phylogenetic Relationships

We constructed a phylogeny of 100 representative isolates of CC464 and associated clonal complexes using 1,643 wgMLST loci (Figure 3; Appendix Table 2). As predicted, ST5136 clustered within CC464; CC353, CC354, and CC574 isolates clustered, in that order, distal to CC464. ST5136 isolates were a single clonal MDR group closely related to the MDR isolates of ST5812 (CC464) reported in Guernsey in 2012 and now also found in England and Scotland (Figure 3). We observed the clustering of CC353, CC354, and CC574 around CC464 at the wgMLST level at which CC353 and CC354 strains had a high prevalence of ciprofloxacin resistance, whereas CC574 had a lower prevalence of ciprofloxacin resistance. Our findings concur with those reported earlier (*36*). Strain ST6209 (isolate no. 72, resistant only to β-lactam) clustered around ST464 isolates (nos. 73, 74, 75) that had genotypes encoding low levels of resistance (Figure 3).

Discussion

The UK broiler sector is the fourth-largest poultry meat producer in the European Union, making the United Kingdom ≈75% self-sufficient in poultry meat. The industry has benefited from the continuing consumer choice of leaner meat, reflected in the 12% rise in poultry meat sales in 2017. The widespread presence of antimicrobial resistance in *Campylobacter* spp. in retail poultry in the United Kingdom suggests horizontal transfer and mutational events within and between broiler farms and environmental conditions leading to proliferation of antibiotic-resistant lineages (*37,38*). This process is observed in ST5136, which was first detected in 2010 as an ST464 variant isolated from chicken meat (C0112) and clinical stool samples (ARI0509) from North East Scotland and Oxford (OXC5459). In 2012, ST5136 was reported from turkey meat (C0469) in North East Scotland, and in all cases from both years these isolates were MDR. ST5136 was never detected before 2010 in any of the 15,365 isolates submitted to the PubMLST *Campylobacter* database, but since its discovery, it has been found in ≥18 abattoirs across the United Kingdom. ST5136 has only ever been isolated from poultry (chicken 90%, turkey 10%, duck <1%) and never from cattle, sheep, pigs, or wild birds (*39*). Thus, human acquisition is probably exclusively from poultry meat.

The evolutionary ancestry of ST5136 shows that it is a member of CC464 and evolved from a heterogeneous complex of related strains, including ST464, all of which show high prevalence of antimicrobial resistance (Figure 3). ST5136 forms a cluster within CC464, an ancestor of CC353 with CC574 and CC354 associated more distally (Figure 2).

In the PubMLST *Campylobacter* database, the earliest report of ST354 was from a human blood culture in 1984; in 1991 it was isolated from chicken meat in the United Kingdom (*39*). ST574 was isolated in Thailand in 1999 and reported in the United Kingdom from an unspecified human sample in 2000 and chicken meat in 2001 (*39*). The closest ancestor of ST464 is ST353, which was isolated from chicken in 1982 in the United Kingdom, and ST464, reported in 2001 from chicken meat in Northern Ireland (first report of ST464 was from Germany in 2000 from human stool) and then ST5136 reported in 2010 (*39*). We compiled an illustration of the acquisition of resistance markers in ST5136 from ST464 (Figure 1). In the ancestral state, most CC464 isolates harbor *aac3* and then acquire ciprofloxacin resistance, *tet*(O/32/O) and bla_{OXA-61}-like. Subsequently, a mutation in the bla_{OXA-61}-like promoter leads to very high levels of ampicillin resistance. All these events have led to the stepwise selection of increasing drug resistance phenotypes in an intensely antimicrobial-competitive environment (Appendix Table 2). Ancestral to CC464 is CC353 and somewhat more distally CC574 and CC354,

each of which shows progressively lower proportions of antimicrobial-resistant isolates. The commonality of the alleles for genes conferring resistance to the 4 different classes of antimicrobial drugs examined in this article indicates a gradual accumulation of these markers, culminating in ST5136. The most recently observed marker, $gyrA_{57}$, is common to isolates of ST5136 and its cogenitor CC464 and leads to ciprofloxacin resistance, resulting in resistant strains that are related and are fitter than their sensitive counterparts (Figure 2; Appendix Table 2) (*34*). Because most ST5136 isolates show high levels of fluoroquinolone, β-lactam, aminoglycoside, and tetracycline resistance, it is likely that these related isolates evolved from a resistant ST464 ancestor.

The $tet(O/32/O)_7$ and $aac3_{17}$ alleles were most likely acquired previously, before the divergence of CC464 from CC353. Purifying selection of resistances could be occurring in CC464, as $tet(O/32/O)_7$ in this group was increasingly common compared with the ancestral strains. The tetracycline resistance gene $tet(O/32/O)_7$ is found on either plasmids or the chromosome in the ancestral isolates CC464, CC353, CC354, and CC574, but by the appearance of the CC464 ancestor of ST5136, it is found only on the chromosome, suggesting acquisition either via conjugation or by transformation into the last common ancestor. There is a constant flow of resistance genes between plasmids, phages, and bacteria in the farm environment and the chicken gut microbiome. Species such as *Streptococcus* and *Enterococcus* that form a part of the chicken gut microbiome often harbor promiscuous plasmids with resistance genes that can occur in *Campylobacter*; this exchange of genetic determinants will be a contributor to antimicrobial resistance evolution in *Campylobacter* (*40,41*). Similarly, OXA-193 dominated in isolates of CC464 that were closely ancestral to ST5136 and evolved to be more highly resistant to β-lactam antimicrobial drugs as a result of the –10 promoter mutation in the upstream region of $bla_{OXA-193}$.

Superimposed onto this linear phylogeny of antimicrobial resistance in ST5136 are the genetic changes occurring in the other strains of the related lineages (Figure 1). In *Campylobacter* the principal sources of genetic change are intracellular mutational events and the acquisition of extracellular genes from other isolates; these events are estimated as equally common (*34,42*), mutations resulting in a clonal distribution of characters in related isolates and the acquisitions in a mosaic distribution of characters across the species. These sources of genetic diversity lead to the emergence of novel variants in fitter isolates selected by the environment. Thus, for *Campylobacter*, there is a complex distribution of individual genetic markers when viewed in the context of the overall phylogeny of related isolates. This finding is most clearly exemplified in the example of 1 ST5136 isolate (ARI3975), which has a basal level of resistance to ampicillin and no promotor mutation; these aspects could be the result of a spontaneous back mutation or horizontal gene transfer from a sensitive strain of *Campylobacter*.

Resistances are propagated across the clonal population of *Campylobacter*; from these survival-of-the-fittest chains of events in antimicrobial drug–rich environments, such as poultry farms, may emerge more abundant lineages due to the prophylactic or metaphylactic use of drugs (*43*). ST464 has given rise to 2 MDR strains: ST5136 (*uncA* allele 1→3) and ST5812 (*pgm* allele 10→17), which are closely associated with each other. It is unclear whether the spread of ST5136 was better facilitated than that of ST5812 or that other genetic changes in ST5136 made it more likely than ST5812 to survive and colonize in poultry or by chance.

Dissemination of highly related *Campylobacter* strains throughout the poultry industry has occurred previously. In New Zealand, *C. jejuni* ST474 (CC48) was a dominant strain found almost exclusively in isolates from 1 poultry supplier and associated with clinical cases during 2005–2008 (*44*). More recently, tetracycline- and fluoroquinolone-resistant *C. jejuni* ST6944 (CC354, one of the ancestors of CC464) has been reported in human cases and in 3 major poultry suppliers in New Zealand (*45*).

The UK poultry industry has responded well to the current BPC antibiotic stewardship program with a 71% reduction in use of antimicrobial drugs from 2012 to 2016, but it is worth noting that enrofloxacin and difloxacin are authorized fluoroquinolones currently used in the chicken industry in the United Kingdom (*46*). A 1991 study in the Netherlands reported an increase in fluoroquinolone resistance in *Campylobacter* correlated with increasing use of enrofloxacin in the poultry industry and thus the transmission of fluoroquinolone-resistant *Campylobacter* from chickens to humans (*38*). In 2012, the poultry industry phased out use of the modern cephalosporins completely, but a commitment to stop the prophylactic use of fluoroquinolones in day-old chickens was not made until 2016 (*46*). Even then, these antimicrobial drugs may be added to the drinking water of flocks of poultry when no disease is present in most of the birds in a flock (*47*). The continued occurrence of resistant strains that emerge in an antimicrobial-stressed environment but retain their resistance in environments even after the cessation of antibiotic pressure has been reported previously (*48*). Although positive antimicrobial usage may mitigate against the creation of future MDR bacteria, preexisting MDR strains, such as *C. jejuni* ST5136, may never lose their resistance characteristics.

Joshua Lederberg has said, "The future of humanity and microbes likely will unfold as episodes of Our Wits Versus Their Genes" (*49*). The evolution and rise of ST5136 indicates that bacteria can evolve by genetic

adaptation to antibiotic-enriched and -deprived environments, which drives the evolution of environment-favored strains by mutation or gene transfer. It is up to us to use our wits to keep up with these changes.

Acknowledgments

We thank Food Standards Scotland (Contracts S14054, FSS00017) and the Scottish Government's Rural and Environment Science and Analytical Services (RG13588-10) for funding this work. Part of this work was presented at the *Campylobacter*, *Helicobacter* and Related Organisms (CHRO) 2017 Congress, Nantes, France.

About the Author

Dr. Lopes is a postdoctoral research fellow at the University of Aberdeen. His research interests include molecular epidemiology of both foodborne and nosocomial gram-negative pathogens, in particular antimicrobial resistance mechanisms.

References

1. Silva J, Leite D, Fernandes M, Mena C, Gibbs PA, Teixeira P. *Campylobacter* spp. as a foodborne pathogen: a review. Front Microbiol. 2011;2:200. http://dx.doi.org/10.3389/fmicb.2011.00200
2. Tam CC, Rodrigues LC, Viviani L, Dodds JP, Evans MR, Hunter PR, et al.; IID2 Study Executive Committee. Longitudinal study of infectious intestinal disease in the UK (IID2 study): incidence in the community and presenting to general practice. Gut. 2012;61:69–77. http://dx.doi.org/10.1136/gut.2011.238386
3. Winstanley C, Haldenby S, Bronowski C, Nelson C, Hertz-Fowler C, Kenny J, et al. Application of whole-genome sequencing to fully characterize *Campylobacter* isolates from the UK infectious intestinal disease 1 and 2 studies. London: United Kingdom Food Standards Agency; 2015.
4. Green PH, Cellier C. Celiac disease. N Engl J Med. 2007;357:1731–43. http://dx.doi.org/10.1056/NEJMra071600
5. Kittl S, Heckel G, Korczak BM, Kuhnert P. Source attribution of human *Campylobacter* isolates by MLST and *fla*-typing and association of genotypes with quinolone resistance. PLoS One. 2013;8:e81796. http://dx.doi.org/10.1371/journal.pone.0081796
6. Sheppard SK, Dallas JF, Strachan NJ, MacRae M, McCarthy ND, Wilson DJ, et al. *Campylobacter* genotyping to determine the source of human infection. Clin Infect Dis. 2009;48:1072–8. http://dx.doi.org/10.1086/597402
7. Wilson DJ, Gabriel E, Leatherbarrow AJ, Cheesbrough J, Gee S, Bolton E, et al. Tracing the source of campylobacteriosis. PLoS Genet. 2008;4:e1000203. http://dx.doi.org/10.1371/journal.pgen.1000203
8. Luangtongkum T, Jeon B, Han J, Plummer P, Logue CM, Zhang Q. Antibiotic resistance in *Campylobacter*: emergence, transmission, and persistence. Future Microbiol. 2009;4:189–200. http://dx.doi.org/10.2217/17460913.4.2.189
9. Oza AN, McKenna JP, McDowell SW, Menzies FD, Neill SD. Antimicrobial susceptibility of *Campylobacter* spp. isolated from broiler chickens in Northern Ireland. J Antimicrob Chemother. 2003;52:220–3. http://dx.doi.org/10.1093/jac/dkg333
10. US Food and Drug Administration. Withdrawal of enrofloxacin for poultry (homepage). 2017 [cited 2019 Apr 25]. https://www.fda.gov/AnimalVeterinary/SafetyHealth/RecallsWithdrawals/ucm042004.htm
11. Veterinary Medicines Directorate. UK veterinary antibiotic resistance and sales surveillance report 2016. 2017 [cited 2019 Apr 25]. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/655403/_1274590_VARSS_2016_report.PDF
12. British Poultry Council. The BPC antibiotic stewardship scheme: leading the way in the responsible use of antibiotics. 2016 [cited 2019 Apr 25]. https://www.nfuonline.com/assets/61352
13. Holmes AH, Moore LS, Sundsfjord A, Steinbakk M, Regmi S, Karkey A, et al. Understanding the mechanisms and drivers of antimicrobial resistance. Lancet. 2016;387:176–87. http://dx.doi.org/10.1016/S0140-6736(15)00473-0
14. Magiorakos AP, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant, and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18:268–81. http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x
15. Benjak A, Avanzi C, Singh P, Loiseau C, Girma S, Busso P, et al. Phylogenomics and antimicrobial resistance of the leprosy bacillus *Mycobacterium leprae.* Nat Commun. 2018;9:352. http://dx.doi.org/10.1038/s41467-017-02576-z
16. Mateus A, Takahashi EA, Elkholly DA, Crotta M, Ekiri A, Wylie C, et al. A systematic review to assess the significance of the food chain in the context of antimicrobial resistance (AMR) with particular reference to pork and poultry meat, dairy products, seafood, and fresh produce on retail sale in the UK. London: Food Standards Agency; 2016.
17. Ford G. State of the poultry industry. Presented at: Agriculture and Horticulture Development Board Outlook Conference 2015; February 11, 2015; Westminster, London, UK [cited 2019 Apr 25]. https://pork.ahdb.org.uk/media/72845/outlook-2015-gary-ford-state-of-the-poultry-industry.pdf
18. Lopes BS, Forbes KJ, Rotariu O, Strachan NJC. i-CaMPS-3 impact of interventions—*Campylobacter* MLST project in Scotland. Aberdeen (Scotland, UK): Food Standards Scotland; 2016.
19. Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. Genome Res. 2008;18:821–9. http://dx.doi.org/10.1101/gr.074492.107
20. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11:595. http://dx.doi.org/10.1186/1471-2105-11-595
21. Sheppard SK, Jolley KA, Maiden MC. A gene-by-gene approach to bacterial population genomics: Whole genome MLST of *Campylobacter.* Genes (Basel). 2012;3:261–77. http://dx.doi.org/10.3390/genes3020261
22. Zeng X, Brown S, Gillespie B, Lin J. A single nucleotide in the promoter region modulates the expression of the β-lactamase OXA-61 in *Campylobacter jejuni.* J Antimicrob Chemother. 2014;69:1215–23. http://dx.doi.org/10.1093/jac/dkt515
23. Ren GWN, Wang Y, Shen Z, Chen X, Shen J, Wu C. Rapid detection of point mutations in domain V of the 23S rRNA gene in erythromycin-resistant *Campylobacter* isolates by pyrosequencing. Foodborne Pathog Dis. 2011;8:375–9. http://dx.doi.org/10.1089/fpd.2010.0676
24. Luo Y, Sahin O, Dai L, Sippy R, Wu Z, Zhang Q. Development of a loop-mediated isothermal amplification assay for rapid, sensitive, and specific detection of a *Campylobacter jejuni* clone. J Vet Med Sci. 2012;74:591–6. http://dx.doi.org/10.1292/jvms.11-0462
25. Gibson MK, Forsberg KJ, Dantas G. Improved annotation of antibiotic resistance determinants reveals microbial resistomes cluster by ecology. ISME J. 2015;9:207–16. http://dx.doi.org/10.1038/ismej.2014.106
26. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, et al. The RAST server: Rapid annotations using subsystems technology. BMC Genomics. 2008;9:75,2164–9-75.

27. Gundogdu O, Bentley SD, Holden MT, Parkhill J, Dorrell N, Wren BW. Re-annotation and re-analysis of the *Campylobacter jejuni* NCTC11168 genome sequence. BMC Genomics. 2007;8:162. http://dx.doi.org/10.1186/1471-2164-8-162
28. Kumar S, Stecher G, Tamura K. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol. 2016;33:1870–4. http://dx.doi.org/10.1093/molbev/msw054
29. Chang YC, Tien N, Yang JS, Lu CC, Tsai FJ, Huang TJ, et al. Class 1 integrons and plasmid-mediated multiple resistance genes of the *Campylobacter* species from pediatric patient of a university hospital in Taiwan. Gut Pathog. 2017;9:50. http://dx.doi.org/10.1186/s13099-017-0199-4
30. Matuschek E, Veldman K, Hakanen A, Bengtsson S, Lehtopolku M, Mevius D, et al. EUCAST standardised disk diffusion methodology for *Campylobacter jejuni* and *C. coli*. European Society of Clinical Microbiology and Infectious Diseases. 2012 [cited 2019 May 2]. https://www.escmid.org/escmid_publications/escmid_elibrary/material/?mid=2694
31. Lopes BS. The conundrum of naming resistance gene determinants. J Antimicrob Chemother. 2016;71:3623–4. http://dx.doi.org/10.1093/jac/dkw281
32. Brocchieri L, Karlin S. Protein length in eukaryotic and prokaryotic proteomes. Nucleic Acids Res. 2005;33:3390–400. http://dx.doi.org/10.1093/nar/gki615
33. Matuschek E, Brown DF, Kahlmeter G. Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories. Clin Microbiol Infect. 2014;20:O255–66. http://dx.doi.org/10.1111/1469-0691.12373
34. Luo N, Pereira S, Sahin O, Lin J, Huang S, Michel L, et al. Enhanced in vivo fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. Proc Natl Acad Sci U S A. 2005;102:541–6. http://dx.doi.org/10.1073/pnas.0408966102
35. van Hoek AH, Mayrhofer S, Domig KJ, Flórez AB, Ammor MS, Mayo B, et al. Mosaic tetracycline resistance genes and their flanking regions in *Bifidobacterium thermophilum* and *Lactobacillus johnsonii.* Antimicrob Agents Chemother. 2008;52:248–52. http://dx.doi.org/10.1128/AAC.00714-07
36. Cody AJ, McCarthy NM, Wimalarathna HL, Colles FM, Clark L, Bowler IC, et al. A longitudinal 6-year study of the molecular epidemiology of clinical *campylobacter* isolates in Oxfordshire, United kingdom. J Clin Microbiol. 2012;50:3193–201. http://dx.doi.org/10.1128/JCM.01086-12
37. Wimalarathna HM, Richardson JF, Lawson AJ, Elson R, Meldrum R, Little CL, et al. Widespread acquisition of antimicrobial resistance among *Campylobacter* isolates from UK retail poultry and evidence for clonal expansion of resistant lineages. BMC Microbiol. 2013;13:160,2180-13-160.
38. Endtz HP, Ruijs GJ, van Klingeren B, Jansen WH, van der Reyden T, Mouton RP. Quinolone resistance in *campylobacter* isolated from man and poultry following the introduction of fluoroquinolones in veterinary medicine. J Antimicrob Chemother. 1991;27:199–208. http://dx.doi.org/10.1093/jac/27.2.199
39. Jolley KA, Bray JE, Maiden MC. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. 2018 [cited 2019 May 2]. https://wellcomeopenresearch.org/articles/3-124/v1
40. Werner G, Hildebrandt B, Witte W. Aminoglycoside-streptothricin resistance gene cluster *aadE-sat4-aphA-3* disseminated among multiresistant isolates of *Enterococcus faecium.* Antimicrob Agents Chemother. 2001;45:3267–9. http://dx.doi.org/10.1128/AAC.45.11.3267-3269.2001
41. Brown-Jaque M, Calero-Cáceres W, Muniesa M. Transfer of antibiotic-resistance genes via phage-related mobile elements. Plasmid. 2015;79:1–7. http://dx.doi.org/10.1016/j.plasmid.2015.01.001
42. Vegge CS, Brøndsted L, Ligowska-Marzęta M, Ingmer H. Natural transformation of *Campylobacter jejuni* occurs beyond limits of growth. PLoS One. 2012;7:e45467. http://dx.doi.org/10.1371/journal.pone.0045467
43. Stokes HW, Gillings MR. Gene flow, mobile genetic elements and the recruitment of antibiotic resistance genes into Gram-negative pathogens. FEMS Microbiol Rev. 2011;35:790–819. http://dx.doi.org/10.1111/j.1574-6976.2011.00273.x
44. Müllner P, Collins-Emerson JM, Midwinter AC, Carter P, Spencer SE, van der Logt P, et al. Molecular epidemiology of *Campylobacter jejuni* in a geographically isolated country with a uniquely structured poultry industry. Appl Environ Microbiol. 2010;76:2145–54. http://dx.doi.org/10.1128/AEM.00862-09
45. French NP, Williamson DA, Biggs R, Biggs PJ, Bloomfield S, Dyet K, et al. Emergence of *Campylobacter jejuni* ST-6964 in poultry and humans in New Zealand: A new twist in the campy story. Wellington (New Zealand): Crown Copyright—Ministry for Primary Industries; 2016.
46. Veterinary Medicines Directorate. UK veterinary antibiotic resistance and sales surveillance report. 2015 [cited 2019 Apr 25]. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/582341/1051728-v53-UK-VARSS_2015.pdf
47. Rose E, Nunan C. Antibiotic use in the UK poultry sector. Alliance to Save Our Antibiotics (Basel). 2016 [cited 2019 Apr 25]. http://www.saveourantibiotics.org/media/1763/antibiotic-use-in-the-uk-poultry-sector.pdf
48. Ward MJ, Goncheva M, Richardson E, McAdam PR, Raftis E, Kearns A, et al. Identification of source and sink populations for the emergence and global spread of the East-Asia clone of community-associated MRSA. Genome Biol. 2016;17:160. http://dx.doi.org/10.1186/s13059-016-1022-0
49. Lederberg J. Infectious history. Science. 2000;288:287–93. http://dx.doi.org/10.1126/science.288.5464.287

Address for correspondence: Bruno S. Lopes, University of Aberdeen, Medical Microbiology, 0:025 Polwarth Building, Aberdeen City, Aberdeen AB25 2ZD, Scotland, UK; email: bruno.lopes@abdn.ac.uk

High-Complexity *Plasmodium falciparum* Infections, North Central Nigeria, 2015–2018

Bitrus Yakubu, Ishaya Yohanna Longdet, Tony Horsefield, Dinchi Tyem Davou, Emmanuel Obishakin

The mass migration that occurred during 2009–2013 and after the insurgency in northeastern Nigeria could have increased malaria incidence and *Plasmodium falciparum* genetic diversity in North Central Nigeria. To determine *P. falciparum* sequence diversity in this region, we screened 282 samples collected in regional clinics during 2015–2018 for *Plasmodium* spp. and, with positive samples, determined *P. falciparum* infection complexity and allele diversity using PCR. Of 34 *P. falciparum*–positive samples, 39 *msp1*, 31 *msp2*, and 13 *glurp* alleles were detected, and 88% of infections were polyclonal. We identified trimorphic and dimorphic allele combinations in a high percentage of samples, indicative of a high infection complexity in the study population. High genetic diversity is a catalyst for the evolution of drug resistant-alleles. Improved measures (e.g., better drug quality, diagnostics) are needed to control *P. falciparum* transmission and reduce the potential for the emergence of drug resistance in Nigeria.

Malaria is endemic in Nigeria; its national prevalence among children 6–59 months of age is 27% (*1*). The World Health Organization World Malaria Report 2017 indicated that Nigeria accounted for 27% of the global malaria cases and 30% of the deaths in 2016 (*2*). The insurgency that occurred during 2009–2013 in northeastern Nigeria has been marked as one of the key factors responsible for driving this increased number of malaria cases and deaths; the insurgency disrupted the healthcare system, and up to 2.1 million persons were displaced (*3*–*6*). Added to the insurgency burden were communal conflicts between internally displaced persons (IDPs) and locals, which predominantly occurred in the states of Plateau, Benue, Taraba, Kaduna, and Nasarawa (*3*). Published reports established that 3 patterns of IDP movement transpired in Nigeria: migration to North Central Nigeria (defined as the states of Kwara, Niger, Nasarawa, Plateau, Benue, and Kaduna, and the Federal Capital Territory), economic migration from rural to urban areas, and secondary displacement of host communities because of communal conflicts (*5*).

The population displacement that occurred in North Central Nigeria was likely a key factor affecting the epidemiology of malaria transmission in this region. A high level of new clones of *Plasmodium falciparum* probably would have been introduced into the host communities of towns and cities experiencing influxes of IDPs. Immigration of infected persons into new areas can increase the rate of malaria transmission (*7*) and might subsequently result in infections of higher complexity and pathogens of more extensive genetic diversity in the host communities. In other malaria-endemic areas, population displacement, political unrest, and insurgency have been found to affect malaria epidemiology (*8*–*10*).

Meiotic recombination of *P. falciparum* parasites in *Anopheles* sp. mosquitoes has been proposed as the origin of the generation of novel alleles leading to new *P. falciparum* strains, and this cycle is likely to continue as long as there are vectors, parasites, and human hosts (*11*–*13*). Treatment failures occur more often in patients infected with higher numbers (≥3 vs. <3) of *P. falciparum* strains (*14*,*15*). Drug resistance and treatment failures are envisaged to be among the challenges to achieving elimination of malaria in Nigeria (*16*,*17*). Ajayi et al. (*18*) reported 3 cases of artemisinin-based combination treatment failure of *P. falciparum* infection that were later adequately cleared with quinine. However, health practitioners and professionals in Nigeria have determined that the failure to clear parasites and resolve clinical disease after drug treatment is the result of many factors (drug nonpotency, incorrect diagnosis, noncompliance with dosing regimen duration, use of substandard drugs, and drug interactions). These reports spurred us to investigate the complexity of *P. falciparum* infections and *P. falciparum* genetic diversity (allele frequencies) in the North Central region of Nigeria.

Merozoite surface protein 1 (*msp1*), merozoite surface protein 2 (*msp2*), and glutamate-rich protein (*glurp*) gene-based studies of *P. falciparum* genetic diversity and infection complexity have been extensively carried out in other parts

Author affiliations: National Veterinary Research Institute Vom, Jos, Nigeria (B. Yakubu, D.T. Davou, E. Obishakin); University of Jos, Jos (I.Y. Longdet, T. Horsefield)

DOI: https://doi.org/10.3201/eid2507.181614

of sub-Saharan Africa, but only a few studies have taken place in regions of Nigeria (*19*–*24*). Research is scant on the genetic diversity of *P. falciparum* in North Central Nigeria, and this knowledge is critical for the implementation of successful control measures (*13*). Earlier reports by Jelinek et al. (*25*) and Meyer et al. (*12*) showed that increased genetic diversity of circulating malaria parasites in a population increases the potential for the selection of drug resistance. In our study, we investigated *P. falciparum* genetic diversity and the complexity of *P. falciparum* infections by assessing *msp1*, *msp2*, and *glurp* allele frequencies and genetic diversity in densely populated areas of North Central Nigeria.

Materials and Methods

Study Design

During August 2015–April 2018, whole blood samples for this study were collected from patients treated at 6 randomly selected healthcare centers in 5 locations of North Central Nigeria: Jos (Plateau State), Karu (Nasarawa State), Kafanchan (Kaduna State), Lokoja (Kogi State), and Abuja (Federal Capital Territory) (Figure 1). We chose these locations because previously published reports indicated that IDP movement occurred in these areas during the peak periods of insurgency, communal conflicts, and economic migration. After collection, whole blood samples were stored at 4°C until we performed PCR analyses at the Department of Biochemistry in the Faculty of Medical Sciences, University of Jos (Jos, Nigeria). Our target was to analyze ≥50 representative archived whole blood samples from each of the 6 healthcare centers. We received permission to collect and analyze these samples from the National Code for Health Research Ethics Committee.

PCR Screening for *P. falciparum*

We characterized *P. falciparum* isolates using the nested PCR methods of Snounou et al. (*26*) as modified by Singh

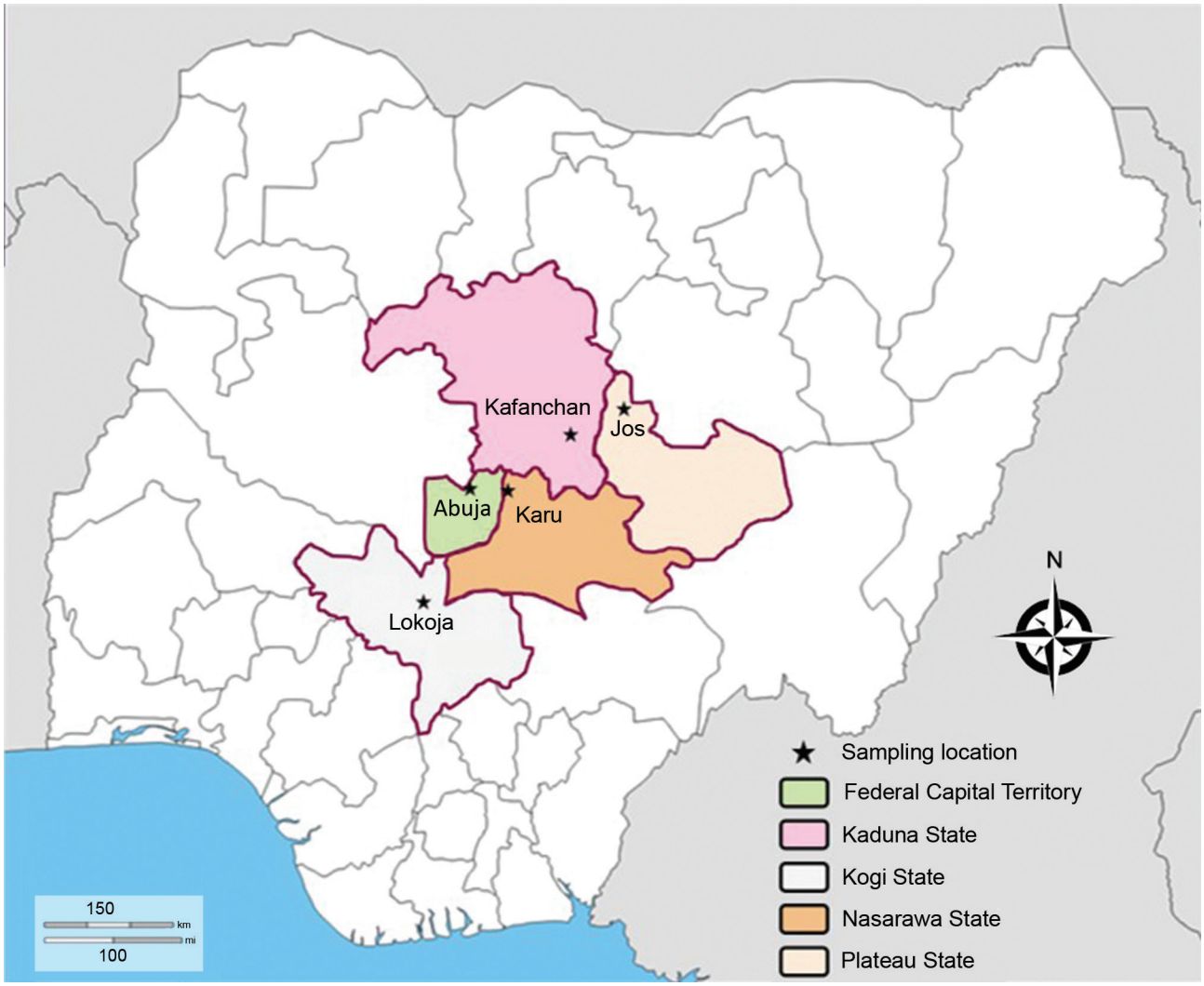


Figure 1. Sampling site locations in study of genetic diversity of *Plasmodium falciparum* spp., North Central Nigeria, 2015–2018. The Jabi region was the sampling site in Abuja.

et al. (*27*). These modifications included the use of a different genus-specific primer combination (rPLU-1 with rPLU-5 instead of rPLU-5 with rPLU-6) and the use of the nest-1 PCR product as the template for the genus-specific (nest-2) PCR. The primers for these PCRs (Table 1) were designed by using the *Plasmodium* small subunit rRNA genes (*29*) and synthesized by Inqaba Biotec (https://www.inqababiotec.co.za). We performed the nest-1 PCR, then the nest-2 PCR (to identify the samples positive for *Plasmodium* spp.), and then the *P. falciparum* species–specific PCR.

The nest-1 reaction mix contained 5 μL of DNA template, 25 μL of One Taq Quick-Load 2× Master Mix (New England BioLabs, https://www.neb.com), 1 μL of each primer (10 μmol/L rPLU1 and rPLU5), and 18 μL nuclease-free water. We used the following cycling program for the nest-1 PCR: initial denaturation at 94°C for 4 min; 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 60 s; and a final extension at 72°C for 4 min. The nest-2 reaction mix was the same as the nest-1 reaction mix, except that we used 2 μL of the nest-1 PCR product as the template and different primers (rPLU3 and rPLU4). The *P. falciparum* species–specific reaction mix was the same as the nest-2 PCR reaction mix, except that we used different primers (rFAL1 and rFAL2). The cycling programs we used for the nest-2 and *P. falciparum* species–specific PCRs were the same as the one used for the nest-1 PCR, except that we used different annealing temperatures (62°C for the nest-2 PCR and 58°C for the *Plasmodium* species–specific PCR). We performed these amplifications (as well as the genotyping PCR described in the next section) in the GeneAmp 9700 (Applied Biosystems, https://www.thermofisher.com).

Genotyping by Nested PCR

With *P. falciparum*–positive samples, we performed primary and genotype-specific PCRs for *msp1*, *msp2*, and *glurp* according to the modified method of Snounou and Färnert (*28*). The primary PCRs amplified the block 2 variable region (for *msp1*), block 3 central repeat region (for *msp2*), or the glutamate-rich protein region (for *glurp*). These regions have repetitive segments and motifs that are variable in length among strains; this variability occurs because of the intragenic recombination in *Anopheles* mosquitoes. PCRs targeting these regions amplify sequences of different lengths, which are the basis of clonality in *P. falciparum*. The primary reagent mixture included 5 μL of genomic DNA extract; 25 μL One Taq Quick-Load 2× Master Mix; 1.0 μL of each of the *msp1*, *msp2*, or *glurp* first reaction primers (10 μmol/L) (Table 1); and 14 μL nuclease-free water. We performed amplifications using an initial denaturation at 95°C for 5 min, followed by 25 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min and then a final extension at 72°C for 5 min. We used the products of the primary PCRs as the template for the subsequent genotype-specific PCRs, which were used to determine *msp1* (*K1*, *MAD20*, *RO33*), *msp2* (*FC-27*, *IC/3D7*), and *glurp* allele type. The reaction mix and conditions we used for the genotype-specific amplifications were the same as the ones we used for the primary amplification, except that the primers

Table 1. Primer sequences used for PCRs to screen and genotype samples collected in study of genetic diversity of *Plasmodium falciparum* parasites, North Central Nigeria, 2015–2018*

PCR description	Primer name or type: sequence, 5′→3′	Size, bp	Reference
Nest-1	rPLU1: TCAAAGATTAAGCCATGCAAGTGA rPLU5: CCTGTTGTTGCCTTAAACTCC	620	(*27*)
Nest-2, genus-specific PCR	rPLU3: TTTTTATAAGGATAACTACGGAAAAGCTGT rPLU4: TACCCGTCATAGCCATGTTAGGCCAATACC	240	(*27*)
Plasmodium falciparum species–specific PCR	rFAL1: TTAAACTGGTTTGGGAAAACCAAATATATT rFAL2: ACACAATGAACTCAATCATGACTACCCGTC	205	(*27*)
msp1, primary reaction	Forward: CTAGAAGCTTTAGAAGATGCAGTATTG Reverse: CTTAAATAGTATTCTAATTCAAGTGGATCA	Variable	(*28*)
K1	Forward: AAATGAAGAAGAAATTACTACAAAAGGTGC Reverse: GCTTGCATCAGCTGGAGGGCTTGCACCAGA	Variable	(*28*)
MAD20	Forward: AAATGAAGGAACAAGTGGAACAGCTGTTAC Reverse: ATCTGAAGGATTTGTACGTCTTGAATTACC	Variable	(*28*)
RO33	Forward: TAAAGGATGGAGCAAATACTCAAGTTGTTG Reverse: CATCTGAAGGATTTGCAGCACCTGGAGATC	Variable	(*28*)
msp2, primary reaction	Forward: ATGAAGGTAATTAAAACATTGTCTATTATA Reverse: CTTTGTTACCATCGGTACATTCTT	Variable	(*28*)
FC27	Forward: AATACTAAGAGTGTAGGTGCARATGCTCCA Reverse: TTTTATTTGGTGCATTGCCAGAACTTGAAC	Variable	(*28*)
IC/3D7	Forward: AGAAGTATGGCAGAAAGTAAKCCTYCTACT Reverse: GATTGTAATTCGGGGGATTCAGTTTGTTCG	Variable	(*28*)
glurp, primary reaction	Forward: TGAATTTGAAGATGTTCACACTGAAC Reverse: GTGGAATTGCTTTTTCTTCAACACTAA	Variable	(*28*)
glurp	Forward: TGTTCACACTGAACAATTAGATTTAGATCA Reverse: GTGGAATTGCTTTTTCTTCAACACTAA	Variable	(*28*)

**glurp*, glutamate-rich protein; *msp1*, merozoite surface protein 1; *msp2*, merozoite surface protein 2.

(Table 1), annealing temperature (61°C), and DNA template (2 μL) were different.

Statistical Analysis

We used GraphPad Prism version 7.03 (https://www.graphpad.com) for allele frequency graph construction. We also used this tool to compare allele frequencies by paired *t* test and analyze the polyclonal distribution of allele families.

Results

P. falciparum Nested PCR

We obtained 282 archived whole blood samples from the 6 healthcare centers participating in the study. Many of the samples originated from patients who visited the centers for general medical checkups, genotyping, and screening for hepatitis viruses. Various clinical laboratory tests were performed on the collected blood samples as requested by the medical doctors for determining diagnosis or as requested by the patient.

Of 282 blood samples, 54 were positive for a *Plasmodium* species (240-bp PCR product) by nested PCR and 34 samples were positive for *P. falciparum* (205-bp PCR product; Figure 2). The estimated prevalence of *P. falciparum* among positive samples was 63% (34/54), unusually low compared with the national *P. falciparum* prevalence of >95% in 2015 (*1*). However, this finding represents a limited number of samples and cannot truly reflect the national average. The estimated prevalence of 37% for non–*P. falciparum* malaria species can probably be attributed to the circulation of ≥2% of the other *Plasmodium* species that infect humans.

Allele Diversity and Complexity of Infections

In genotyping PCRs, 32 persons were positive for *msp1*, 29 for *msp2*, and 31 for *glurp*. We found more genetic variation (i.e., more alleles) for the *msp1* gene than the *msp2* or *glurp* genes. We detected a total of 39 *msp1* (16 *K1*, 13 *MAD20*, and 10 *RO33*), 31 *msp2* (16 *FC27* and 15 *IC/3D7*), and 13 *glurp* clones in *P. falciparum*–positive blood samples (Tables 2, 3). A high percentage of the population had evidence of polyclonal infections; ≥2 different-sized PCR products (i.e., ≥2 alleles) were present on agarose gel for the *msp1*, *msp2*, or *glurp* genes in 88% of the samples. Similar studies conducted previously in Nigeria (mainly in the southwestern part) during 2004–2014 showed less genetic diversity (Table 3). In those studies, the total number of clones detected were 4 for *K1*, 2 for *MAD20*, and 4 for *RO33* of the *msp1* gene and 9 for *FC27* and 4 for *IC/3D7* of the *msp2* gene. Polyclonal *P. falciparum* infections were more prevalent in our study (multiplicity of infection [MOI], i.e., parasite clones per sample, 2.4) than in the previous reports (MOIs 1.1 and 1.4; Table 3).

In our study, fragment sizes were 100–1,200 bp for *msp1*, 150–1,200 bp for *msp2*, and 200–1,550 bp for *glurp* (Figures 3–5). The highest number of clones seen for a single allele in a single sample was 8 (with 8 clear bands of the *FC27* allele); this sample came from a patient in Kafanchan. A number of samples contained only 1 clone: 8 samples contained a single clone of *K1*, 8 a single clone of *RO33*, 6 a single clone of *MAD20*, 7 a single clone of *FC27*, and 9 a single clone of *IC/3D7*.

The combinations of *msp1* family alleles observed were *K1*, *MAD20*, and *RO33* (63%, 20/32); *K1* and *MAD20* (3%, 1/32); *K1* and *RO33* (6%, 2/32); and *MAD20* and *RO33* (3%, 1/32). Infections were predominantly trimorphic (63%), and fewer were dimorphic (12%); 25% were monomorphic. For the *msp2* allele family, dimorphic infections (*FC27* + *IC/3D7*) occurred in 50% of the population; 35% of the population was positive for only *IC/3D7* and 15% for only *FC27*. Many patients had infections with high MOIs. The mean MOI per allele family was 2.4 for *msp1* and *msp2* and 1.0 for *glurp* (Table 2). The wide occurrence of trimorphic and dimorphic allele combinations is indicative of high complexity of infection in the study population.

We assessed the frequency of the occurrence of each allele in the population. We categorized clones into molecular weight groups differing by 50 bp for clear discrimination from other clones and elimination of errors that would result from estimating the molecular weight on agarose gels. For the *msp1* allele family, the *K1* allele of size 200–250 bp was detected at the highest frequency (n = 18), followed by the *MAD20* allele of fragment size 150–200 bp (n = 11) and the *RO33* alleles of fragment sizes 100–150

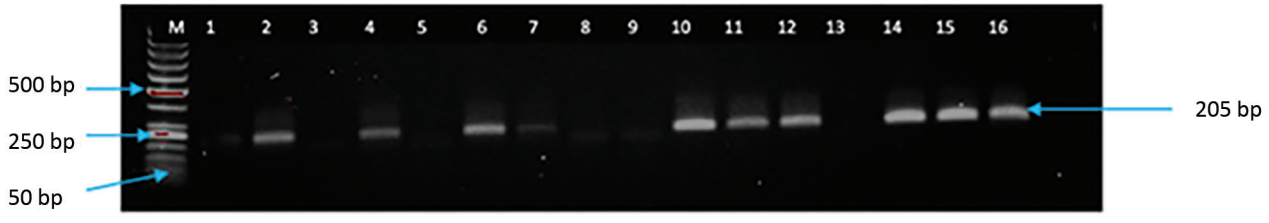


Figure 2. Screening PCR results of persons with *Plasmodium falciparum* parasite infections, North Central Nigeria, 2015–2018. Lane M, 50-bp DNA marker (ThermoFisher Scientific, https://www.thermofisher.com); lanes 1–4, archived blood samples from Nisa Premier Hospital (Jabi, Federal Capital Territory, Nigeria); lanes 5–15, archived blood samples from Kogi Specialist Hospital (Lokoja, Kogi State, Nigeria); lane 16, positive control. Samples positive for *P. falciparum* had a PCR product size of 205 bp.

Table 2. Alleles of genes detected in *Plasmodium falciparum* isolates, North Central Nigeria, 2015–2018*

Allele family	No. positive by PCR	No. alleles	Allele size range, bp	Mean MOI†	% Polyclonal
msp1	32				
K1	32	16	100–916	2.63	68
MAD20	21	13	100–1,200	2.38	73
RO33	22	10	100–1,100	2.23	64
msp2	29				
FC27	17	16	150–1,130	2.52	53
IC/3D7	28	15	300–1,200	2.21	68
glurp	31	13	200–1,550	1.03	23

*glurp, glutamate-rich protein; MOI, multiplicity of infection; *msp1*, merozoite surface protein 1; *msp2*, merozoite surface protein 2.
†Defined as number of parasite clones per sample.

bp (n = 9) and 650–700 bp (n = 9) (Figure 3). For the *msp2* allele family, the most frequently detected alleles were the *FC27* allele of size 250–300 bp (n = 9) and the *IC/3D7* allele of size 400–450 bp (n = 9) (Figure 4). The *glurp* allele with the highest frequency (n = 11) was 900–950 bp in size (Figure 5).

We did not attempt to compare the distribution patterns of the allele families by location because the number of *P. falciparum*–positive samples in some locations was low. However, an analysis of the seasonal patterns of diversity showed that 60% of the infections that occurred during the dry season (November–March) and 50% of those that occurred during the wet season (April–October) were polyclonal. Paired *t* tests showed that the temporal distribution of polyclonal infections and the frequency of clones were statistically significant ($p<0.05$). During the dry season, the person infected with the highest number of clones (8 clones) was sampled, as well as others infected with 4–5 clones. The finding that polyclonal infections were more common in the dry season was unexpected. However, the increased transmission during this period can be explained by the open gutter and sewage systems present in urban areas of Nigeria; this factor has led to the mosquitoes that potentiate malaria transmission being prevalent year-round.

Discussion

In this prospective cross-sectional study, we investigated *P. falciparum* genetic diversity and the complexity of *P. falciparum* infection in North Central Nigeria because a high level of genetic diversity and infection complexity was a likely aftermath of the influx of IDPs into the region. We hypothesized that a high level of genetic diversity would be found in the region partly as a result of immigration of *P. falciparum*–infected IDPs into the region. High genetic diversity of malaria parasites circulating in a population could serve as a risk factor for genetic recombination and the generation of novel alleles (*11*–*13*). We found that 63% of the *Plasmodium* spp.–positive samples were positive for *P. falciparum*. Our PCR screening results cannot be used as an estimate of the prevalence of *P. falciparum* in North Central Nigeria, but this finding has revealed that non–*P. falciparum* malaria species are circulating in this region and infecting humans. The finding of 37% positivity for non–*P. falciparum Plasmodium* spp. is high compared with previous reports of low-level prevalence (0.4%–6.3%) in other parts of Nigeria (*30*–*33*). An epidemiologic study of non–*P. falciparum* malaria species circulating in the North Central region would be vital for national malaria control. Treatment failures reported by some authors (*16*,*17*) in Nigeria could be related to incorrect diagnosis and hence inappropriate drug administration. Artemisinin-based combination therapy is recommended for the treatment of *P. falciparum* malaria, but chloroquine plus primaquine is the first-line regimen for *P. vivax* malaria (*34*).

We detected the *msp1*, *msp2*, and *glurp* alleles in most patients positive for *P. falciparum* parasites, and these alleles had high genetic variability. Many samples from infected patients contained multiple alleles, and the genetic variation was more extensive with the *msp1* and *msp2* allele families than with the *glurp* allele. Overall, 88% of the

Table 3. Distribution of *msp 1*, *msp 2*, and *glurp* clones detected in previous studies conducted in southwestern Nigeria, 2004–2014, and North Central Nigeria, 2015–2018*

Study	Region (state or territory)	No. samples	No. alleles†						Mean MO‡
			msp1			*msp2*			
			K1	*MAD20*	*R033*	*FC27*	*IC/3D7*	*glurp*	
Happi et al. (*30*)	Southwestern (Ogun)	47	4	2	4	9	4	5	–
Olasehinde et al. (*31*)	Southwestern (Ogun)	100	4	3	1	3	3	–	1.1
Oyebola et al. (*32*)	Southwestern (Lagos)	100	3	2	1	3	3	–	1.4
Bamidele Abiodun et al. (*33*)	Southwestern (Lagos)	78	2	2	1	–	–	–	1.4
This study	North Central (Plateau, Nasarawa, Kogi, Kaduna, FCT)	54	16	13	10	16	15	13	2.4

*FDT, Federal Capital Territory; *glurp*, glutamate-rich protein; MOI, multiplicity of infection; *msp1*, merozoite surface protein 1; *msp2*, merozoite surface protein 2.
†Total number alleles found, regardless of size.
‡Defined as number of parasite clones per sample.

population had evidence of polyclonal infection with ≥1 of the allelic forms. We discovered 10–16 *msp1* clones and 15–16 *msp2* clones. This finding does not corroborate results of similar previous studies from southwestern Nigeria, in which 1–4 *msp1* clones and 3–9 *msp2* clones were found (Table 3). We detected a total of 39 *msp1*, 31 *msp2*, and 13 *glurp* alleles, which is quite high compared with the reported values in other regions of Nigeria (*30*,*32*) and other countries of West Africa (*21*). Infections with pathogens harboring high numbers of alleles could influence the emergence of resistance (*35*), considering that, in this scenario, multiple alleles are exposed to the same chemotherapeutic agents. The high level of trimorphic, dimorphic, and monomorphic infections in the population is evidence of extensive genetic diversity. Our work revealed a higher frequency of occurrence of polyclonal infections and infections with *P. falciparum* parasites of multiple genotypes, as well as infections of higher MOIs, compared with previous studies (*30*,*33*,*36*).

The question yet to be answered, however, is whether polyclonal infection is a frequent occurrence in Nigeria. The frequent occurrence of this phenomenon in a single geographic area has been determined to be an early indicator for the emergence of alleles associated with drug resistance (*11*,*35*). The high number of polyclonal infections seen in North Central Nigeria could be attributed to a high level of genetic recombination and high evolutionary pressure on the *msp1*, *msp2*, and *glurp* genes in this region, which could partially be attributed to the high level

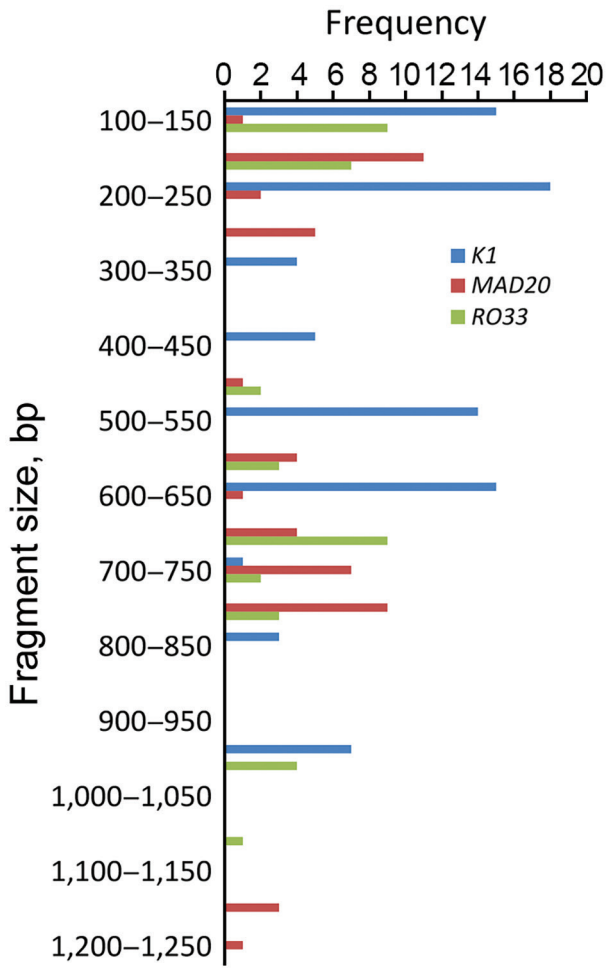


Figure 3. Allele frequency of *msp1* in persons with *Plasmodium falciparum* infection, North Central Nigeria, 2015–2018. The K1 allele of size 200–250 bp was detected at the highest frequency (n = 18). The next highest detected were the *MAD20* allele of fragment size 150–200 bp (n = 11) and the *RO33* alleles of fragment sizes 100–150 bp (n = 9) and 650–700 bp (n = 9).

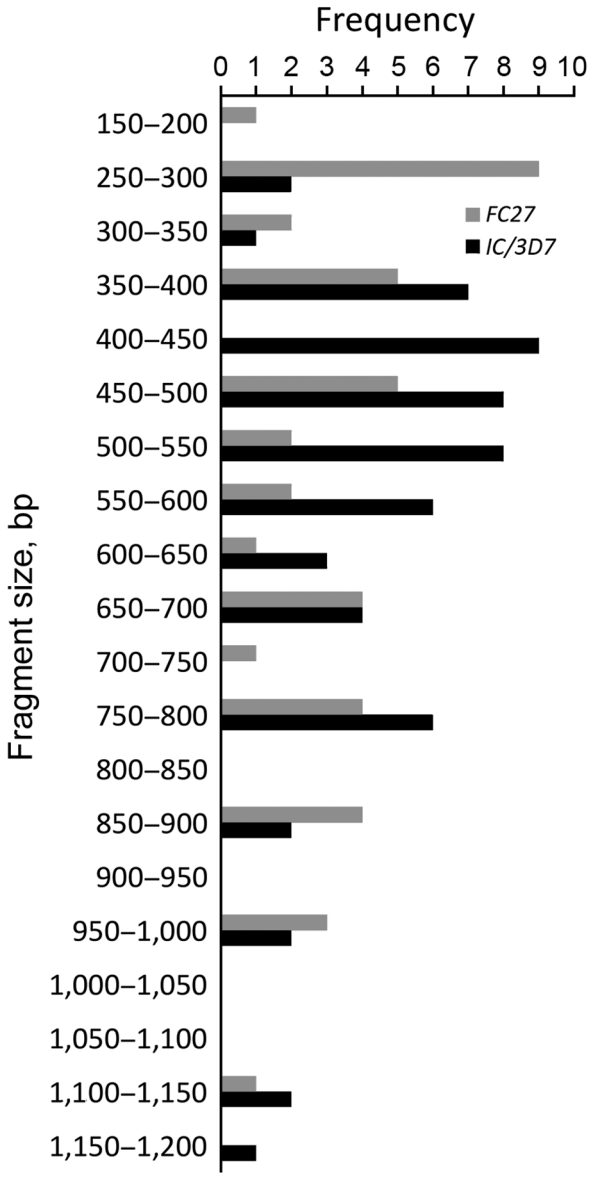


Figure 4. Allele frequency of *msp2* in persons with *Plasmodium falciparum* infection, North Central Nigeria, 2015–2018. The most frequently detected alleles were the *FC27* allele of size 250–300 bp (n = 9) and the *IC/3D7* allele of size 400–450 bp (n = 9).

of movement into the North Central region (the economic hub of the nation) for commercial reasons. Drug resistance of *P. falciparum* parasites is a highly complex mechanism involving multiple genes, *pfk13* being a gene responsible (*37*). Work by Oboh et al. (*36*) and Ebenebe et al. (*37*) indicated that in 2018 drug resistance of malaria pathogens to artemisinin-based combination therapy was not an immediate public health threat for southwestern Nigeria. Nonetheless, a nationwide study should be performed to comprehensively evaluate for the presence of *P. falciparum* drug resistance genes with techniques such as next-generation sequencing and genome walking (*38*,*39*). Oboh et al. (*36*) were able to detect synonymous mutations (not validated AA mutations of the *pfk13* gene), such as the nucleotide change from CCG to CCA in the *pfk13* gene in *P. falciparum* from southwestern Nigeria, and called for close monitoring of parasites in Nigeria. A nationwide study should also be conducted to comprehensively determine the genetic diversity of *P. falciparum* and complexity of infections in Nigeria. Determining the genetic diversity of malaria parasites in the North Central region, as well as for the entire nation of Nigeria in general, is needed for the design of a national treatment policy, vaccine development, and immunogenicity studies.

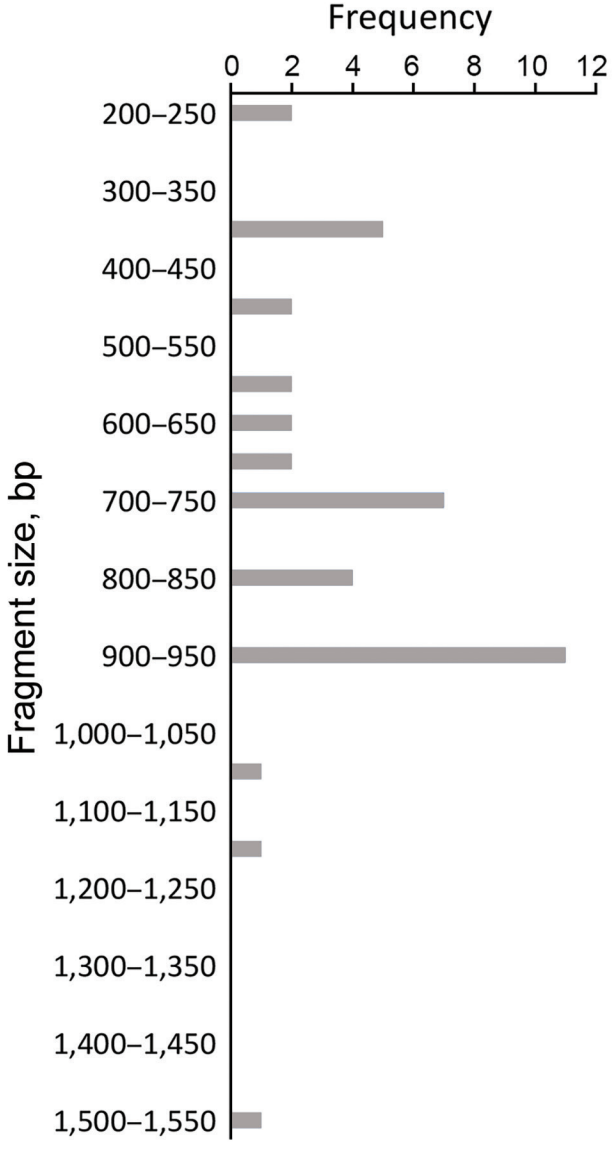


Figure 5. Allele frequency of *glurp* in persons with *Plasmodium falciparum* infection, North Central Nigeria, 2015–2018. The allele with the highest frequency (n = 11) was 900–950 bp in size.

The frequency of polyclonal infections in southwestern Nigeria during 2004–2014 can be described as low and not extensive compared with the level discovered in this study. The period of this study (2015–2018) falls within peak periods of communal conflicts in Nigeria (2009–2017), which resulted in the displacement of persons in the North Central region (*40*,*41*). Our findings suggest a relationship between the high number of polyclonal infections discovered in North Central Nigeria and the movements of IDPs into the region, although a previous study of the *msp2* genotype by Oyedeji et al. (*42*) in Lafia (Nasarawa State) showed a high level of genetic diversity in 2005–2006. A high level of introduction of new *P. falciparum* clones could have occurred in the host communities in the towns and cities that experienced IDP influxes.

A major limitation of this study is the lack of a comparison of the genetic diversity of *Plasmodium* spp. in patients in other regions of the country. However, previous studies conducted in southwestern Nigeria provided a baseline for comparison to determine whether the level of *Plasmodium* spp. genetic diversity discovered in this study was different and thus potentially associated with the movement of IDPs. The correlation would have been more comprehensive if we had also included comparison groups from other regions and specific IDP camps in our investigation.

In summary, this study revealed a high level of *P. falciparum* genetic diversity and infection complexity in North Central Nigeria and suggested that this complexity might be a result of the insurgency and movement of IDPs that occurred in Nigeria during 2009–2017. A deliberate effort is needed to control malaria and eliminate the risk for the evolution of resistance alleles across Nigeria, but particularly in this region of Nigeria, which is the economic hub of the nation and at highest risk for distributing new strains worldwide. The information we generated on *Plasmodium* spp. epidemiology and genetic diversity could serve as a source in a database to be used for policy development on nationwide malaria control and intervention programs for Nigeria. Our work also led to the establishment of a laboratory capable of performing PCR methods to evaluate malaria epidemiology in the region, which could be used for future comprehensive malaria control measures.

Acknowledgments

We sincerely thank Bitrus Matawal for his valuable assistance in securing the national code for ethics approval to conduct this healthcare research. We also appreciate the medical laboratory scientists in the selected healthcare centers who provided aliquots of archived blood samples analyzed in this study.

This study was sponsored by the Biotechnology Development Center of the University of Jos.

About the Author

Dr. Yakubu is a research fellow at the Biotechnology Division of the National Veterinary Research Institute Vom in Jos, Nigeria. He specializes in PCR methodology and diagnostics and has research interests in DNA barcoding, vaccinology, and protein biotechnology.

References

1. National Malaria Elimination Programme, National Population Commission, and National Bureau of Statistics, Federal Republic of Nigeria; ICT International. Nigeria malaria indicator survey 2015. 2016 Aug [cited 2018 Oct 18]. https://dhsprogram.com/pubs/pdf/MIS20/MIS20.pdf
2. World Health Organization. World malaria report 2017. Geneva: The Organization; 2017 [cited 2018 Oct 18]. https://www.who.int/malaria/publications/world-malaria-report-2017
3. United Nations Office for the Coordination of Humanitarian Affairs. Situation report no.1, 2014. 2014 [cited 2018 Oct 18]. http://www.unocha.org/nigeria
4. Aribodor DN, Ugwuanyi IK, Aribodor OB. Challenges to achieving malaria elimination in Nigeria. Am J Public Health Res. 2016;4:38–41.
5. Internal Displacement Monitoring Centre. GRID 2016. Global report on internal displacement. 2016 May [cited 2018 Oct 18]. http://www.internal-displacement.org/globalreport2016
6. Emmanuelar I. Insurgency and humanitarian crisis in northern Nigeria: the case of Boko Haram. Afr J Pol Sci Int Relat. 2015;9:284–96. http://dx.doi.org/10.5897/AJPSIR2015.0789
7. Martens P, Hall L. Malaria on the move: human population movement and malaria transmission. Emerg Infect Dis. 2000;6:103–9. http://dx.doi.org/10.3201/eid0602.000202
8. Greenwood B, Mutabingwa T. Malaria in 2002.Nature. 2002;415:670–2. http://dx.doi.org/10.1038/415670a
9. Gu W, Killeen GF, Mbogo CM, Regens JL, Githure JI, Beier JC. An individual-based model of *Plasmodium falciparum* malaria transmission on the coast of Kenya. Trans R Soc Trop Med Hyg. 2003;97:43–50. http://dx.doi.org/10.1016/S0035-9203(03)90018-6
10. Osorio L, Todd J, Pearce R, Bradley DJ. The role of imported cases in the epidemiology of urban *Plasmodium falciparum* malaria in Quibdó, Colombia. Trop Med Int Health. 2007;12:331–41. http://dx.doi.org/10.1111/j.1365-3156.2006.01791.x
11. Conway DJ, Roper C, Oduola AMJ, Arnot DE, Kremsner PG, Grobusch MP, et al. High recombination rate in natural populations of *Plasmodium falciparum.* Proc Natl Acad Sci U S A. 1999;96:4506–11. http://dx.doi.org/10.1073/pnas.96.8.4506
12. Meyer CG, May J, Arez AP, Gil JP, Do Rosario V. Genetic diversity of *Plasmodium falciparum*: asexual stages. Trop Med Int Health. 2002;7:395–408. http://dx.doi.org/10.1046/j.1365-3156.2002.00875.x
13. Kiwanuka GN. Genetic diversity in *Plasmodium falciparum* merozoite surface protein 1 and 2 coding genes and its implications in malaria epidemiology: a review of published studies from 1997-2007. J Vector Borne Dis. 2009;46:1–12.
14. Lee SA, Yeka A, Nsobya SL, Dokomajilar C, Rosenthal PJ, Talisuna A, et al. Complexity of *Plasmodium falciparum* infections and antimalarial drug efficacy at 7 sites in Uganda. J Infect Dis. 2006;193:1160–3. http://dx.doi.org/10.1086/501473
15. Kyabayinze DJ, Karamagi C, Kiggundu M, Kamya MR, Wabwire-Mangen F, Kironde F, et al. Multiplicity of *Plasmodium falciparum* infection predicts antimalarial treatment outcome in Ugandan children. Afr Health Sci. 2008;8:200–5.
16. Amodu OK, Adeyemo AA, Ayoola OO, Gbadegesin RA, Orimadegun AE, Akinsola AK, et al. Genetic diversity of the *msp-1* locus and symptomatic malaria in south-west Nigeria. Acta Trop. 2005;95:226–32. http://dx.doi.org/10.1016/j.actatropica.2005.06.017
17. Amodu OK, Oyedeji SI, Ntoumi F, Orimadegun AE, Gbadegesin RA, Olumese PE, et al. Complexity of the *msp2* locus and the severity of childhood malaria, in south-western Nigeria. Ann Trop Med Parasitol. 2008;102:95–102. http://dx.doi.org/10.1179/136485908X252340
18. Ajayi NA, Ukwaja KN. Possible artemisinin-based combination therapy-resistant malaria in Nigeria: a report of three cases. Rev Soc Bras Med Trop. 2013;46:525–7. http://dx.doi.org/10.1590/0037-8682-0098-2013
19. Ferreira MU, Kaneko O, Kimura M, Liu Q, Kawamoto F, Tanabe K. Allelic diversity at the merozoite surface protein-1 (*msp-1*) locus in natural *Plasmodium falciparum* populations: a brief overview. Mem Inst Oswaldo Cruz. 1998;93:631–8. http://dx.doi.org/10.1590/S0074-02761998000500013
20. Ferreira MU, Ribeiro WL, Tonon AP, Kawamoto F, Rich SM. Sequence diversity and evolution of the malaria vaccine candidate merozoite surface protein-1 (MSP-1) of *Plasmodium falciparum.* Gene. 2003;304:65–75. http://dx.doi.org/10.1016/S0378-1119(02)01180-0
21. Mwingira F, Nkwengulila G, Schoepflin S, Sumari D, Beck H-P, Snounou G, et al. *Plasmodium falciparum msp1*, *msp2*, and *glurp* allele frequency and diversity in sub-Saharan Africa. Malar J. 2011;10:79. http://dx.doi.org/10.1186/1475-2875-10-79
22. Manske M, Miotto O, Campino S, Auburn S, Almagro-Garcia J, Maslen G, et al. Analysis of *Plasmodium falciparum* diversity in natural infections by deep sequencing. Nature. 2012;487:375–9. http://dx.doi.org/10.1038/nature11174
23. Irawati N, Jamsari, Wirasti Y. Genetic diversity of merozoite surface protein-1 in *Plasmodium falciparum* field isolates from a mountain and coastal area in West Sumatera, Indonesia. J Pharm Biomed Sci. 2013;30(30):1061–4.
24. Mawili-Mboumba DP, Mbondoukwe N, Adande E, Bouyou-Akotet MK. Allelic diversity of *msp1* gene in *Plasmodium falciparum* from rural and urban areas of Gabon. Korean J Parasitol. 2015;53:413–9. http://dx.doi.org/10.3347/kjp.2015.53.4.413
25. Jelinek T, Kilian AH, Westermeier A, Pröll S, Kabagambe G, Nothdurft HD, et al. Population structure of recrudescent *Plasmodium falciparum* isolates from western Uganda. Trop Med Int Health. 1999;4:476–80. http://dx.doi.org/10.1046/j.1365-3156.1999.00428.x
26. Snounou G, Viriyakosol S, Zhu XP, Jarra W, Pinheiro L, do Rosario VE, et al. High sensitivity of detection of human malaria parasites by the use of nested polymerase chain reaction. Mol Biochem Parasitol. 1993;61:315–20. http://dx.doi.org/10.1016/0166-6851(93)90077-B
27. Singh B, Bobogare A, Cox-Singh J, Snounou G, Abdullah MS, Rahman HA. A genus- and species-specific nested polymerase chain reaction malaria detection assay for epidemiologic studies. Am J Trop Med Hyg.1999;60:687–92. http://dx.doi.org/10.4269/ajtmh.1999.60.687

28. Snounou G, Färnert A. Genotyping of *Plasmodium falciparum* parasites by PCR. In: Moll K, Ljungström I, Perlmann H, Scherf A, Wahlgren M, editors. Methods in malaria research. Manassas (VA): American Type Culture Collection; 2008. p. 238–42 [cited 2018 Oct 18]. https://ki.se/sites/default/files/methods_in_malaria_research.pdf
29. Waters AP, McCutchan TF. Rapid, sensitive diagnosis of malaria based on ribosomal RNA. Lancet. 1989;333:1343–6. http://dx.doi.org/10.1016/S0140-6736(89)92800-6
30. Happi CT, Gbotosho GO, Sowunmi A, Falade CO, Akinboye DO, Gerena L, et al. Molecular analysis of *Plasmodium falciparum* recrudescent malaria infections in children treated with chloroquine in Nigeria. Am J Trop Med Hyg. 2004;70:20–6. http://dx.doi.org/10.4269/ajtmh.2004.70.20
31. Olasehinde GI, Yah CS, Singh R, Ojuronbge OO, Ajayi AA, Valecha N, et al. Genetic diversity of *Plasmodium falciparum* field isolates from south western Nigeria. Afr Health Sci. 2012;12:355–61.
32. Oyebola MK, Idowu ET, Olukosi YA, Iwalokun BA, Agomo CO, Ajibaye OO, et al. Genetic diversity and complexity of *Plasmodium falciparum* infections in Lagos, Nigeria. Asian Pac J Trop Biomed. 2014;4(Suppl 1):S87–91. http://dx.doi.org/10.12980/APJTB.4.2014C1301
33. Bamidele Abiodun I, Oluwadun A, Olugbenga Ayoola A, Senapon Olusola I. *Plasmodium falciparum* merozoite surface proein-1 polymorphisms among asymptomatic sickle cell anemia patients in Nigeria. Acta Med Iran. 2016;54:44–53.
34. Sam Wobo SO, Adekunle NO, Adeleke MA, Dedeke GA, Oke OA, Abimbola WA, et al. Epidemiological factors in prevalence of malaria parasites in primary health facilities attendants, Ogun State, Nigeria. Malar Chemother Control Elimin. 2014;3:1000111.
35. Cui L, Mharakurwa S, Ndiaye D, Rathod PK, Rosenthal PJ. Antimalarial drug resistance: literature review and activities and findings of the ICEMR network. Am J Trop Med Hyg.2015;93(Suppl):57–68. http://dx.doi.org/10.4269/ajtmh.15-0007
36. Oboh MA, Ndiaye D, Antony HA, Badiane AS, Singh US, Ali NA, et al. Status of artemisinin resistance in malaria parasite *Plasmodium falciparum* from molecular analysis of *kelch13* gene in southwestern Nigeria. BioMed Res Int. 2018;2018:2305062. http://dx.doi.org/10.1155/2018/2305062
37. Ebenebe JC, Ntadom G, Ambe J, Wammanda R, Jiya N, Finomo F, et al.; for the Antimalarial Therapeutic Efficacy Monitoring Group National Malaria Elimination Programme The Federal Ministry Of Health Abuja Nigeria. Efficacy of artemisinin-based combination treatments of uncomplicated falciparum malaria in under-five year-old Nigerian children ten years following adoption as first line antimalarials. Am J Trop Med Hyg. 2018;99:649–64. http://dx.doi.org/10.4269/ajtmh.18-0115
38. Flannery EL, Wang T, Akbari A, Corey VC, Gunawan F, Bright AT, et al. Next-generation sequencing of *Plasmodium vivax* patient sample shows evidence of direct evolution in drug-resistance genes. ACS Infect Dis. 2015;1:367–79. http://dx.doi.org/10.1021/acsinfecdis.5b00049
39. Nag S, Dalgaard MD, Kofoed PE, Ursing J, Crespo M, Andersen LO, et al. High throughput resistance profiling of *Plasmodium falciparum* infections based on custom dual indexing and Illumina next generation sequencing-technology. Sci Rep. 2017;7:2398. http://dx.doi.org/10.1038/s41598-017-02724-x
40. International Organization for Migration. Displacement tracking matrix (DTM) round 12 report-October 2016. 2016 Oct [cited 2018 Oct 18]. https://reliefweb.int/report/nigeria/displacement-tracking-matrix-dtm-round-12-report-october-2016
41. International Committee of the Red Cross. Internal displacement in northeastern Nigeria. Kampala Convention. Supra note 6. Article 5(1). 2016 Dec [cited 2018 Oct 18]. http://www.icrc.org/where-we-work/africa/nigeria
42. Oyedeji SI, Awobode HO, Anumudu C, Kun J. Genetic diversity of *Plasmodium falciparum* isolates from naturally infected children in north-central Nigeria using the merozoite surface protein-2 as molecular marker. Asian Pac J Trop Med. 2013;6:589–94. http://dx.doi.org/10.1016/S1995-7645(13)60102-9

Address for correspondence: Bitrus Yakubu, National Veterinary Research Institute, Biotechnology Division, PMB 01 Vom, Jos, Plateau State 00931, Nigeria; email: minitsa65@gmail.com

Hospital-Associated Multicenter Outbreak of Emerging Fungus *Candida auris*, Colombia, 2016

Paige A. Armstrong,[1] Sandra M. Rivera,[1] Patricia Escandon, Diego H. Caceres, Nancy Chow, Matthew J. Stuckey, Jorge Díaz, Adriana Gomez, Norida Vélez, Andres Espinosa-Bode, Soraya Salcedo, Adriana Marin, Indira Berrio, Carmen Varón, Angel Guzman, Jairo E. Pérez-Franco, Julian D. Escobar, Nohora Villalobos, Juan M. Correa, Anastasia P. Litvintseva, Shawn R. Lockhart, Ryan Fagan, Tom M. Chiller, Brendan Jackson,[2] Oscar Pacheco[2]

Medscape EDUCATION ACTIVITY

In support of improving patient care, this activity has been planned and implemented by Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is jointly accredited by the Accreditation Council for Continuing Medical Education (ACCME), the Accreditation Council for Pharmacy Education (ACPE), and the American Nurses Credentialing Center (ANCC), to provide continuing education for the healthcare team.

Medscape, LLC designates this Journal-based CME activity for a maximum of 1.00 ***AMA PRA Category 1 Credit(s)™***. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

Successful completion of this CME activity, which includes participation in the evaluation component, enables the participant to earn up to 1.0 MOC points in the American Board of Internal Medicine's (ABIM) Maintenance of Certification (MOC) program. Participants will earn MOC points equivalent to the amount of CME credits claimed for the activity. It is the CME activity provider's responsibility to submit participant completion information to ACCME for the purpose of granting ABIM MOC credit.

All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test with a 75% minimum passing score and complete the evaluation at http://www.medscape.org/journal/eid; and (4) view/print certificate. For CME questions, see page 1443.

Release date: June 13, 2019; Expiration date: June 13, 2020

Learning Objectives

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

- Assess the clinical characteristics and outcomes of a hospital-associated *Candida auris* outbreak in Colombia in 2015 to 2016, based on a clinical case series
- Determine the likely mode of transmission and associated findings in a hospital-associated *C. auris* outbreak in Colombia in 2015 to 2016, based on a clinical case series
- Evaluate treatment and other clinical and public health implications of a hospital-associated *C. auris* outbreak in Colombia in 2015 to 2016, based on a clinical case series

CME Editor

Deborah Wenger, MBA, Copyeditor, Emerging Infectious Diseases. *Disclosure: Deborah Wenger, MBA, has disclosed no relevant financial relationships.*

CME Author

Laurie Barclay, MD, freelance writer and reviewer, Medscape, LLC. *Disclosure: Laurie Barclay, MD, has disclosed no relevant financial relationships.*

Authors

Disclosures: ***Paige A. Armstrong, MD, MHS; Sandra Rivera, BN, PGrad Dip; Patricia Escandon, MSc; Diego H. Caceres, MSc,; Nancy A. Chow, PhD; Matthew J. Stuckey, PhD, MPH; Jorge Luis Díaz, MD; Adriana Leonor Gomez, MS; Norida Natally Vélez, MSc; Andres Espinosa-Bode, MD; Soraya Salcedo, MD; Adriana Marin, MSc; Carmen Varón, BN, RN; Angel Alfonso Guzman, MD; Jairo E. Pérez-Franco, MD; Nohora Constanza Villalobos, MS; Juan Manuel Correa, MD, MSc; Anastasia P. Litvintseva, PhD; Shawn R. Lockhart, PhD; Ryan Fagan, MD, MPH&TM; Tom Chiller, MD, MPH&TM; Brendan R. Jackson, MD, MPH;*** *and* ***Oscar Pacheco, MD,*** *have disclosed no relevant financial relationships.* ***Indira Berrio, MD,*** *has disclosed the following relevant financial relationships: served as an advisor or consultant for MSD, Pfizer; served as a speaker or a member of a speakers bureau for Gilead, MSD, Pfizer.* ***Julian David Escobar, MSc,*** *has disclosed the following relevant financial relationships: employed by a commercial interest, MSD Colombia.*

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (P.A. Armstrong, D.H. Caceres, N. Chow, M.J. Stuckey, A. Espinosa-Bode, A.P. Litvintseva, S.R. Lockhart, R. Fagan, T.M. Chiller, B. Jackson); Instituto Nacional de Salud, Bogotá, Colombia (S.M. Rivera, P. Escandon, J. Díaz, A. Gomez, N. Vélez, O. Pacheco); Clínica General del Norte, Barranquilla, Colombia (S. Salcedo, A. Marin); Clínica El Rosario, Medellín, Colombia (I. Berrio); Corporación para Investigaciones Biológicas (CIB), Medellín (I. Berrio); Hospital General de Medellín, Medellín (I. Berrio); Hospital Doña Pilar, Cartagena, Colombia (C. Varón, A. Guzman); Hospital Militar Central, Bogotá (J.E. Pérez-Franco, J.D. Escobar); Clínica los Nogales, Bogotá (N. Villalobos, J.M. Correa)

DOI: https://doi.org/10.3201/eid2507.180491

[1]These authors contributed equally to this article.

[2]These authors contributed equally to this article.

Candida auris is an emerging multidrug-resistant fungus that causes hospital-associated outbreaks of invasive infections with high death rates. During 2015–2016, health authorities in Colombia detected an outbreak of *C. auris*. We conducted an investigation to characterize the epidemiology, transmission mechanisms, and reservoirs of this organism. We investigated 4 hospitals with confirmed cases of *C. auris* candidemia in 3 cities in Colombia. We abstracted medical records and collected swabs from contemporaneously hospitalized patients to assess for skin colonization. We identified 40 cases; median patient age was 23 years (IQR 4 months–56 years). Twelve (30%) patients were <1 year of age, and 24 (60%) were male. The 30-day mortality was 43%. Cases clustered in time and location; axilla and groin were the most commonly colonized sites. Temporal and spatial clustering of cases and skin colonization suggest person-to-person transmission of *C. auris*. These cases highlight the importance of adherence to infection control recommendations.

Candida auris is an emerging multidrug-resistant fungus that has been implicated in recent hospital-associated outbreaks of invasive infections with high death rates (*1*). *C. auris* from an external ear isolate in Japan was described in 2009; the fungus has since been identified as the cause of outbreaks in other countries, with rapid spread globally (*2*–*5*). Of particular concern, *C. auris* isolates have demonstrated resistance to multiple classes of antifungal drugs (*2*,*6*). In addition, whereas *Candida* is often considered a commensal organism that most commonly colonizes the gastrointestinal tract, the potential for person-to-person spread of *C. auris* has raised concern for widespread outbreaks (*7*,*8*).

Accurate identification of *C. auris* is challenging because phenotypic methods do not correctly identify this species. It is most commonly misidentified as *C. haemulonii*, especially when using the VITEK 2 system (bioMérieux, https://www.biomerieux-diagnostics.com), but is also misidentified as *C. famata*, *C. sake*, *Rhodotorula glutinis*, and other *Candida* species (*9*). Specialized techniques, such as matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry and DNA sequencing, are needed for reliable identification.

During January 2015–September 2016, the Colombian Instituto Nacional de Salud (INS) and the Secretarias de Salud (Ministries of Health) of Barranquilla, Bogotá, and Cartagena, Colombia, identified an increasing number of reported bloodstream infections with *C. haemulonii*, an otherwise rare yeast isolated in only a few invasive human infections (*10*). In May 2016, a total of 27 isolates initially identified as *C. haemulonii* by phenotypic identification methods were sent to the US Centers for Disease Control and Prevention (CDC), where 24 were confirmed to be *C. auris* by MALDI-TOF mass spectrometry (Microflex; Bruker Daltonics, https://www.bruker.com) and DNA sequencing. Through case findings and review of national surveillance data, an additional 16 cases of *C. haemulonii* reported to INS were identified as *C. auris* by MALDI-TOF mass spectrometry and confirmed at CDC with sequencing. The cases were reported from 4 large referral hospitals in Colombia. Two of these hospitals were located along the northern coast: 1 in Barranquilla, which serves both pediatric and adult patients, and the other a specialized pediatric facility in Cartagena. The other 2 hospitals were located in Bogotá and serve adult patients. No transfers took place between the involved facilities, and all follow national guidelines for infection control practices (*11*). These findings prompted an investigation to further characterize the epidemiology, transmission mechanisms, and environmental reservoirs of this organism to guide prevention measures.

Methods

In September 2016, a team consisting of clinicians, epidemiologists, and microbiologists from INS, CDC, and the Ministries of Health of Barranquilla, Bogotá, and Cartagena conducted an outbreak investigation in 1 acute-care hospital serving adult and pediatric patients in Barranquilla, 2 adult hospitals in Bogotá, and 1 pediatric hospital in Cartagena. These facilities all had confirmed cases of *C. auris* candidemia after January 2015.

Descriptive Epidemiology

We defined a case as *C. auris* confirmed by MALDI-TOF mass spectrometry isolated from a patient's blood during January 2015–September 2016. We abstracted medical records using a standardized case report form to collect data on concurrent conditions, location and duration of hospital stay, administration of antimicrobial drugs, exposure to invasive procedures, use of indwelling catheters, and outcome. We also interviewed infection control personnel, healthcare workers, and key informants at each site to trace exposures and locations of patients throughout their hospital stays.

Sampling and Laboratory Analysis

To better understand the role of colonization and transmission, we obtained samples from the 7 patients who were hospitalized at the time of the investigation with *C. auris* isolated or suspected in a specimen. We used premoistened swabs (Fisherfinest Transport Swabs, https://www.fishersci.com) to sample the following 10 body sites: bilateral nares, ears, axillae, and groin; oral cavity; and rectum. These locations were determined to be high-yield sites for colonization, and sampling was intended to provide initial insight into the utility of each. We collected fecal and urine specimens when possible.

Specimens were processed at the INS Microbiology Laboratory using protocols developed by CDC's Mycotic Diseases Branch (National Center for Emerging and Zoonotic Infectious Diseases, Division of Foodborne, Waterborne,

and Environmental Diseases). In brief, the swabs were cultured in Sabouraud dextrose enrichment broth with high salt content (10% wt/vol NaCl) at elevated temperature (40°C), shaken (250 rpm) for up to 7 days, and plated onto Sabouraud dextrose agar (*12*). Yeast isolates recovered from the selective agar were initially screened using CHROMagar Candida (Becton Dickinson, http://www.bd.com); white and pink colonies were typed using the Microflex database version MBT 6903 MSP Library (no. 1829023) using CDC MicrobeNet's supplemental MALDI database library.

Antifungal susceptibility testing was performed at CDC by broth microdilution using custom-made frozen panels; susceptibility testing for amphotericin B was performed using Etest (bioMérieux). We used the following breakpoints, based on published data: fluconazole ≥32 μg/mL, amphotericin B ≥2 μg/mL, caspofungin ≥4 μg/mL, and anidulafungin ≥2 μg/mL (*13*).

Statistical Analysis

We calculated medians and ranges for continuous variables and frequencies and percentages for categorical variables. We used 30-day mortality as outcome of interest for analysis. To compare characteristics between patients who died and those who survived, we applied a *t*-test (equal or unequal variance as appropriate) for continuous variables and a χ^2 test for categorical variables. For categorical variables having cell sizes <5, we used the Fisher exact test. Sample size did not allow for multivariate analysis. When comparing medians, we used Wilcoxon rank, as distributions were not normal. We performed all data analyses in SAS version 9.3 (https://www.sas.com) and considered a p value <0.05 significant.

CDC and INS determined that this was an emergency public health investigation. Therefore, it did not meet the criteria for research.

Results

We identified 40 cases of *C. auris* candidemia occurring during January 2015–September 2016. In 3 hospitals, cases clustered in May–July 2016, whereas in the fourth hospital, cases occurred throughout the 21-month period (Figure 1). The median patient age was 23 years (interquartile range [IQR] 4 months–56 years); 12 patients (30%) were <1 year of age. Most patients (24; 60%) were male. Thirty-five (88%) patients had a documented concurrent condition before hospitalization; the most commonly reported conditions were hemodialysis for renal failure (23%); diabetes (18%); and immunocompromising conditions (16%), including cancer and transplant.

More than half (58%) of patients died while hospitalized; the overall 30-day mortality was 43%. The median length of admission was 46 days (IQR 34–69 days). For the 38 patients with known intensive care unit (ICU) admission, median length of ICU stay was 36 days (IQR 25–58 days). All case-patients were exposed to an invasive procedure. Central venous catheter (CVC) placement was present in all 40 patients (100%); other common procedures were intubation (35; 97%), surgical procedures (28; 70%), and hemodialysis (15; 38%) (Table 1).

Median time from admission to having a blood culture positive for *C. auris* was 22 days (IQR 18–31 days). The median time from placement of CVC to positive blood culture was 12 days (IQR 5–21 days). The most common treatments received for *C. auris* candidemia were fluconazole (16; 43%) and caspofungin (13; 34%). Nineteen patients (45%) received >1 antifungal drug; the median number of antimicrobial drugs administered to patients was 3.5 (range 1–6) (Table 1). The most frequent laboratory abnormalities were mild leukocytosis (median leukocyte count 12.7×10^9 cells/L) and anemia (median hemoglobin 9.8 g/dL).

The median age of the 17 patients who died within 30 days of positive culture was 36 years (IQR 6 months–65 years). Neither age group (p = 0.18), sex (p = 0.9), nor preexisting concurrent condition (p = 0.30) were associated with 30-day mortality. Diabetes was associated with 30-day mortality (odds ratio [OR] 12, 95% CI 1.28–112.42; p = 0.03). The median length of hospital stay was longer for surviving case-patients (63 days, IQR 45–95 days) than

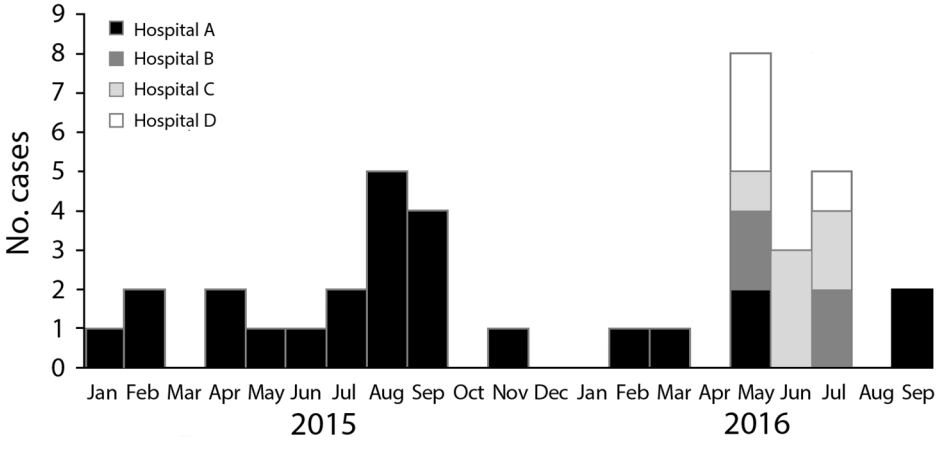


Figure. Epidemic curve for cases of *Candida auris* candidemia in Colombia, by hospital, January 2015–September 2016.

Table 1. Characteristics of 40 patients with *Candida auris* bloodstream infection, Colombia, 2015–2016*

Characteristic	Value	Data missing
Median age (IQR)	23 y (4 mo–56 y)	0
Infant up to 1 y	12 (30)	0
Child 1–18 y	8 (20)	0
Adult 18–65 y	14 (35)	0
Adult >65 y	6 (15)	0
Sex		
M	24 (60)	0
F	16 (40)	0
Concurrent conditions	35 (88)	0
Chronic renal disease	9 (23)	0
Hemodialysis dependent	9 (23)	0
Diabetes	7 (18)	2
Immunosuppressive condition	6 (16)	3
Cancer	4 (11)	3
Solid tumor	2 (5)	4
Hematologic malignancy	2 (5)	4
Transplant	2 (5)	4
Neuromuscular condition	1 (3)	0
Outcome at 30 d		
Deceased	17 (43)	0
Alive	21 (53)	0
Hospitalized	2 (5)	0
In-hospital deaths	23 (58)	6
Admitted to hospital	40 (100)	0
Median inpatient stay, d (IQR)	46 (34–69)	3
Admitted to ICU	38 (100)	2
Median ICU stay, d (IQR)	36 (25–58)	10
Transferred from another facility	16 (40)	0
Previously hospitalized in the 90 d before admission		
Yes	11 (28)	1
No	23 (59)	1
Unknown	5 (13)	1
Previous exposure to antifungal drugs		
No	22 (67)	7
Unknown	11 (33)	7
Treatments and procedures		
CVC	40 (100)	0
Vasopressors	31 (82)	2
Respiratory support	36 (100)	4
Intubation	35 (97)	4
Bilevel positive airway pressure	1 (3)	4
Surgical procedure	28 (70)	0
Total parenteral nutrition	19 (50)	2
Corticosteroids	18 (47)	2
Hemodialysis	15 (38)	1
Bronchoscopy	7 (19)	4
Chemotherapy	4 (11)	3
Median time from admission to *C. auris* positive culture, d (IQR)	22 (18–31)	0
Median time from CVC to *C. auris* positive culture, d (IQR)	12 (5–21)	1
Treatment for *C. auris* bloodstream infection		0
Fluconazole	16 (42)	
Caspofungin	13 (34)	
Amphotericin B	8 (21)	
Voriconazole	1 (3)	
No antifungal	2 (5)	
>1 antifungal	19 (48)	
No. antibacterial drugs administered, median (range)	3.5 (1–6)	

*Values are no. (%) patients except as indicated. CVC, central venous catheter; ICU, intensive care unit; IQR, interquartile range.

for those who died (36 days, IQR 29–45 days; p<0.01). The median length of ICU admission was 32 days (IQR 27–44 days) for case-patients who died and 51 days (IQR 14–76 days) for those who survived (p = 0.3). No procedures were found to be associated with 30-day mortality. The median time from admission to positive blood culture was 22 days (IQR 21–37 days) for the patients who died and 21 days (IQR 12–28 days) in those who survived (p = 0.23). The median time from positive blood culture to death in those who died was 7.5 days (IQR 6–17 days). We were unable to ascertain whether death was attributable to *C. auris* fungemia.

Among the 12 cases in infants, median age was 34 days (IQR 17–107 days) and 9 (75%) were male; 6 (50%) died in the hospital, and 5 (42%) died within 30 days of *C. auris* culture. Five (50%) were preterm (<37 weeks' gestation), and 6 (50%) had congenital heart disease (Table 2). Four (80%) of 5 premature infants survived.

We performed antifungal susceptibility testing on 34 available clinical isolates from 34 unique case patients at 3 hospitals. Six (18%) isolates were resistant to fluconazole, 10 (29%) to amphotericin B, and none to the echinocandin class (anidulafungin, caspofungin). Ten (50%) patients who died had been treated with fluconazole, 9 (43%) received caspofungin, 1 received amphotericin B, and 1 received voriconazole; 2 patients who died did not receive an antifungal drug. Of the 6 case-patients with isolates resistant to fluconazole, 4 (67%) died by 30 days. Of the 10 case-patients with isolates resistant to amphotericin B, 4 (40%) died by 30 days. Resistance to fluconazole was not

Table 2. Characteristics of 12 infants <1 y of age with *Candida auris* bloodstream infection, Colombia, 2015–2016*

Characteristic	Value	Data missing
Median age, d (IQR)	34 (17–107)	0
Sex		
M	9 (75)	0
F	3 (25)	0
30-day mortality	5 (42)	0
In-hospital mortality		
Died	6 (50)	0
Alive	4 (33)	0
Still hospitalized, 2016 Sep 30	2 (17)	0
Concurrent conditions	12 (100)	0
Prematurity	5 (50)	2
Median gestational age at birth, wk (IQR)	36 (29–39)	2
Congenital heart disease	6 (50)	0
Surgical procedure	9 (75)	0
Treatments and procedures		
Central venous catheter	12 (100)	0
Intubation	11 (100)	1
Total parenteral nutrition	11 (92)	0
Feeding tube	5 (71)	5
Corticosteroids	4 (33)	0
Hemodialysis	1 (8)	0

*Values are no. (%) patients except as indicated. CVC, central venous catheter; IQR, interquartile range.

associated with outcome (p = 0.21), nor was resistance to amphotericin B (p = 0.85) (Table 3). Fluconazole-resistant isolates were seen at all 3 hospitals and amphotericin B-resistant isolates at 2 hospitals, in Barranquilla and Cartagena (northern region of Colombia).

Patient Sampling

We collected skin and rectal swab specimens from 7 patients admitted at the time of the investigation: 3 patients with *C. auris* bloodstream infection, 2 with *C. auris* cultured from a body site other than blood, and 2 without a clinical *C. auris* culture (but who at the time had a clinical culture growing yeast suspected to be *C. auris*). Five patients yielded positive samples, 4 of whom had a clinical culture with *C. auris*. One patient with a bloodstream infection had no positive samples. Samples were collected 3 weeks to 3 months after positive clinical culture (Table 4). The case isolates showed a high degree of relatedness on whole-genome sequencing within hospitals and regional clustering, supporting in-hospital and person-to-person transmission (*14*).

We identified spatial and temporal clustering of cases in 1 operating room and multiple intensive care units at all 4 sites. Hospital A (Figure) experienced the largest number of cases over the longest period. Few case-patients shared the same room, and cases occurred throughout different floors and units. For the remaining hospitals, all cases occurred within a 3-month period. At hospital B (Figure), all 4 cases were exposed to the same operating room over a 3-month period. In hospitals C and D, patients also overlapped in time and ICUs (Figure).

Discussion

Cases of *C. auris* bloodstream infection in Colombia were associated with nearly 60% all-cause, in-hospital deaths and a 43% 30-day mortality, which is higher than that reported in the United States. Cases occurred primarily in patients in ICUs who had central venous catheters and other invasive devices, continuing to support the findings that these cases occur in patients with long stays involving multiple procedures (*13*). In contrast to the United States, where most cases have occurred in older adults, nearly one third of cases in Colombia were in infants, and the median patient age was 23 years (*15*). Although cases are occurring in vulnerable extremes of age populations, our findings suggest that no age group is unaffected and that any given hospital could be affected by an outbreak.

Clustering of patients in time and space, the findings of skin colonization, and the highly clonal nature of the

Table 3. Characteristics of patients who died at 30 d compared with those who survived *Candida auris* bloodstream infection, Colombia, 2015–2016*

Characteristic	Total	Died	Survived	Crude odds ratio (95% CI)	p value†
All	40 (100)	17 (43)	23 (58)	NA	NA
Age, y					0.18
<1	12 (30)	5 (29)	7 (30)	0.95 (0.24–3.75)	0.94
1–17	8 (20)	2 (12)	6 (26)	0.24 (0.04–1.45)	0.12
18–65	14 35)	5 (29)	9 (39)	0.65 (0.17–2.47)	0.53
>65	6 (15)	5 (29)	1 (4)	9.17 (0.96–87.79)	0.05
Sex				0.92 (0.26–3.30)	0.9
M	24 (60)	10 (59)	14 (61)		
F	16 (40)	7 (41)	9 (39)		
Concurrent conditions	35 (88)	16 (94)	19 (83)	3.37 (0.34–33.26)	0.30
Chronic renal disease	9 (23)	3 (18)	6 (26)	0.61 (0.13–2.88)	0.53
Hemodialysis dependent	9 (23)	3 (18)	6 (26)	0.61 (0.13–2.88)	0.53
Diabetes	7 (18)	6 (35)	1 (4)	12 (1.28–112.42)	0.03
Immunosuppressive condition	6 (16)	4 (24)	2 (9)	3.23 (0.52–20.20)	0.21
Prematurity, n = 12	5 (42)	1 (6)	4 (17)	0.30 (0.03–2.93)	0.30
Median length of hospital stay, d (IQR)	46 (34–69)	36 (29–45)	63 (45–95)	NA	<0.01
Median length of ICU stay, d (IQR)	36 (25–58)	32 (27–44)	51 (14–76)	NA	0.3
Central venous catheter	40 (100)	17 (100)	23(100)	NA	NA
Respiratory support	36 (90)	17 (100)	19 (83)	NA	0.12
Vasopressors	31 (82)	16 (94)	15 (65)	8.53 (0.95–76.63)	0.06
Surgical procedure	28 (70)	13 (76)	15 (65)	1.73 (0.43–7.11)	0.45
Total parenteral nutrition	19 (50)	7 (41)	12 (52)	0.64 (0.18–2.28)	0.49
Corticosteroids	18 (47)	8 (47)	10 (43)	1.16 (0.33–4.07)	0.82
Hemodialysis	15 (38)	8 (47)	7 (30)	2.03 (0.55–7.47)	0.29
Chemotherapy	4 (11)	2 (12)	2 (9)	1.4 (0.18–11.08)	0.74
Median time from admission to *C. auris* positive culture, d (IQR)	22 (18–31)	22 (21–37)	21 (12–28)	NA	0.23
Isolates resistant to fluconazole, n = 34	6 (18)	4 (24)	2 (9)	3.23 (0.52–20.2)	0.21
Isolates resistant to amphotericin B, n = 34	10 (29)	4 (24)	6 (26)	0.87 (0.20–3.74)	0.85

*Values are no. (%) patients except as indicated. ICU, intensive care unit; IQR, interquartile ratio; NA, not applicable.
†Fisher exact test, χ^2, *t*-test, or Wilcoxon rank as appropriate.

Table 4. Patient sampling in suspected or known cases of *Candida auris* fungemia, Colombia, 2016*

Patient no.	Primary specimen type positive	No. body sites sampled for colonization	No. (%) body sites positive for *C. auris*
1	Blood	11	1 (9%): rectum
2	Blood	10	0
3	Blood	11	7 (64%): ears, axilla, left nostril, rectum, fecal material
4	Urine	10	1 (10%): left groin
5	Sputum	11	1 (9%): right groin
6	None	10	2 (20%): axillae

*Sampling from a seventh patient, who had an unidentified yeast growing in blood culture at the time of testing, did not yield *C. auris*.

isolates all strongly support person-to-person transmission occurring in the healthcare setting. Although *Candida* is often considered a commensal organism that most commonly colonizes the gastrointestinal tract, *C. auris* behaves more similarly to *C. parapsilosis* in its propensity to colonize the skin, which provides an opportunity for person-to-person spread. Outbreaks of *C. parapsilosis* have occurred in both adult and neonatal ICUs (*16*,*17*). In our investigation, 2 NICUs were involved; in at least 1 NICU, healthcare workers shared time between patients, providing an opportunity for transmission. The propensity of *C. auris* to colonize the skin may also explain the strong association with intubation, catheters, and feeding tubes in infants. The ability of *C. auris* to form biofilms may further enhance its ability to migrate into the bloodstream when provided a conduit through the skin (*18*). In this investigation, central lines were in place for a mean of 12 days at the time of positive culture. The tendency for skin colonization, biofilm production, and ability to cause invasive infection further emphasizes the need for diligent central line care.

The high death rate associated with *C. auris* infections, coupled with potential for nosocomial transmission, including among high-risk neonates, underscores the importance of infection control to prevent its spread. Current recommendations focus on early notification, hand hygiene, disinfection with a US Environmental Protection Agency–registered hospital-grade disinfectant effective against *Clostridium difficile* spores, and use of standard and contact precautions (*19*).

The characteristics of patients with *C. auris* candidemia were similar, but not identical, to those reported for candidemia caused by other species. Patients with *C. auris* bloodstream infection shared exposures such as ICU stay, central venous catheters, and surgical procedures (*7*). Whereas immunosuppressive conditions, including solid organ tumors, hematologic malignancy, and transplants are known risk factors for candidemia, they were uncommon in patients with *C. auris* candidemia (*18*). The reason for this may be that whereas other *Candida* species invade when there is a shift in the physiology of the host, *C. auris* is not a commensal, but rather a new exposure. Thirty-day mortality rates for *C. auris* candidemia in Colombia appear to be slightly higher (43%) than those seen for candidemia from other species, although the small sample size in our investigation limits the comparison (*20*). Half the infants in our cohort died, although age was not significantly associated with death. Furthermore, we compared characteristics between case-patients who survived and those who died and found few to be associated with outcome. Preterm birth was more common in those who survived, and only diabetes was associated with 30-day mortality. The high death rate is likely multifactorial but is most clearly related to long hospital stays, multiple invasive procedures, and lack of clinician awareness of resistance profiles.

In September 2016, a national laboratory alert was released in Colombia to increase reporting and surveillance catchment, as well as to enhance awareness of *C. auris* and the other species with which it was most commonly confused (*21*). According to the national guidelines for surveillance of healthcare-associated infections, aerobic and anaerobic blood cultures should be performed on any patient with signs of bloodstream infection (*11*). However, the capacity to perform blood cultures may not be available in all facilities; thus, it is possible that cases may be missed. The alert coincided with the initiation of our investigation and thus did not contribute heavily to our case counts, but it did increase reporting nationally.

Directed treatment is key to successful outcomes in fungemia. Resistance is another noteworthy feature of *C. auris*. Among *C. auris* isolates collected from 4 world regions, nearly all (93%) were resistant to fluconazole, and 41% were resistant to ≥2 classes of antifungal drugs (*13*). In our investigation, fluconazole was the most common antifungal drug administered, and treatment failure may have played a role in death rates. Of the isolates in this investigation, only about one fifth were resistant to fluconazole, but nearly one third were resistant to amphotericin B. The low proportion resistant to fluconazole was surprising; medical practices surrounding administration likely play a role. Forty-three percent of patients received fluconazole around the time of blood culture yielding *C. auris*, and of these, 57% died. The mortality rate was even higher (83%) among the patients with isolates resistant to fluconazole. Most *C. auris* isolates have been susceptible to

echinocandin drugs, which remain the recommended first-line treatment for *C. auris* candidemia (*9*,*13*,*22*). However, the often high cost of these drugs can be an obstacle to their use in resource-limited settings.

Because of the retrospective nature of this investigation, data on all variables were not available for each case-patient. Data on previous exposure to antifungal drugs and laboratory values were the most commonly missing. These limitations, along with the small sample size, may have prevented the detection of associations between risk factors and outcome.

The findings of our investigation highlight the necessity of adherence to infection control recommendations, especially aspects of careful central line care and maintenance, hand hygiene, proper disinfection of medical equipment, and use of standard and contact precautions (https://www.cdc.gov/fungal/diseases/candidiasis/recommendations.html). *C. auris* remains an emerging pathogen with the potential for high levels of resistance to a limited body of antifungal drugs. Its propensity to colonize skin provides a means for person-to-person transmission and elevates the concern for healthcare-associated outbreaks. Further understanding of its comportment and attention to infection control and prevention recommendations are paramount to prevent further spread.

Acknowledgments

We acknowledge Claudia Parra and the Pontificia Universidad Javeriana for diagnostic support during the course of this investigation; Martha Lucia Ospina and Maritza Gonzalez for support in the development this study; and Reina Turcios and Loren Cadena. We also acknowledge contributions from Maria Victoria Ovalle; the infection control teams of the Ministries of Health of Bogotá, Cartagena, and Barranquilla; and Cary Hilbert for their assistance in data collection and chart review. Paige Armstrong and Matthew Stuckey acknowledge the Epidemic Intelligence Service, as the investigation took place during their participation in the program. Diego Caceres acknowledges Oak Ridge Institute for Science and Education (ORISE).

This work was supported by the Centers for Disease Control and Prevention and the Instituto Nacional de Salud de Colombia.

About the Author

Dr. Armstrong is a medical epidemiologist in the Division of Vector-Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA; her research focuses on characterizing epidemiologic trends in infectious diseases and early intervention. Ms. Rivera is a nurse epidemiologist with training in infection control who currently works at the National Institute of Health of Colombia and is responsible for national surveillance and infection control for healthcare-associated infections.

References

1. Calvo B, Melo ASA, Perozo-Mena A, Hernandez M, Francisco EC, Hagen F, et al. First report of *Candida auris* in America: clinical and microbiological aspects of 18 episodes of candidemia. J Infect. 2016;73:369–74. http://dx.doi.org/10.1016/j.jinf.2016.07.008
2. Morales-López SE, Parra-Giraldo CM, Ceballos-Garzón A, Martínez HP, Rodríguez GJ, Álvarez-Moreno CA, et al. Invasive infections with multidrug-resistant yeast *Candida auris*, Colombia. Emerg Infect Dis. 2017;23:162–4. http://dx.doi.org/10.3201/eid2301.161497
3. Chowdhary A, Sharma C, Duggal S, Agarwal K, Prakash A, Singh PK, et al. New clonal strain of *Candida auris*, Delhi, India. Emerg Infect Dis. 2013;19:1670–3. http://dx.doi.org/10.3201/eid1910.130393
4. Magobo RE, Corcoran C, Seetharam S, Govender NP. *Candida auris*-associated candidemia, South Africa. Emerg Infect Dis. 2014;20:1250–1. http://dx.doi.org/10.3201/eid2007.131765
5. Ruiz Gaitán AC, Moret A, López Hontangas JL, Molina JM, Aleixandre López AI, Cabezas AH, et al. Nosocomial fungemia by *Candida auris*: first four reported cases in continental Europe. Rev Iberoam Micol. 2017;34:23–7. http://dx.doi.org/10.1016/j.riam.2016.11.002
6. Chowdhary A, Anil Kumar V, Sharma C, Prakash A, Agarwal K, Babu R, et al. Multidrug-resistant endemic clonal strain of *Candida auris* in India. Eur J Clin Microbiol Infect Dis. 2014;33:919–26. http://dx.doi.org/10.1007/s10096-013-2027-1
7. Kullberg BJ, Arendrup MC. Invasive candidiasis. N Engl J Med. 2015;373:1445–56. http://dx.doi.org/10.1056/NEJMra1315399
8. Cleveland AA, Farley MM, Harrison LH, Stein B, Hollick R, Lockhart SR, et al. Changes in incidence and antifungal drug resistance in candidemia: results from population-based laboratory surveillance in Atlanta and Baltimore, 2008–2011. Clin Infect Dis. 2012;55:1352–61. http://dx.doi.org/10.1093/cid/cis697
9. Centers for Disease Control and Prevention. *Candida auris* interim recommendations for healthcare facilities and laboratories. 2016 [cited 2017 Feb 3]. https://www.cdc.gov/fungal/diseases/candidiasis/recommendations.html
10. Cendejas-Bueno E, Kolecka A, Alastruey-Izquierdo A, Theelen B, Groenewald M, Kostrzewa M, et al. Reclassification of the *Candida haemulonii* complex as *Candida haemulonii* (*C. haemulonii* group I), *C. duobushaemulonii* sp. nov. (*C. haemulonii* group II), and *C. haemulonii* var. *vulnera* var. nov.: three multiresistant human pathogenic yeasts. J Clin Microbiol. 2012;50:3641–51. http://dx.doi.org/10.1128/JCM.02248-12
11. Sandra MRV, Liliana IBG, Andrea PVR. Infecciones asociadas a dispositivos; protocolo de vigilancia en salud publica. Instituto Nacional de Salud Colombia. 2017 [cited 2018 Dec 10]. http://www.ins.gov.co/buscador-eventos/ZIKA%20Lineamientos/PRO%20Infecciones%20aso%20dispositivos_.pdf
12. Welsh RM, Bentz ML, Shams A, Houston H, Lyons A, Rose LJ, et al. Survival, persistence, and isolation of the emerging multidrug-resistant pathogenic yeast *Candida auris* on a plastic health care surface. J Clin Microbiol. 2017;55:2996–3005. http://dx.doi.org/10.1128/JCM.00921-17
13. Lockhart SR, Etienne KA, Vallabhaneni S, Farooqi J, Chowdhary A, Govender NP, et al. Simultaneous emergence of multidrug-resistant *Candida auris* on 3 continents confirmed by whole-genome sequencing and epidemiological analyses. Clin Infect Dis. 2017;64:134–40. http://dx.doi.org/10.1093/cid/ciw691
14. Escandón P, Chow NA, Caceres DH, Gade L, Berkow EL, Armstrong P, et al. Molecular epidemiology of *Candida auris* in Colombia reveals a highly related, countrywide colonization with regional patterns in amphotericin B resistance. Clin Infect Dis. 2019;68:15–21.
15. Vallabhaneni S, Kallen A, Tsay S, Chow N, Welsh R, Kerins J, et al. Investigation of the first seven reported cases of *Candida*

auris, a globally emerging invasive, multidrug-resistant fungus—United States, May 2013–August 2016. Am J Transplant. 2017;17:296–9. http://dx.doi.org/10.1111/ajt.14121
16. Pinhati HMS, Casulari LA, Souza ACR, Siqueira RA, Damasceno CMG, Colombo AL. Outbreak of candidemia caused by fluconazole resistant *Candida parapsilosis* strains in an intensive care unit. BMC Infect Dis. 2016;16:433. http://dx.doi.org/10.1186/s12879-016-1767-9
17. Hernández-Castro R, Arroyo-Escalante S, Carrillo-Casas EM, Moncada-Barrón D, Alvarez-Verona E, Hernández-Delgado L, et al. Outbreak of *Candida parapsilosis* in a neonatal intensive care unit: a health care workers source. Eur J Pediatr. 2010;169:783–7. http://dx.doi.org/10.1007/s00431-009-1109-7
18. Lortholary O, Renaudat C, Sitbon K, Madec Y, Denoeud-Ndam L, Wolff M, et al.; French Mycosis Study Group. Worrisome trends in incidence and mortality of candidemia in intensive care units (Paris area, 2002–2010). Intensive Care Med. 2014;40:1303–12. http://dx.doi.org/10.1007/s00134-014-3408-3
19. Centers for Disease Control and Prevention. Recommendations for infection control for *Candida auris*; infection control recommendations for inpatient settings (acute care hospitals, long-term acute care hospitals, and nursing homes). 2017 [cited 2017 Oct 2]. https://www.cdc.gov/fungal/diseases/candidiasis/c-auris-infection-control.html
20. Ortíz Ruiz G, Osorio J, Valderrama S, Álvarez D, Elías Díaz R, Calderón J, et al. Risk factors for candidemia in non-neutropenic critical patients in Colombia. Med Intensiva. 2016;40:139–44.
21. Instituto Nacional de Salud Colombia. Alerta por emergencia global de infecciones invasivas causadas por la levadura multirresistente, *Candida auris*. 2016 [cited 2018 Dec 10]. https://www.minsalud.gov.co/sites/rid/Lists/BibliotecaDigital/RIDE/IA/INS/ins-alerta-colombia-candida-auris.pdf
22. Pappas PG, Kauffman CA, Andes DR, Clancy CJ, Marr KA, Ostrosky-Zeichner L, et al. Executive summary: clinical practice guideline for the management of candidiasis: 2016 update by the Infectious Diseases Society of America. Clin Infect Dis. 2016;62:409–17. http://dx.doi.org/10.1093/cid/civ1194

Address for correspondence: Paige A. Armstrong, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Mailstop C-17, Atlanta, GA 30329-4027, USA; email: yzu9@cdc.gov; Sandra Milena Rivera Vargas, Instituto Nacional de Salud, Av calle 26 N 51-20, Bogotá, Colombia; email: srivera@ins.gov.co

Environmental Hotspots for Azole Resistance Selection of *Aspergillus fumigatus*, the Netherlands

Sijmen E. Schoustra, Alfons J.M. Debets, Antonius J.M.M. Rijs,[1] Jianhua Zhang, Eveline Snelders, Peter C. Leendertse, Willem J.G. Melchers, Anton G. Rietveld, Bas J. Zwaan, Paul E. Verweij

Azole resistance is a major concern for treatment of infections with *Aspergillus fumigatus*. Environmental resistance selection is a main route for *Aspergillus* spp. to acquire azole resistance. We investigated the presence of environmental hotspots for resistance selection in the Netherlands on the basis of the ability of *A. fumigatus* to grow and reproduce in the presence of azole fungicide residues. We identified 3 hotspots: flower bulb waste, green waste material, and wood chippings. We recovered azole-resistant *A. fumigatus* from these sites; all fungi contained *cyp51A* tandem repeat–mediated resistance mechanisms identical to those found in clinical isolates. Tebuconazole, epoxiconazole, and prothioconazole were the most frequently found fungicide residues. Stockpiles of plant waste contained the highest levels of azole-resistant *A. fumigatus*, and active aerobic composting reduced *Aspergillus* colony counts. Preventing plant waste stockpiling or creating unfavorable conditions for *A. fumigatus* to grow in stockpiles might reduce environmental resistance burden.

Aspergillus fumigatus is a saprophytic mold whose natural habitat is decaying plant material (*1*). This fungus can tolerate high temperatures (>50°C) that can occur in heaps of decaying plant material. The fungus releases large amounts of aerial asexual spores. Humans might inhale hundreds of *A. fumigatus* spores daily, but aspergillosis generally does not develop in healthy persons because the spores are eliminated by the innate immune response (*1*). However, in immunosuppressed patients, the fungus can cause a range of clinical syndromes ranging from allergic conditions to acute and chronic invasive pulmonary aspergillosis. Invasive aspergillosis is a life-threatening infection that has a mortality rate of up to 60% (*2*).

Triazoles are the main class of drugs for treatment of aspergillus diseases. Clinically licensed anti-*Aspergillus* compounds include itraconazole, voriconazole, posaconazole, and isavuconazole. The triazoles are recommended for prevention of invasive aspergillosis in high-risk patients (posaconazole), for treatment of chronic pulmonary aspergillosis (itraconazole), and for treatment of invasive aspergillosis (voriconazole and isavuconazole). Alternative treatment options are limited to liposomal amphotericin B or echinocandins for specific aspergillus diseases and host groups.

In 1998, triazole-resistant *A. fumigatus* isolates were found in the Netherlands; since then, resistance has been reported from many countries worldwide (*3*,*4*). It is generally accepted that resistance can develop through patient treatment (patient route) and through exposure of *A. fumigatus* to azole fungicides in the environment (environmental route) (*2*–*9*). Environmental resistance mutations commonly are composed of a tandem repeat (TR) in the promoter region of the *cyp51A* gene in combination with single or multiple point mutations in the CYP51A protein (TR_{34}/L98H; TR_{53}; TR_{46}/Y121F/T289A) (*3*,*6*,*10*,*11*). Although *A. fumigatus* is not a phytopathogen and thus not a target for fungicide application, many azole fungicides show in vitro activity against *A. fumigatus* (*12*,*13*). These fungicides include various classes of azoles, such as triazoles (e.g., epoxiconazole), imidazoles (e.g., prochloraz and imazalil), and triazolinthiole (e.g., prothioconazole).

We previously demonstrated that 5 azole fungicides from the triazole class were highly active against wild-type *A. fumigatus* but showed no activity against resistant isolates with TR_{34}/L98H (*12*). These 5 azole fungicides showed similarities with the molecular structure of medical triazoles and cross-resistance. This finding complements other studies, which showed that various azole fungicides can induce cross-resistance to medical triazoles because all of these

Author affiliations: Wageningen University and Research, Wageningen, the Netherlands (S.E. Schoustra, A.J.M. Debets, J. Zhang, E. Snelders, B.J. Zwaan); Radboud University Medical Center; Nijmegen, the Netherlands (A.J.M.M. Rijs, W.J.G. Melchers, P.E. Verweij); Center for Agriculture and Environment, Culemborg, the Netherlands (P.C. Leendertse); Center of Expertise in Mycology Radboudumc/CWZ, Nijmegen (W.J.G. Melchers, P.E. Verweij); National Institute for Public Health and the Environment, Bilthoven, the Netherlands (A.G. Rietveld)

DOI: https://doi.org/10.3201/eid2507.181625

[1]Deceased.

compounds affect the CYP51A enzyme that is central in the ergosterol biosynthesis pathway (*13*,*14*). Patients with triazole-resistant aspergillus disease have a high probability of treatment failure (*15*). The day 42 mortality rate for patients with voriconazole-resistant invasive aspergillosis was found to be 21% higher than for patients with voriconazole-susceptible infections; mortality rates were up to 90% (*16*).

Because genetic diversity, population size, and selection pressure are critical for adaptation of fungi to new environments, we hypothesized that sites that support the growth, reproduction, and genetic variation of *A. fumigatus* and contain residues of azole fungicides would facilitate the emergence, amplification, and spread of triazole-resistance mutations. Locations that meet these 2 criteria were considered hotspots for azole resistance of *A. fumigatus.* In this study, we investigated hotspots as possible sources for selection and reservoirs of triazole-resistant *A. fumigatus* in the environment and aimed to identify and characterize environmental sites that facilitate triazole-resistance selection in *A. fumigatus*.

Methods

An expert panel suggested potential sites meeting hotspot criteria. The panel included experts from organizations representing husbandry, agriculture, composting, sustainable farming, drinking-water research, fungicide authorization, public health, and medicine. The areas of expertise included pesticides, biocides, *Aspergillus* taxonomy, evolutionary biology, fungal genetics, livestock farming, human and animal *Aspergillus* disease, and plant bulb diseases. The experts formulated that, to support growth of *A. fumigatus*, dead plant biomass should be present because this is the preferred substrate. *A. fumigatus* can grow at a wide range of temperatures (12°C–65°C; 35°C is considered the optimal growth temperature); it prefers high relative humidity (85%–100% is optimal); it is not affected by low pH (3.7–7.6); and it has potential for dispersal from the hotspot of environmental spread of conidia. To enable genetic diversity to arise, we anticipated that sufficient time for reproduction to take place would be a factor that might also affect the ability to select for resistance. In addition, the presence of azole-fungicide residues was considered to be a critical factor to impose selection pressure for azole resistance. The azole residues should furthermore exhibit activity against *A. fumigatus*. Several potential sites fulfilling the criteria were listed and ranked according to the expert estimation of resistance selection risk.

Sampling of Potential Hotspots

Sites that were identified as potential hotspots were sampled 2 or 3 times (Table). If possible, contrasting sites were selected and compared (i.e., those with known application of azole fungicides and similar sites where no azole fungicides were used).

To recover *A. fumigatus* isolates from the outdoor environment, we dissolved 2 g of sample in 8 mL of 0.2 mol/L NaCl, 1% Tween 20, as described (*5*). From this suspension, we plated 100 μL on Sabouraud dextrose agar, as well as on agar plates supplemented with 4 mg/L of itraconazole and incubated at 37°C. We compared the number of colonies on itraconazole-containing and itraconazole-free agar. If colonies were present, we collected 20 colonies from each plate.

We used molecular methods for strain identification and determination of the resistance mechanism as described (*17–20*). We determined the full coding sequence of the *cyp51A* gene by PCR amplification and sequencing (*14*). We used the *cyp51A* sequence (GenBank accession no. AF338659) for comparison in detecting mutations.

We sent samples to Eurofins Laboratorium Zeeuws-Vlaanderen B.V. (https://www.eurofinsdiscoveryservices.com) for detection and characterization of fungicide residues and metabolites. The following fungicides were analyzed, which covered commonly and less commonly used azole compounds: azaconazole, bromuconazole, cyproconazole, difenoconazole, epoxiconazole, flusilazole, flutriafole, metconazole, penconazole, propiconazole, prothiconazole, tebuconazole, thiabendazole, cyazofamid, fenamidone, iprodione, triazoxide, imazalil, and prochloraz. In addition, metabolites of prochloraz (2,4,6-trichlorophenol and prochloraz-desimidazole-amino) and prothioconazole (prothioconazole-desthio) were analyzed.

Results

We selected potential hotspots on the basis of preset criteria. These potential hotspots included waste from flower bulbs, residential household waste, green material, wood chippings, exotic fruit, regional fruit, wheat cereal, horse manure, poultry manure, cattle manure, and maize silage.

Resistance Levels at Sampling Sites

We sampled 11 sites in duplicate or triplicate (total 41 samples). *A. fumigatus* was detected at levels $>10^4$ CFU/g in waste from flower bulbs, green material, and wood chippings. One sample of household waste contained high levels of CFUs of *A. fumigatus*, which was not confirmed on sampling at another site. Azole fungicides and their residues were detected in all but 9 samples (Table).

Wheat Cereals

Wheat cereals are sprayed with azole fungicides in conventional farming. The grain is collected and stored in warehouses. The straw is partly used in animal stables and mixed with manure. Different animal manure was collected. We analyzed organic and conventional grain and straw. *A. fumigatus* was not found in grain (dry or moist) or

manure from different locations and animals. Azole fungicides were present in some samples (Table).

Maize

Maize has been occasionally sprayed with azole fungicides in conventional farming since 2014 and is stored in a silage after harvesting. In this study, we sampled 2 types of silage: sprayed (conventional) with azole fungicides and unsprayed. No *A. fumigatus* was found in maize silage (with or without azole fungicides) (Table). This finding can be explained by anoxic fermentation that normal maize silage undergoes, which creates unfavorable conditions for *A. fumigatus* to grow in. Therefore, maize silage is not considered a hotspot.

Regional Fruit Waste and Exotic Fruit Waste

Regional fruit and exotic fruit are sprayed with azole fungicides in conventional farming and commonly follow separate waste chains. Some of this fruit may begin to rot during transportation or storage; this fruit is then separated from healthy fruit in waste heaps. We analyzed organic and conventional fruit waste (regional and exotic). *A. fumigatus* was not found (with or without azole fungicides) (Table).

Household Waste

Household waste (consisting of vegetable, fruit, and garden waste) might contain azole fungicides. This green waste was collected by using household containers, taken to collection stations, and processed by using hydrolysis steps. We sampled household waste at the central collection station simultaneously at 3 positions in the hydrolysis process: just before the start of the process, during the process (1 week into the process), and at the end of the process (3 weeks). This household waste contained *A. fumigatus* at the start (2.3×10^3 CFU total counts and 200 azole-resistant CFUs), but low *A. fumigatus* counts remained at the end of

Table. Characteristics of sampling sites in environmental hotspots tested for azole resistance in *Aspergillus fumigatus*, the Netherlands*

Sampling site	Type	Total, CFU/g	Azole-resistant, CFU/g	Resistance mutations (no. colonies)	Azole fungicide (mg/L)†
Household waste, privately owned	R	6.4×10^4	1.5×10^3	TR_{34}/L98H (10); TR_{34}/L98H/S302N (1)	NT
Flower bulb waste					
Location 1	R	2×10^4	4.3×10^3	TR_{34}/L98H (6); TR_{46}/Y121F/T289A (7)	Prothioconazole (6.4); prothiconazoledestio (0.15); prochloraz (0.11); prochloraz-desimidazole-amino (0.085)
	O + R	1.2×10^4	2.5×10^3	TR_{34}/L98H (7); TR_{34}/L98H/F495I (1); TR_{46}/Y121F/T289A (2); TR_{92}/Y121F/M172I/T289A/G448S (1)	Prothioconazole (1.9); prochloraz (0.09); prochloraz-desimidazole-amino (0.086); prothiconazole-destio (0.07)
Location 2	O + R	2.9×10^3	180	TR_{34}/L98H (4); TR_{34}/L98H/S297T/F495I (1); TR_{46}/Y121F/T289A/I36 4V (1)	Prothioconazole (0.43); prochloraz (0.044); prothiconazole-destio (0.026); epoxiconazole; azaconazole
	O + R	2.7×10^4	3.9×10^3	TR_{34}/L98H (5); TR_{46}/Y121F/T289A (3)	Prochloraz (0.18); 2,4,6-trichloorphenol (0.17); prothioconazole (0.033); prothiconazole-destio (0.01); tebuconazole (0.022) prochloraz (0.011)
Location 2, 1 y later	O + R	9.4×10^5	2.3×10^5	TR_{34}/L98H (5); TR_{46}/Y121F/T289A (6); TR_{92}/Y121F/M172I/T289A/G448S (1)	Prothioconazole (1.3); prothiconazoledestio (0.033); difenoconazole; epoxiconazole; penconazole; propriconazole; azaconazole; tebuconazole
Green waste, location A, 2015					
0–1 wk old	R	9.4×10^4	8×10^3	TR_{34}/L98H (12)	Tebuconazole
5–6 wk old	R	1.9×10^6	8.4×10^4	TR_{34}/L98H (2)	Tebuconazole (0.004)
7 wk old	R	2.7×10^5	3.6×10^3	TR_{34}/L98H (10); TR_{34}/L98H/L343H (1); TR_{34}/L98H/E356V (1)	Tebuconazole (0.001)
Wood chippings waste, location 1, 2015	R	6.2×10^4	3.6×10^3	TR_{34}/L98H (18)	Azaconazole (0.53); propioconazole (0.036); tebuconazole (0.013)

*Only sampling sites in which resistance mutations were found are shown. An expanded version of this table is available online (https://wwwnc.cdc.gov/EID/article/25/7/18-1625-T1.htm). O, organic cultivation; NT, not tested; O + R, cultivation in transition from regular to organic; R, regular cultivation.
†If no fungicide concentration is shown, the concentration could not be quantified (i.e., <0.001 mg/L).

the hydrolysis process (3 weeks) (Table). Residues of various azole fungicides were detected in these samples.

Private garden owners sometimes use household organic waste (consisting of vegetable, fruit, and garden waste) in compost heaps. These heaps might contain residues of azole fungicides. We sampled 2 of these heaps and, from 1, recovered >1 × 10^4 *A. fumigatus* CFUs, including 1.5 × 10^3–1.8 × 10^3 azole-resistant colonies, but no azole fungicide residues were detected (Table). The presence of azole-resistant *A. fumigatus* in the absence of azole residues might be caused by azole-resistant *A. fumigatus* in the materials that were added to the heap, rather than an indication that this sample type is a source of azole resistance. Furthermore, the second privately owned compost heap showed no azole-resistant *A. fumigatus*.

Flower Bulb Waste

Flower bulbs are sprayed with or dipped in azole fungicides in conventional farming. Bulb waste (peals, bulb, and leaf waste) are collected and stored before composting. We sampled different bulb waste heaps from an organic grower and a conventional grower. *A. fumigatus* and azole fungicides were found in the flower bulb waste heaps in high quantities; high counts of azole-resistant *A. fumigatus* ranged from 180 to 2.3 × 10^5 CFU/g (Table). The heap from the organic flower bulb grower also contained high levels of azole fungicides. This grower indicated that he had just started the conversion of his farm to organic flower bulb production and therefore the bulb waste still contained fungicides (from applications in the previous season), which showed that azole fungicides have a high chemical stability. We conducted a second sampling and analysis for the heaps 1 year later and the initial findings were confirmed, in terms of presence of azoles and of *A. fumigatus*.

Green Material Waste

Organic waste originating from landscaping (including grass, leaves, shrubs and trees) is used for compost, which is professionally produced in large composting plants at several locations in the Netherlands. Stockpiling of the various waste materials precedes active composting. These stockpiling materials contained residues of azole fungicides, and *A. fumigatus* was found in high quantities; counts of azole-resistant *A. fumigatus* ranged from 60 to 8.4 × 10^4 CFU/g (Table). Repeated sampling after 5–6 weeks and 7 weeks indicated persistence of high levels of *A. fumigatus*. After grinding, woody material is blended with leaves and grass and monitored for temperature during composting. To ensure even heating and oxygenation, the material is turned at regular intervals. This procedure is known as the pathogen-reduction phase. During the consecutive composting stages, *A. fumigatus* CFUs were reduced to approximately or below detectable limits, and no azole-resistant *A. fumigatus* was found in mature compost.

Wood Chippings

Waste of processed wood is collected and stored at some of the professional composting plants. This woody material is a mixture of several kinds of wood (e.g., railway sleepers, wooden boxes, and wooden fences). The processed wood contained traces of azole fungicides, and *A. fumigatus* was found in the wood waste in high quantities: counts of azole-resistant *A. fumigatus* were ≤6 × 10^3 CFU/g (Table). We conducted a second sampling and analysis in this stored wood 1 year later at the same location and at another location, and results of this sampling confirmed the initial findings.

Azole Fungicide Measurements

We analyzed 41 samples for azole fungicides, of which 32 (78%) contained azole residues (Table). The number of compounds detected in individual samples ranged from 1 to 8 (median 2.5) fungicides/sample. The most azole fungicides were found in bulb waste (range 3–7 different compounds/sample). In the 32 positive samples, a fungicide was detected 97 times, including 11 different compounds and 3 metabolites. Tebuconazole was detected most frequently (23 samples), followed by epoxiconazole (11), prothioconazole (10), azaconazole (8), and propiconazole (7) (Table). For 33 of 97 positive results, a fungicide could be detected but not quantified (represented as <0.001 mg/kg). For 64 measurements, the fungicide concentration could be quantified and ranged from 0.001 mg/kg to 6.4 mg/kg (mean 0.3 mg/kg; median 0.036 mg/kg). The 3 hotspots contained different fungicides; prothioconazole was found predominantly in flower bulb waste, tebuconazole mostly in green material waste, and azaconazole in wood chippings.

Genetic Basis of Resistance

The *cyp51A* gene was characterized for 105 azole-resistant *A. fumigatus* isolates from 10 samples; 5 from flower bulb waste at 3 sites; 3 from organic waste; 1 from household green waste; and 1 from wood chippings compost (Table). We analyzed 50 isolates recovered from flower compost, 37 from green material waste, and 18 from wood chippings waste. All isolates contained *cyp51A* TR-mediated resistance mutations; 19 (18%) isolates contained TR_{46} (1 additional repeat of a 46-bp region in the promoter); 2 isolates (2%) contained TR_{92} (2 additional repeats of a 46-bp region in the promoter), and the remaining isolates contained TR_{34} (1 repeat of a 34-bp region in the promoter) (80%) (Table). We observed additional polymorphisms in the *cyp51A* gene for 5 isolates that contained TR_{34}/L98H and for 1 TR_{46}/Y121F/T289A isolate (Table). TR_{34} was recovered from all samples, and TR_{46} and TR_{92} were recovered only from flower bulb waste.

Discussion

We identified 3 hotspots for azole-resistance selection in *A. fumigatus*: waste originating from flower bulbs, green material, and wood chippings. Repeated sampling confirmed growth of *A. fumigatus* and azole-resistant *A. fumigatus* and residues of azole fungicides. Household waste was not classified as a hotspot because resistance and azole residues were not consistently found.

These hotspots contained the highest proportions of azole-resistant *A. fumigatus* isolates and azole fungicide residues. The azole fungicides that were detected in our environmental samples are known to show in vitro activity against *A. fumigatus*; these fungicides include azaconazole, epoxiconazole, tebuconazole, prothioconazole, difenoconazole, propiconazole, cyproconazole, prochloraz, and imazalil (*12*). Although to date numerous studies have investigated environmental samples for the presence of azole-resistant *A. fumigatus*, the presence of azole fungicides in the sample is generally not measured (*21–24*). Studies that measured azole fungicide concentrations included a study on the presence of azole-resistant *A. fumigatus* in sawmills and that found a major association between the number of resistant colonies recovered and the concentration of propiconazole (*25*). Another study measured levels of 4 azole fungicides from samples taken in flower fields in Colombia and linked these levels to the presence of azole-resistant *A. fumigatus* (*26*).

Although recovery of azole-resistant *A. fumigatus* from environmental samples indicated the presence of resistance, it does not directly implicate that the resistance emerged in that sample; it is possible that already resistant types had colonized the material and were able to proliferate. To gain more insight into resistance selection, we believe that it is critical to determine *A. fumigatus* growth and phenotype, as well as azole residues simultaneously. This study showed that *A. fumigatus* does not grow in some of the sample types we tested, and that the risk for resistance selection is considered low in these environments at that stage of composting, even when azole fungicides are present. Further studies are needed to determine how resistant mutants arise in a hotspot (i.e., through de novo acquisition or by selection of already existing resistance mutations).

We found that azole fungicides were widely present in samples we tested, indicating a broad selection pressure for *A. fumigatus*. In general, low levels of residues were detected, which challenges the concept of high exposure as the main driver for resistance selection or maintenance, as postulated by Gisi (*27*). We previously compared the resistance dynamics in 2 compost heaps with and without azole exposure (*14*) and found that, in the presence of the azole, the dominant phenotype of the *A. fumigatus* populations differed from predominantly wild-type in the absence of azoles to predominantly resistant in the presence of azoles (*14*). Exposure to low concentrations of azoles might pose a greater risk for resistance selection than exposure to high concentrations because at lower concentrations, a larger population of fungal cells will be available to produce progeny (*28*). However, a larger population might purport greater competition for nutrients, underscoring the need to further investigate the dynamics of competition between resistant and wild-type strains in these environments that contain low amounts of azoles. Furthermore, in all samples taken from a hotspot, we recovered wild-type *A. fumigatus*, in addition to resistant phenotypes, indicating that the wild-type population can survive in an environment containing azoles. It is highly likely that the azole fungicide concentrations will vary considerably inside a plant-waste heap and will also vary over time, suggesting a dynamic environment.

Our study indicated that the risk for resistance selection varies depending on the local conditions. Thus, analysis of the entire fungicide application cycle is needed. Aerobic composting is efficient in destroying many harmful microorganisms that are pathogenic to humans or plants. We found that *A. fumigatus* does not survive aerobic composting, possibly because of the high (>70°C) temperatures that are reached. Although *A. fumigatus* is a thermotolerant fungus, asexual spores do generally not survive temperatures >60°C. In contrast, the on-farm stockpiling of plant waste at flower bulb farms before industrial composting treatment occurred was found to be a hotspot.

All azole-resistant *A. fumigatus* isolates harbored CYP51A TR-mediated resistance mutations, which are also the dominant mutations found in patients with azole-resistant invasive aspergillosis in the Netherlands (*16*,*29*). National surveillance of clinical isolates in the Netherlands showed that TR-mediated resistance mutations accounted for >80% of the resistance mutations found in the previous 5 years (*29*). The identical resistance mutations found in the environment and in patients suggests a role for these hotspots in patient infections. However, we cannot exclude that other hotspots exist.

Although our chemical analysis covered a wide range of commonly and less commonly used azole fungicides and their residues, we might have missed relevant azole compounds present in sites that contained azole-resistant *A. fumigatus*. Thus, we were unable to determine which proportion of patients are infected through either 1 of the 3 hotspots that we have identified. Such information will be useful in supporting new policies aimed at reducing the environmental burden of resistance. TR_{46} and TR_{92} mutations were recovered only from flower bulb waste, which might be caused by use of specific (combinations of) azole fungicides or concentration of azole residues. Therefore, future studies should include quantification of aerial dispersal of

A. fumigatus conidia from hotspots and investigate occupational exposure of agricultural workers to azole-resistant *A. fumigatus*.

Azoles are useful in management of *Aspergillus* diseases and also play a major role in control of fungi during global food production and as biocides in nonfood materials. Therefore, a full ban of the use of this class of antifungal compounds is undesirable, but actions are needed to retain the class for both applications. Our study indicates that composting practices (i.e., stockpiling of plant waste) are key to resistance selection in *A. fumigatus*, rather than mere application of azole fungicides to protect against phytopathogenic fungi. One option would be to create conditions that preclude the growth of *A. fumigatus* and thus to turn the hotspot into a coldspot. The climate in the Netherlands provides favorable conditions for *A. fumigatus* to grow in plant waste material, but favorable conditions for *A. fumigatus* might be prevented (e.g., if the heap was shielded from the outdoor environment and stored under dry conditions). Furthermore, we found no *A. fumigatus* in fruit waste that underwent hydrolysis. These conditions in fruit waste might be unfavorable for *A. fumigatus* because of its anaerobic nature, absence of specific nutrients, or competition with unicellular microorganisms. Further studies are needed to systematically compare different composting techniques in relation to the growth of *A. fumigatus* and to design strategies that prevent resistance selection/maintenance in the environment. In addition, the currently available fungicides should be used prudently and require antifungal stewardship in hospitals and for nonmedical applications, thus preventing unnecessary exposure of fungi, including *A. fumigatus*. Similar interventions have been successful for bacterial resistance in reducing resistance rates (*30*). Furthermore, the possibility of replacing azole fungicides with other fungicide classes that do not cause cross-resistance to medical triazoles should be investigated for different applications.

Although we took great care in selecting which sites were sampled as potential hotspots, our study was limited by the number of sites sampled. Other hotspots that might be present in the environment might contribute to the overall extent of environmental resistance. We believe that our definition of a hotspot and the concept of analyzing the full application cycle of fungicides is broadly applicable and will help to identify additional potential hotspots. Research into azole-resistance selection requires a multidisciplinary approach and can be studied from a One Health perspective. Clarifying in greater detail the factors that drive resistance selection, the dynamics of resistance selection in *A. fumigatus* populations, and the link between hotspot and resistant disease in humans is critical for designing preventive measures.

Acknowledgments

We thank the experts on this research for participating in panel discussions and owners of different sampling locations for allowing sampling on their properties.

This study was supported by an unrestricted grant from the Dutch Ministry of Health, Welfare and Sport.

About the Author

Dr. Schoustra is an assistant professor in the Laboratory of Genetics at Wageningen University and Research, Wageningen, the Netherlands. His primary research interest is evolution of azole resistance in *A. fumigatus*.

References

1. Latgé J-P. *Aspergillus fumigatus* and aspergillosis. Clin Microbiol Rev. 1999;12:310–50. http://dx.doi.org/10.1128/CMR.12.2.310
2. Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? Clin Infect Dis. 2016;62:362–8. http://dx.doi.org/10.1093/cid/civ885
3. Denning DW, Venkateswarlu K, Oakley KL, Anderson MJ, Manning NJ, Stevens DA, et al. Itraconazole resistance in *Aspergillus fumigatus*. Antimicrob Agents Chemother. 1997;41:1364–8. http://dx.doi.org/10.1128/AAC.41.6.1364
4. Snelders E, van der Lee HA, Kuijpers J, Rijs AJ, Varga J, Samson RA, et al. Emergence of azole resistance in *Aspergillus fumigatus* and spread of a single resistance mechanism. PLoS Med. 2008;5:e219. http://dx.doi.org/10.1371/journal.pmed.0050219
5. Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. Appl Environ Microbiol. 2009;75:4053–7. http://dx.doi.org/10.1128/AEM.00231-09
6. Verweij PE, Snelders E, Kema GH, Mellado E, Melchers WJ. Azole resistance in *Aspergillus fumigatus*: a side-effect of environmental fungicide use? Lancet Infect Dis. 2009;9:789–95. http://dx.doi.org/10.1016/S1473-3099(09)70265-8
7. Zhang J, Debets AJ, Verweij PE, Melchers WJ, Zwaan BJ, Schoustra SE. Asexual sporulation facilitates adaptation: The emergence of azole resistance in *Aspergillus fumigatus.* Evolution. 2015;69:2573–86. http://dx.doi.org/10.1111/evo.12763
8. Camps SM, van der Linden JW, Li Y, Kuijper EJ, van Dissel JT, Verweij PE, et al. Rapid induction of multiple resistance mechanisms in *Aspergillus fumigatus* during azole therapy: a case study and review of the literature. Antimicrob Agents Chemother. 2012;56:10–6. http://dx.doi.org/10.1128/AAC.05088-11
9. Howard SJ, Cerar D, Anderson MJ, Albarrag A, Fisher MC, Pasqualotto AC, et al. Frequency and evolution of azole resistance in *Aspergillus fumigatus* associated with treatment failure. Emerg Infect Dis. 2009;15:1068–76. http://dx.doi.org/10.3201/eid1507.090043
10. van der Linden JW, Snelders E, Kampinga GA, Rijnders BJ, Mattsson E, Debets-Ossenkopp YJ, et al. Clinical implications of azole resistance in *Aspergillus fumigatus*, the Netherlands, 2007–2009. Emerg Infect Dis. 2011;17:1846–54. http://dx.doi.org/10.3201/eid1710.110226
11. van der Linden JW, Camps SM, Kampinga GA, Arends JP, Debets-Ossenkopp YJ, Haas PJ, et al. Aspergillosis due to voriconazole highly resistant *Aspergillus fumigatus* and recovery of genetically related resistant isolates from domiciles. Clin Infect Dis. 2013;57:513–20. http://dx.doi.org/10.1093/cid/cit320

12. Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, van der Lee HA, et al. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus.* PLoS One. 2012;7:e31801. http://dx.doi.org/10.1371/journal.pone.0031801
13. Faria-Ramos I, Farinha S, Neves-Maia J, Tavares PR, Miranda IM, Estevinho LM, et al. Development of cross-resistance by *Aspergillus fumigatus* to clinical azoles following exposure to prochloraz, an agricultural azole. BMC Microbiol. 2014;14:155. http://dx.doi.org/10.1186/1471-2180-14-155
14. Zhang J, van den Heuvel J, Debets AJ, Verweij PE, Melchers WJ, Zwaan BJ, et al. Evolution of cross-resistance to medical triazoles in *Aspergillus fumigatus* through selection pressure of environmental fungicides. Proc Biol Sci. 2017;284:20170635. http://dx.doi.org/10.1098/rspb.2017.0635
15. Meis JF, Chowdhary A, Rhodes JL, Fisher MC, Verweij PE. Clinical implications of globally emerging azole resistance in *Aspergillus fumigatus.* Philos Trans R Soc Lond B Biol Sci. 2016;371:20150460. http://dx.doi.org/10.1098/rstb.2015.0460
16. Lestrade PP, Bentvelsen RG, Schauwvlieghe AF, Schalekamp S, van der Velden WJ, Kuiper EJ, et al. Voriconazole resistance and mortality in invasive aspergillosis: a multicenter retrospective cohort study. Clin Infect Dis. 2018. [Epub ahead of print]. http://dx.doi.org/10.1093/cid/ciy859
17. Schoustra SE, Debets AJM, Slakhorst M, Hoekstra RF. Mitotic recombination accelerates adaptation in the fungus *Aspergillus nidulans.* PLoS Genet. 2007;3:e68. http://dx.doi.org/10.1371/journal.pgen.0030068
18. Samson RA, Visagie CM, Houbraken J, Hong SB, Hubka V, Klaassen CH, et al. Phylogeny, identification and nomenclature of the genus *Aspergillus.* Stud Mycol. 2014;78:141–73. http://dx.doi.org/10.1016/j.simyco.2014.07.004
19. Debets AJ. Parasexuality in fungi: mechanisms and significance in wild populations. In: Bridge PD, Couteaudier Y, Clarkson JM, editors. Molecular variability of fungal pathogens. Wallingford (UK): CAB International; 1998. p. 41–52.
20. Schoustra S, Hwang S, Krug J, de Visser JA. Diminishing-returns epistasis among random beneficial mutations in a multicellular fungus. Proc Biol Sci. 2016;283:e20161376. http://dx.doi.org/10.1098/rspb.2016.1376
21. Bader O, Tünnermann J, Dudakova A, Tangwattanachuleeporn M, Weig M, Groß U. Environmental isolates of azole-resistant *Aspergillus fumigatus* in Germany. Antimicrob Agents Chemother. 2015;59:4356–9. http://dx.doi.org/10.1128/AAC.00100-15
22. Lavergne R-A, Morio F, Favennec L, Dominique S, Meis JF, Gargala G, et al. First description of azole-resistant *Aspergillus fumigatus* due to TR_{46}/Y121F/T289A mutation in France. Antimicrob Agents Chemother. 2015;59:4331–5. http://dx.doi.org/10.1128/AAC.00127-15
23. Toyotome T, Fujiwara T, Kida H, Matsumoto M, Wada T, Komatsu R. Azole susceptibility in clinical and environmental isolates of *Aspergillus fumigatus* from eastern Hokkaido, Japan. J Infect Chemother. 2016;22:648–50. http://dx.doi.org/10.1016/j.jiac.2016.03.002
24. Santoro K, Matić S, Gisi U, Spadaro D, Pugliese M, Gullino ML. Abundance, genetic diversity and sensitivity to demethylation inhibitor fungicides of *Aspergillus fumigatus* isolates from organic substrates with special emphasis on compost. Pest Manag Sci. 2017;73:2481–94. http://dx.doi.org/10.1002/ps.4642
25. Jeanvoine A, Rocchi S, Reboux G, Crini N, Crini G, Millon L. Azole-resistant *Aspergillus fumigatus* in sawmills of eastern France. J Appl Microbiol. 2017;123:172–84. http://dx.doi.org/10.1111/jam.13488
26. Alvarez-Moreno C, Lavergne R-A, Hagen F, Morio F, Meis JF, Le Pape P. Azole-resistant *Aspergillus fumigatus* harboring TR_{34}/L98H, TR_{46}/Y121F/T289A and TR_{53} mutations related to flower fields in Colombia. Sci Rep. 2017;7:45631. http://dx.doi.org/10.1038/srep45631
27. Gisi U. Assessment of selection and resistance risk for demethylation inhibitor fungicides in *Aspergillus fumigatus* in agriculture and medicine: a critical review. Pest Manag Sci. 2014;70:352–64. http://dx.doi.org/10.1002/ps.3664
28. Van de Bosch F, Paveley N, Shaw M, Hobbelen P, Oliver R. The dose debate: does the risk of fungicide resistance increase or decrease with dose? Plant Pathology. 2011;60:597–606. http://dx.doi.org/10.1111/j.1365-3059.2011.02439.x
29. DeGreef S, Mouton J. NethMap 2017: consumption of antimicrobial agents and antimicrobial resistance among medically important bacteria in the Netherlands. MARAN 2017: monitoring of antimicrobial resistance and antibiotic usage in animals in the Netherlands in 2016 [cited 2019 May 16]. https://www.rivm.nl/publicaties/nethmap-2017-consumption-of-antimicrobial-agents-and-antimicrobial-resistance-among
30. Speksnijder DC, Graveland H, Eijck IAJM, Schepers RW, Heederik DJ, Verheij TJ, et al. Effect of structural animal health planning on antimicrobial use and animal health variables in conventional dairy farming in the Netherlands. J Dairy Sci. 2017;100:4903–13. http://dx.doi.org/10.3168/jds.2016-11924

Address for correspondence: Sijmen E. Schoustra, Laboratory of Genetics, Wageningen University and Research, Droevendaalsesteeg 1, Wageningen 6708 PB, the Netherlands; email: sijmen.schoustra@wur.nl

Asymptomatic Dengue Virus Infections, Cambodia, 2012–2013

Sowath Ly,[1] Camille Fortas,[1] Veasna Duong, Tarik Benmarhnia, Anavaj Sakuntabhai, Richard Paul, Rekol Huy, Sopheak Sorn, Kunthy Nguon, Siam Chan, Souv Kimsan, Sivuth Ong, Kim Srorn Kim, Sowathy Buoy, Lim Voeung, Philippe Dussart, Philippe Buchy,[1,2] Arnaud Tarantola[1]

We investigated dengue virus (DENV) and asymptomatic DENV infections in rural villages of Kampong Cham Province, Cambodia, during 2012 and 2013. We conducted perifocal investigations in and around households for 149 DENV index cases identified through hospital and village surveillance. We tested participants 0.5–30 years of age by using nonstructural 1 rapid tests and confirmed DENV infections using quantitative reverse transcription PCR or nonstructural 1–capture ELISA. We used multivariable Poisson regressions to explore links between participants' DENV infection status and household characteristics. Of 7,960 study participants, 346 (4.4%) were infected with DENV, among whom 302 (87.3%) were <15 years of age and 225 (65.0%) were ≤9 years of age. We identified 26 (7.5%) participants with strictly asymptomatic DENV infection at diagnosis and during follow-up. We linked symptomatic DENV infection status to familial relationships with index cases. During the 2-year study, we saw fewer asymptomatic DENV infections than expected based on the literature.

Annually, ≈390 million people in >100 countries are infected with dengue virus (DENV); 70% of cases occur in countries in Asia (*1*). DENV is a flavivirus transmitted by *Aedes aegypti* and *Ae. albopictus* anthropophilic female mosquitoes. DENV has 4 distinct serotypes, DENV-1–4 (*2*); DENV infections can range from asymptomatic to life-threatening.

In Cambodia, the national dengue surveillance system reported 60,000 cases and 135 deaths attributed to DENV in 2012 and 2013 (*3*). Syndromic surveillance and random testing of dengue-like cases in referral pediatric hospitals in Cambodia likely underestimate the true disease burden (*4*). By definition, syndromic surveillance does not detect asymptomatic DENV infections, which increase vector transmission potential (*5*). Mammen et al. used both dengue-positive and dengue-negative index cases of febrile children to initiate perifocal investigations and found no cases in proximity to dengue-negative index cases (*6*). To maximize the number of recruited cases, we investigated homes around preidentified, dengue-positive index cases, as per a previous study (*7*). Our objectives were to document the proportion of strictly asymptomatic infections in this region of Cambodia; characterize human, sociodemographic, household-level, and mosquito control–related factors associated with DENV infection; and identify factors associated with asymptomatic DENV infection.

Methods

Ethics Considerations

The study protocol was approved by the Cambodian National Ethics Committee on Health Research. We obtained informed consent from participants or their guardians documented during hospital or village surveillance or perifocal investigations.

Study Site

We conducted a study in rural villages of Kampong Cham Province, 120 km northeast of Cambodia's capital, Phnom Penh. The study area included 368 villages with ≈60,000 households and 3 hospitals within a 30-km radius. Dengue is endemic in the region and mainly affects children ≤15 years of age during the annual rainy season (June–October).

Identification of Dengue Index Cases in Hospitals and Villages

During June 1–October 31, 2012 and 2013, we identified DENV index cases in 3 referral hospitals and 26 villages

Author affiliations: Institut Pasteur du Cambodge, Phnom Penh, Cambodia (S. Ly, C. Fortas, V. Duong, S. Sorn, K. Nguon, S. Chan, S. Kimsan, S. Ong, P. Dussart, P. Buchy, A. Tarantola); University of California, San Diego, California, USA (T. Benmarhnia); Institut Pasteur, Paris, France (A. Sakuntabhai, R. Paul); Malaria National Center, Phnom Penh (R. Huy); Kampong Cham Provincial Hospital, Kampong Cham, Cambodia (K.S. Kim); Prey Chhor District Referral Hospital, Kampong Cham (S. Buoy); Tboung Khmum District Referral Hospital, Thoung Khmum, Cambodia (L. Voeung)

DOI: https://doi.org/10.3201/eid2507.181794

[1]These authors contributed equally to this article.

[2]Current affiliation: GlaxoSmithKline Vaccines Research and Design, Singapore.

under active surveillance for febrile illness (*5*). We targeted persons 0.5–30 years of age. In the 3 hospitals, blood samples were drawn at admission and discharge for all patients suspected of having DENV infection on the basis of clinical assessment and platelet count. In the 26 villages, volunteers monitored eligible residents weekly, measuring axillary body temperature using a digital thermometer to identify persons with temperatures ≥38°C. Blood samples were drawn 1–2 days after fever onset, as described elsewhere (*4*,*8*,*9*). All samples were screened for DENV infection by using a nonstructural (NS) 1 IgM/IgG combination rapid test. We confirmed DENV by using quantitative reverse transcription PCR (qRT-PCR) or NS1-capture ELISA and included case-patients with confirmed DENV infection as index cases in the study.

Perifocal Investigations

Within 1–2 days of identifying an index case, whether from village or hospital surveillance, we began a perifocal investigation of the index case-patient's village of origin (*7*). For each perifocal investigation, we used a rapid dengue test kit to screen eligible residents in the index case-patient's household for DENV and completed a baseline questionnaire on individual symptoms, socioeconomic status, and household characteristics. We did the same in 20 households in a 100-meter radius of the index case-patient's household. We included persons 0.5–30 years of age who consented or whose guarantor consented. We tested adults >20 years of age during the first year of the study but found no DENV-positive cases and did not test this age group during the second year. All consecutive cases were eligible for inclusion. To avoid bias through overlapping investigations of a potentially common source of infection, we did not conduct a perifocal investigation within 1 week of a previous investigation for ≥2 index cases consecutively detected from the same village.

DENV Testing and Case Definitions

To screen for DENV infection during surveillance and perifocal investigations, investigators tested all blood samples on-site using SD BIOLINE Dengue Duo kit (Standard Diagnostics, https://www.alere.com), according to the manufacturer's instructions. Investigators interpreted results within 15–20 minutes and ruled out possible cases if the control band was negative. Blood samples from DENV-positive participants were sent to Institut Pasteur du Cambodge (Phnom Penh, Cambodia) for qRT-PCR testing, as described previously (*10*,*11*), or confirmation using an NS1-capture ELISA (*11*,*12*) with positive controls diluted to the limit of detection, negative, and nontemplate controls used during extraction and PCR steps to reduce inaccuracies (*10*). We considered cases confirmed when a blood sample tested positive by NS1 rapid test and was confirmed by NS1-capture ELISA or qRT-PCR. During the first year, we also tested participants for Japanese encephalitis virus (JEV) and chikungunya virus (CHIKV) IgM antibodies by ELISA and confirmed IgM-positive results using specific RT-PCR to ensure that symptoms were not related to CHIKV, JEV, or co-infections (*11*–*13*).

Symptomatic DENV-confirmed case-patients had fever, muscle or joint pain, rash, bleeding, prolonged headaches, or digestive signs. We asked participants whether they had taken antipyretics in the previous 24 hours. We termed afebrile all symptomatic DENV-positive participants without a fever and no antipyretic use. We considered participants asymptomatic when they had confirmed DENV infection, no antipyretic use, and no signs or symptoms, including fever. Participants who were symptomatic at initial diagnosis on day 0 received follow-up monitoring on days 2 and 7. We monitored asymptomatic participants daily on days 0–7 and again on day 10 using a questionnaire to document signs and symptoms of DENV. In our analyses, we recategorized participants who were asymptomatic at baseline to symptomatic if they reported any symptoms during the follow-up period.

Statistical Analysis

We described DENV infection attack rates for perifocal investigations and the proportion of asymptomatic cases among all DENV infections and circulating serotypes. To explore participant- and household-level factors associated with DENV infection, we conducted a multivariable Poisson regression estimating attack rate ratios (ARRs) (*14*), excluding index cases. We built explanatory models around each participant-level and household-level factor, with and without adjusting for covariates. Participant-level factors included age, sex, occupation or schooling, and relationship to an index case-patient. Because we found collinearity between age and occupation, we adjusted only for age. We placed participants 0.5–1 year of age into a specific category to account for differences in immunity and exposure to vectors due to reduced mobility. Household-level factors included the main source of income, source of water, measures against mosquitoes, and environmental factors favorable to mosquito development. We further divided the source of water into 2 categories: piped water (from indoor or outdoor taps with a tube well and pump) or nonpiped water (from a pond, river, lake, or a well without pump).

Considering the limited flight range of a female *Aedes* mosquito, we assumed that the probability of DENV transmission would be higher within a household than across households. To account for this factor and measure potential clustering, we developed a random-effects multilevel model. We computed the intraclass correlation coefficient as the proportion of the variability in the probability of infection attributable to differences between households versus differences within households (*15*). We excluded

19 participants with missing covariates or predictors from the regression analyses. We explored associations between asymptomatic DENV infection and DENV serotype, participant-level factors, and the main source of income as socioeconomic indicators. We used the Fisher exact test for comparing proportions, the Student *t* test for means, and an empty multilevel model to search for a cluster effect. We conducted analyses using Stata version 13 (StataCorp, https://www.stata.com).

Results

Dengue Surveillance for Index Case Identification

We identified 1,294 suspected DENV-infected persons, 834 (64.5%) among hospital inpatients and 460 (35.5%) through febrile illness surveillance in villages. Our testing confirmed 555 (66.5%) DENV-positive cases among hospital patients and 36 (7.8%) DENV-positive cases through febrile illness surveillance in villages.

Perifocal Investigations

From the 591 DENV-positive patients, we selected 149 (25.2%) consecutive cases for which we conducted perifocal investigations: 131 from hospital patients, termed PI-H, and 18 from village febrile surveillance, termed PI-V. Perifocal investigations took place in 104 villages over the 2 rainy seasons and documented 7,960 participants, 6,811 (86%) male and 1,149 (14%) female, in 2,988 households (Figure).

We found 346 (4.3%) persons who were positive for DENV infection, 225 (65.0%) of whom were <9 years of age. We determined attack rates of 14.7/1,000 participants (14/952) in PI-V and 47.4/1,000 (332/7,008) in PI-H ($p<0.05$). The attack rate over the 2 outbreak seasons increased marginally from 37/1,000 persons 0.5–30 years of age during the 2012 season to 46/1,000 among those 0.5–20 years of age during 2013 ($p = 0.056$). Only 26 (7.5%) of 346 DENV-positive participants remained strictly asymptomatic during the 10-day follow-up, an asymptomatic DENV-infection attack rate of 3.3/1,000 over the 2 years of our study. The proportion of asymptomatic infections was 21.4% (3/14) in PI-V and 6.9% (23/332) in PI-H.

Besides headache and fever, symptomatic case-patients mainly experienced muscle, retro-orbital, and joint pain. Although fever is considered a typical symptom of DENV infection, careful interview, rigorous clinical assessment, and follow-up interviews showed that participants remained afebrile in 110 (31.8%) of the 320 symptomatic DENV infections, even without antipyretics. Only 6 (1.7%) of the DENV-positive case-patients required hospitalization, 2 with bleeding.

The 2 annual outbreaks were dominated by DENV-1. However, DENV-2 and DENV-4 emerged in 2013, and we detected DENV-3 sporadically (Table 1). During the first year of the study, samples from all symptomatic and asymptomatic DENV cases were negative for CHIKV by MAC-ELISA. Because we diagnosed no CHIKV in year 1, and our national surveillance system also did not detect any CHIKV cases (data not shown), we did not perform CHIKV testing during year 2. Of 26 asymptomatic cases confirmed by qRT-PCR or NS1-capture ELISA, 6 had positive JEV serology and also were positive for DENV IgM. We could not conclude whether JEV-positive results were indicative of a recent or acute JEV co-infection or the result of cross-reaction among flaviviruses. Among hospitalized patients, 2 had positive JEV results without detectable DENV IgM, even though qRT-PCR or NS1-capture ELISA was positive. These results could suggest a recent or acute JEV co-infection. During perifocal investigations, 42 participants tested positive for JEV by MAC-ELISA

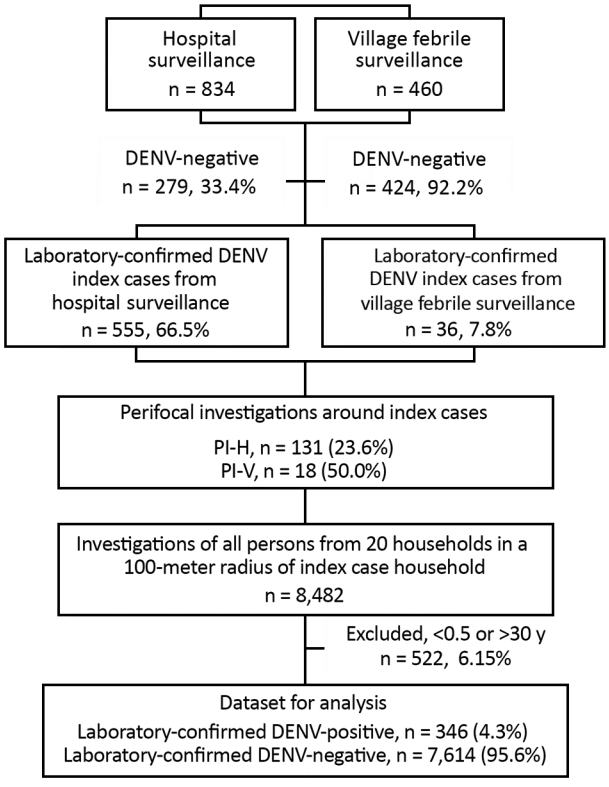


Figure. Participant screening and data flowchart for perifocal investigations for asymptomatic DENV infection, Cambodia, 2012–2013. Initial DENV screening of febrile cases was conducted using nonstructural (NS) 1 IgM/IgG combo rapid test. Perifocal investigations took place in villages of index cases; we screened all persons in 20 households within a 100-m radius of an index case household. We excluded persons <0.5 and >30 years of age. Laboratory confirmation of DENV was conducted through quantitative reverse transcription PCR or NS1-capture ELISA. DENV, dengue virus; PI-H, perifocal investigations conducted for index cases identified through hospital surveillance; PI-V, perifocal investigations conducted for index cases identified through village febrile surveillance.

with negative DENV results, NS1, and qRT-PCR, supporting evidence of JEV co-circulation in the country (*16*).

Screened participants had a mean age (± SD) of 11.7 (± 7.9; median 10; interquartile range 6–16); 6,207 (77.9%) were schoolchildren, university students, or nonschooled children. The main sources of household income were planting crops (61.0%), working in a factory (14.3%), and keeping a shop (13.4%). Participants reported low use of protective measures against mosquitoes, including mosquito coils in 787 (26.3%) households, insecticide sprays in 557 (18.6%) households, and larvicidal temephos in 374 (12.5%) households. Our investigation found uncovered water jars in 1,867 (62.7%) households and mosquito larvae in water containers of 1,663 (55.7%) households (Table 2).

Among DENV-positive cases, boys and girls were equally affected at a mean (± SD) age of 8.5 (± 5.7) years. Compared with persons 15–30 years of age, we found that children 1–10 years of age had a higher ARR of DENV infection (ARR 4.04 [95% CI 2.72–5.98] for those 1–5 years of age and ARR 3.83 [95% CI 2.59–5.67] for those 6–10 years of age). Siblings and cousins of index case-patients were more prone to DENV infection than neighbors were; siblings were 2.24 (95% CI 1.42–3.53) times and cousins 1.40 (95% CI 1.02–1.90) times more at risk for infection than neighbors. Participants who used piped water had a higher risk for DENV infection (ARR 1.35 [95% CI 1.06–1.71]) than did those who used nonpiped water. Households in which the main source of income was fishing, farming, or animal husbandry also had higher risks for infection (ARR 2.02 [95% CI 1.18–3.45]). Households reporting mosquito control–related parameters did not have a lower risk for DENV infection (Table 2).

The main source of income was similarly distributed between households with ≥1 case and households with no cases (p = 0.272). Our multilevel model showed a notable clustering effect at the household level after adjustment (intraclass correlation coefficient 40.8%).

We found 26 (7.5%) case-patients, 17 (65.4%) male and 9 (34.6%) female, who were positive for DENV infection but remained asymptomatic. We found serotypes DENV-1, DENV-2, and DENV-4 in our study group (Table 3). We used a multilevel approach to explore the role of specific serotypes and participant-level factors, such as age, gender, and relationship to the index case-patient, a proxy for common genetic background, with being DENV-positive and asymptomatic. We found that only family relationship to the index case-patient was associated with asymptomatic infection. We did not identify a cluster effect or associated factors.

Discussion

We screened 7,960 participants in communities in Cambodia during 2012 and 2013 and found 346 (4.3%) participants

Table 1. Surveillance data from perifocal investigations for asymptomatic dengue virus, Cambodia, 2012–2013*

Surveillance data	2012	2013	Total
No. participants	2,391	5,569	7,960
No. villages investigated	35	77	104
No. perifocal investigations conducted	47	102	149
Mean no. participants per perifocal investigation	51	55	53
Confirmed infections, no. (%)	88	258	346
Strictly asymptomatic	5 (5.7)	21 (8.1)	26 (7.5)
Afebrile	33 (37.5)	77 (29.8)	110 (31.2)
Symptomatic	83 (94.3)	237 (91.9)	320 (92.5)
Attack rate/1,000 participants, %	36.8	46.3	43.5
Asymptomatic infections	2.1	3.8	3.3
Symptomatic infections	34.7	42.6	40.2
Afebrile infections	13.8	13.8	13.8
Symptoms at diagnosis or follow-up, no. (%)	83	237	320
Fever	55 (66.2)	180 (75.9)	236 (73.8)
Headache	52 (62.7)	169 (71.3)	221 (69.1)
Muscle pain	16 (19.3)	73 (30.8)	89 (27.8)
Retro-orbital pain	17 (20.5)	73 (30.8)	90 (28.1)
Joint pain	17 (20.5)	68 (28.7)	85 (26.5)
Rash	15 (18.1)	53 (22.4)	68 (21.3)
Any bleeding	13 (15.7)	50 (21.1)	63 (19.7)
Hospitalizations, no. (%)	3 (3.5)	8 (3.3)	11 (3.3)
DENV infections	88	258	346
Serotype, no. (%)			
DENV-1	82 (98.8)	188 (72.9)	270 (78.0)
DENV-2	1 (1.2)	36 (13.9)	37 (10.7)
DENV-3	0	2 (0.8)	2 (0.6)
DENV-4	0	31 (12.0)	31 (9.0)
DENV-1 and DENV-2	0	1 (0.4)	1 (0.3)
Missing	5	0	5

*Participants 0.5–30 years of age in 2012 and 0.5–20 years of age in 2013. DENV, dengue virus.

infected by DENV; 26 (7.5%) remained asymptomatic before, during, and after our study. We found comparable attack rates, 37/1,000 persons in 2012 and 46/1,000 persons in 2013, to other community investigations conducted in Cambodia. For instance, another study reported DENV attack rates of 13.4–57.8 cases/1,000 persons during 2006–2008 (*4*). Previous studies only included participants ≤20 years of age, but we included persons 0.5–30 years of age with confirmed DENV infection, even symptomatic but afebrile case-patients, who were 31.8% of the DENV infections in

Table 2. Participant and household characteristics with unadjusted and adjusted attack rate ratios for factors potentially associated with dengue virus infection, Cambodia, 2012–2013*

Characteristics	Infected	Uninfected	Total	Unadjusted ARR (95% CI)	Adjusted ARR (95% CI)
Participants	346	7,614	7,960		
Sex					
M	171	4,103	4,272	Referent	Referent
F	175	3,511	3,686	1.14 (0.92–1.40)	1.01 (0.82–1.24)
Age, y†					
0.5–<1	9 (2.6)	150 (2.0)	159 (2.0)	3.47 (1.65–7.32)	3.53 (1.67–7.46)
1–<5	108 (31.2)	1,701 (22.3)	1,809 (22.7)	3.98 (2.69–5.90)	4.04 (2.72–5.98)
5–<10	126 (36.4)	2,083 (27.4)	2,209 (27.8)	3.79 (2.56–5.60)	3.83 (2.59–5.67)
10–<15	71 (20.5)	1,675 (22.0)	1,746 (21.9)	2.59 (1.70–3.94)	2.55 (1.67–3.88)
15–30	32 (9.3)	2,005 (26.3)	2,037 (25.6)	Referent	Referent
Mean (± SD, median)	8.5 (± 5.7, 7)	11.9 (± 8.0, 10)	11.7 (± 7.9, 10)	–	–
Occupation‡					
Student, school or university	171 (49.8)	3,588 (47.2)	3,759 (47.3)	2.14 (1.35–3.41)	2.14 (1.34–3.41)
Preschool or unschooled	149 (43.2)	2,299 (30.2)	2,448 (30.8)	2.84 (1.79–4.54)	2.84 (1.78–4.54)
Planting crops	20 (5.8)	910 (12.0)	930 (11.7)	Referent	Referent
Other	5 (1.5)	809 (10.6)	814 (10.2)	0.28 (0.10–7.76)	0.28 (0.10–7.76)
Missing	1 (0.2)	8 (0.1)	9 (1.1)		
Relationship to index case-patient§					
Neighbor	260 (75.4)	6,309 (83.0)	6,569 (82.6)	Referent	Referent
Cousin	58 (16.8)	991 (13.0)	1,049 (13.2)	1.38 (1.01–1.89)	1.40 (1.02–1.90)
Sibling	23 (6.7)	251 (3.3)	274 (3.5)	2.11 (1.33–3.34)	2.24 (1.42–3.53)
Other	5 (1.2)	55 (0.7)	59 (0.7)	1.66 (0.59–4.65)	1.76 (0.34–4.90)
Missing	1 (0.2)	8 (0.1)	9 (1.1)		
Households	292¶	2,706	2,988		
Water source#					
Nonpiped	108 (36.3)	1,186 (43.7)	1,284 (43.0)	Referent	Referent
Piped	184 (63.7)	1,520 (56.3)	1,704 (57.0)	1.32 (1.03–1.69)	1.35 (1.06–1.71)
Primary source of income**					
Planting crops	176 (60.9)	1,648 (61.0)	1,824 (61.0)	Referent	Referent
Working in a factory	42 (14.5)	384 (14.2)	426 (14.3)	1.16 (0.84–1.62)	1.20 (0.87–1.66)
Shopkeeping	37 (12.8)	362 (13.4)	399 (13.4)	0.97 (0.67–1.40)	1.03 (0.72–1.48)
Fishing, farming, animal husbandry	14 (4.8)	55 (2.0)	69 (2.3)	1.98 (1.15–3.43)	2.02 (1.18–3.45)
Working in government	5 (1.7)	57 (2.1)	62 (2.1)	0.94 (0.38–2.30)	0.99 (0.41–2.37)
Other	15 (5.2)	193 (7.2)	208 (7.0)	0.76 (0.43–1.32)	0.85 (0.50–1.46)
Mosquito control measures††					
Temephos	26 (9.0)	348 (12.9)	374 (12.5)	0.70 (0.47–1.06)	0.73 (0.48–1.10)
Larvivorous fish	26 (9.0)	214 (7.9)	240 (8.3)	1.14 (0.75–1.74)	1.18 (0.78–1.79)
Treated mosquito netting	27 (9.3)	311 (11.5)	338 (11.3)	0.78 (0.52–1.17)	0.82 (0.55–1.21)
Treated jar cover	3 (1.0)	47 (1.7)	50 (1.7)	0.73 (0.24–2.24)	0.77 (0.26–2.27)
Coils	77 (26.6)	710 (26.3)	787 (26.3)	1.08 (0.82–1.41)	1.16 (0.89–1.51)
Insecticide spray	44 (15.2)	513 (19.0)	557 (18.6)	0.79 (0.57–1.10)	0.88 (0.63–1.22)
Environmental factors**					
Vegetable garden	57 (9.7)	546 (20.2)	603 (20.2)	0.89 (0.66–1.21)	0.89 (0.66–1.20)
Water collection around house	126 (43.6)	1,180 (43.7)	1,306 (44.7)	0.91 (0.71–1.15)	0.88 (0.70–1.12)
Uncovered water jars	178 (61.6)	1,689 (62.6)	1,867 (62.5)	0.96 (0.75–1.22)	0.97 (0.76–1.23)
Larvae in water containers	168 (58.1)	1,495 (55.4)	1,663 (55.7)	1.09 (0.86–1.39)	1.07 (0.85–1.37)
Distance from house to nearest water jar, m (± SD)	1.5 (± 2.2)	1.3 (± 2.0)	1.3 (± 2.0)	1.00 (0.98–1.02)	1.00 (0.98–1.02)
Missing for all items	3	7	10		

*Values are no. or no. (%) except as indicated. ARR, attack rate ratio; DENV, dengue virus.
†Participants 0.5–30 years of age in 2012 and 0.5–20 years of age in 2013.
‡Adjusted for sex.
§Adjusted for age.
¶No. households with ≥1 DENV case.
#Nonpiped water comes from a river, pond, lake, or a well that does not have a pump; piped water comes from indoor or outdoor taps with a tube well and pump.
**Adjusted for sex and occupation.
††Adjusted for age, relationship to index case-patient, occupation, and primary source of income.

Table 3. Univariate tests for associations between sociodemographic factors and infecting serotypes with asymptomatic dengue virus infections, Cambodia, 2012–2013*

Factor	Asymptomatic, n = 26	Symptomatic, n = 320	p value†
Sex			
M	17	154	0.09
F	9	166	
Age, y			
0.5 to <1	0	9 (2.8)	0.976
1–5	9 (34.6)	99 (30.9)	
6–10	9 (34.6)	117 (36.6)	
11–14	5 (19.2)	66 (20.6)	
15–30	3 (11.5)	29 (9.1)	
Mean (± SD, median)	9.2 (± 7.2, 8)	11.0 (± 7.1, 10)	
Relationship to index case-patient			
Neighbor	17 (65.4)	243 (76.0)	0.004
Cousin	5 (19.2)	53 (16.6)	
Sibling	1 (3.9)	22 (6.9)	
Other	3 (11.5)	1 (0.3)	
Source of household income			
Planting crops	14 (53.8)	192 (60.0)	0.812
Working in a factory	3 (11.5)	53 (16.6)	
Shopkeeping	5 (19.2)	36 (11.3)	
Fishing, farming, animal husbandry	1 (3.9)	17 (5.3)	
Working in government	0	6 (1.9)	
Other or missing	3 (11.5)	16 (5.0)	
DENV serotype‡			
DENV-1	21 (80.8)	249 (79.1)	0.892
DENV-2	2 (7.7)	35 (11.1)	
DENV-3	0	2 (0.6)	
DENV-4	3 (11.5)	28 (8.9)	

*Values are no. (%) patients except as indicated. DENV, dengue virus.
†By Fisher test or χ^2 test.
‡Data for 5 symptomatic patients were missing, and another patient was excluded from analysis because of co-infection with DENV-1 and DENV-2.

this study. We noted that attack rates were lower in PI-V, 14.7/1,000 participants (14/952), than in PI-H, 47.4/1,000 participants (332/7,008). Circulation of DENV around febrile index case-patients identified through PI-V was less intense, but with more asymptomatic cases, than around index case-patients identified through PI-H. Aside from possible detection biases (*17*), multiple factors could explain this observation and deserve further research.

Our study documented cases of DENV infection in transmission clusters located around index case-patients. We found that 26.6% of DENV-confirmed case-patients reported clinical symptoms, including headache and muscle pain, but no fever even in the absence of antipyretics, comparable to data from Thailand, where 40.4% of the DENV cases remained afebrile (*17*). The appearance of afebrile DENV-infected patients raises potential concerns for case definitions for detection, especially of imported cases in at-risk countries.

An additional 7.5% of DENV-confirmed case-patients had no symptoms during the 10-day course of clinical monitoring, a considerably lower rate than estimates from other prospective studies (*5*,*18*–*21*). Published sources refer to inapparent infections, often defined as afebrile clinical complaints with biologic evidence of DENV infection, ranging from 20% to 80% of cases (*19*,*22*,*23*). Previous studies used different definitions of asymptomatic infection than ours, but the major difference lies in follow-up monitoring. Other retrospective studies used school or work absenteeism as a basis for follow-up (*19*). Strictly asymptomatic patients, such as those we describe, escape detection by surveillance or control measures, infect mosquitoes, and might disproportionately contribute to DENV transmission (*5*).

The DENV burden documented through hospital-based surveillance of febrile case-patients in Thailand and Vietnam showed a shift to older age groups (*24*,*25*). Our active, systematic case-finding system to identify DENV in villages in Cambodia found the attack rate was highest in children <10 years of age, which is what we expected in a dengue-endemic country with frequent outbreaks demonstrated in other careful studies (*26*). This finding raises concerns because recommendations for the only licensed dengue vaccine are for use in persons 9–45 years of age with demonstrated evidence of past DENV infection (*27*). Our study demonstrates that children in rural Cambodia might have undergone >1 DENV infection before 9 years of age, reducing the potential cost-effectiveness of vaccination.

Few studies have explored the role of socioeconomic status, which might be a proxy for peridomestic environmental management, on DENV infection in Southeast Asia. Often, the direction of the association is unclear and socioeconomic status has entirely different associations depending on the setting (*28*). Our study shows that the adjusted risk for DENV infection was highest in households in which the main source of income was from fishing, farming, or animal husbandry, activities associated with lower average household income in Cambodia.

We found temephos provided no additional protection against DENV infection after adjusting for other factors. Although temephos is effective in reducing *Aedes* spp. larval populations in water storage jars, its use did not correlate with lower DENV transmission in Cambodia or elsewhere (*8*), due to incorrect distribution coverage, dosage, and placement (*29*) or multiple vector breeding sites. In addition, increases in temephos-resistant *A. aegypti* mosquito larvae have been documented in Cambodia (*30*).

Unexpectedly, we found a higher risk for DENV with piped water as a main water source after adjusting for other factors, contrary to a study in Thailand (*6*). However, piped water with suboptimal sanitation in Cambodia might contribute to collection of water in or around households that could become breeding sites for DENV-transmitting mosquitoes.

We found that 40.8% of the variability in probability of being DENV infected was explained by differences between households. Those living in the same household as an index case-patient were 2.11 times more likely to be

infected, consistent with other published sources. A study in Mexico found that the risk for infection for those living with an index case-patient was twice that of someone living in a 50-meter radius of an index case-patient (*31*). This relationship was further described in a cluster study in Thailand that showed decreased risk for infection with increasing distance from the index case household (*31*). This clustering effect around an index case, however, seems to occur only on a short temporal scale, at least in urban settings (*32*).

Rates and severity of illness after infection with the different DENV serotypes differ widely (*33*,*34*). The only notable epidemiologic factor associated with asymptomatic DENV infections in our study was being family-related to the index case-patient. Another study showed that adaptive immune responses against DENV differ between persons with symptomatic and asymptomatic DENV infection (*35*), which might explain our observations. We found no other associated epidemiologic factor, including age or cluster effects. Although the ratio of male to female participants was twice as high among asymptomatic than symptomatic participants, this finding was not statistically significant, likely due to sample size.

Although the size and duration of our study confer strength to our data, it might suffer from biases and limitations, especially due to the small number of strictly asymptomatic DENV-positive participants after stratifying by DENV serotype. We found dengue incidence rates highest in young children. These data might be biased because we focused on investigating clusters around an index case, perhaps overestimating the incidence in the general population. DENV circulation, however, is intense in children in Cambodia, and these figures remain comparable to those found in dengue studies that use different methods, ranging from 20 to 80 per 1,000 person-seasons (*1*). Furthermore, we did not capture cases referred to the private sector, lowering our estimates somewhat. Healthy male workers often were away at the time of the investigations, possibly leading to an overestimation of DENV incidence. These workers, however, are ≥18 years of age, but DENV infections occur mainly in persons <15 years of age in Cambodia (*4*).

Documentation bias might also have pulled our risk factor estimates toward the null. We did not document solid waste disposal in our study, but comparatively high Breteau index values have been reported in Cambodia (*29*). In addition, we could have missed details or misrepresented implementation of mosquito-control measures. Despite the potential misclassification, mosquito-control measures remain nondifferential and likely had no major effect on our risk estimates.

Further, 7.5% of our DENV-infected participants remained strictly asymptomatic. Aside from case definition issues we discuss, our method of screening for DENV around symptomatic cases might have underestimated the number of asymptomatic DENV infections. In addition, we did not enroll persons who tested negative for DENV IgM, NS1-capture ELISA, and qRT-PCR. Some of these persons might have been infected but not yet mounted an IgM response, so that NS1 and viral RNA titers had already receded to undetectable levels when we tested them. This strict case definition might have underestimated the incidence of asymptomatic cases, but a precise retrospective documentation of such cases would be extremely difficult. Similarly, we retrospectively conducted MAC-ELISA on samples collected during perifocal investigations and identified 11 cases of IgM seroconversion in the absence of PCR- or NS1-positive tests. Even in the context of JEV cocirculation, some of these cases could have been true DENV infections, but including them would not have changed the overall estimated attack rate. Previous studies suggested virus serotype might affect severity and types of symptoms and observed that DENV-1 infections more frequently were associated with clinically apparent illness (*36*,*37*). Virus molecular analysis studies are ongoing to determine whether specific strains cause more asymptomatic infection than others. Furthermore, DENV infection in Cambodia occurs mainly in children who might be more likely to answer positively to daily-repeated questions on dengue symptoms, somewhat underestimating asymptomatic cases. Having implemented careful and thorough 10-day clinical assessment of objective symptoms in each asymptomatic DENV-positive participant, we believe these figures reflect the true proportion of strictly asymptomatic DENV infection in our setting. However, we collected our findings mainly in children with DENV-1 infection in Cambodia. Whether these findings are directly applicable to other epidemiologic settings, populations, or virus serotypes or genotypes remains to be determined (*33*).

Finally, vaccination against JEV might have led to cross-protection against symptomatic dengue. Data on JEV vaccination were not collected during perifocal investigations. According to local health centers, however, JEV vaccine has been provided only recently and only for children 9–24 months of age. In our study, only 3 children were DENV-positive in that age category.

Our study demonstrates that systematically relying on fever for DENV case definition can underestimate cases and hinder control efforts in areas with potential vectors and at risk for DENV introduction. We found 7.5% of DENV-infected participants remained strictly asymptomatic, which has wide-ranging epidemiologic consequences. Undetected sources can increase transmission (*5*), a factor that must be taken into account in future vaccine coverage and vaccine effectiveness studies. The attack rate differences observed around febrile index case-patients detected in village surveillance and index case-patients detected in

hospital surveillance deserve further study. In-depth virus (*36*) and human genetic studies could contribute useful insights (*33*,*35*). Our strict definition of asymptomatic DENV infections should be considered when designing studies that aim to elucidate the pathophysiological mechanisms of dengue disease.

Acknowledgments

The authors gratefully acknowledge participating hospitals, villages, and study participants, as well as Tineke Cantaert for her editorial comments and suggestions.

Dengue Framework for Resisting Epidemics in Europe studies were funded by a grant (no. 282378) from the European Union 7th FP.

P.B. is a former head of virology at Institut Pasteur du Cambodge and is an employee of GSK Vaccines, Singapore.

About the Authors

Dr. Ly is a medical doctor and epidemiologist at the Epidemiology & Public Health Department at Institut Pasteur du Cambodge; his primary research interests are epidemiology of endemic and epidemic arboviruses and zoonoses in the Mekong Region. Ms. Fortas is an epidemiologist whose research interests are tropical infectious diseases in low- and middle-income countries.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7. http://dx.doi.org/10.1038/nature12060
2. Simmons CP, Farrar JJ, van Vinh Chau N, Wills B. Dengue. N Engl J Med. 2012;366:1423–32. http://dx.doi.org/10.1056/NEJMra1110265
3. Western Pacific Regional Office of the World Health Organization. Dengue situation updates [cited 2017 Jul 12]. http://www.wpro.who.int/entity/emergencies/wp_dengue_biweekly/en
4. Vong S, Goyet S, Ly S, Ngan C, Huy R, Duong V, et al. Under-recognition and reporting of dengue in Cambodia: a capture-recapture analysis of the National Dengue Surveillance System. Epidemiol Infect. 2012;140:491–9. http://dx.doi.org/10.1017/S0950268811001191
5. Duong V, Lambrechts L, Paul RE, Ly S, Lay RS, Long KC, et al. Asymptomatic humans transmit dengue virus to mosquitoes. Proc Natl Acad Sci U S A. 2015;112:14688–93. http://dx.doi.org/10.1073/pnas.1508114112
6. Mammen MP, Pimgate C, Koenraadt CJM, Rothman AL, Aldstadt J, Nisalak A, et al. Spatial and temporal clustering of dengue virus transmission in Thai villages. PLoS Med. 2008;5:e205. http://dx.doi.org/10.1371/journal.pmed.0050205
7. Dussart P, Baril L, Petit L, Beniguel L, Quang LC, Ly S, et al. Clinical and virological study of dengue cases and the members of their households: the multinational DENFRAME Project. PLoS Negl Trop Dis. 2012;6:e1482. http://dx.doi.org/10.1371/journal.pntd.0001482
8. Huy R, Buchy P, Conan A, Ngan C, Ong S, Ali R, et al. National dengue surveillance in Cambodia 1980–2008: epidemiological and virological trends and the impact of vector control. Bull World Health Organ. 2010;88:650–7. http://dx.doi.org/10.2471/BLT.09.073908
9. Vong S, Khieu V, Glass O, Ly S, Duong V, Huy R, et al. Dengue incidence in urban and rural Cambodia: results from population-based active fever surveillance, 2006–2008. PLoS Negl Trop Dis. 2010;4:e903. http://dx.doi.org/10.1371/journal.pntd.0000903
10. Hue KDT, Tuan TV, Thi HTN, Bich CTN, Anh HHL, Wills BA, et al. Validation of an internally controlled one-step real-time multiplex RT-PCR assay for the detection and quantitation of dengue virus RNA in plasma. J Virol Methods. 2011;177:168–73. http://dx.doi.org/10.1016/j.jviromet.2011.08.002
11. Andries A-C, Duong V, Ngan C, Ong S, Huy R, Sroin KK, et al. Field evaluation and impact on clinical management of a rapid diagnostic kit that detects dengue NS1, IgM and IgG. PLoS Negl Trop Dis. 2012;6:e1993. http://dx.doi.org/10.1371/journal.pntd.0001993
12. Andries A-C, Duong V, Ong S, Ros S, Sakuntabhai A, Horwood P, et al. Evaluation of the performances of six commercial kits designed for dengue NS1 and anti-dengue IgM, IgG and IgA detection in urine and saliva clinical specimens. BMC Infect Dis. 2016;16:201. http://dx.doi.org/10.1186/s12879-016-1551-x
13. Duong V, Andries A-C, Ngan C, Sok T, Richner B, Asgari-Jirhandeh N, et al. Reemergence of chikungunya virus in Cambodia. Emerg Infect Dis. 2012;18:2066–9. http://dx.doi.org/10.3201/eid1812.120471
14. Zou G. A modified Poisson regression approach to prospective studies with binary data. Am J Epidemiol. 2004;159:702–6. http://dx.doi.org/10.1093/aje/kwh090
15. Wu S, Crespi CM, Wong WK. Comparison of methods for estimating the intraclass correlation coefficient for binary responses in cancer prevention cluster randomized trials. Contemp Clin Trials. 2012;33:869–80. http://dx.doi.org/10.1016/j.cct.2012.05.004
16. Tarantola A, Goutard F, Newton P, de Lamballerie X, Lortholary O, Cappelle J, et al. Estimating the burden of Japanese encephalitis virus and other encephalitides in countries of the Mekong region. PLoS Negl Trop Dis. 2014;8:e0002533. https://doi.org/10.1371/journal.pntd.0002533
17. Yoon I-K, Srikiatkhachorn A, Hermann L, Buddhari D, Scott TW, Jarman RG, et al. Characteristics of mild dengue virus infection in Thai children. Am J Trop Med Hyg. 2013;89:1081–7. http://dx.doi.org/10.4269/ajtmh.13-0424
18. Porter KR, Beckett CG, Kosasih H, Tan RI, Alisjahbana B, Rudiman PIF, et al. Epidemiology of dengue and dengue hemorrhagic fever in a cohort of adults living in Bandung, West Java, Indonesia. Am J Trop Med Hyg. 2005;72:60–6. http://dx.doi.org/10.4269/ajtmh.2005.72.60
19. Endy TP, Chunsuttiwat S, Nisalak A, Libraty DH, Green S, Rothman AL, et al. Epidemiology of inapparent and symptomatic acute dengue virus infection: a prospective study of primary school children in Kamphaeng Phet, Thailand. Am J Epidemiol. 2002;156:40–51. http://dx.doi.org/10.1093/aje/kwf005
20. Kosasih H, Alisjahbana B, Nurhayati, de Mast Q, Rudiman IF, Widjaja S, et al. The epidemiology, virology and clinical findings of dengue virus infections in a cohort of Indonesian adults in western Java. PLoS Negl Trop Dis. 2016;10:e0004390. http://dx.doi.org/10.1371/journal.pntd.0004390
21. Yoon I-K, Rothman AL, Tannitisupawong D, Srikiatkhachorn A, Jarman RG, Aldstadt J, et al. Underrecognized mildly symptomatic viremic dengue virus infections in rural Thai schools and villages. J Infect Dis. 2012;206:389–98. http://dx.doi.org/10.1093/infdis/jis357
22. Balmaseda A, Standish K, Mercado JC, Matute JC, Tellez Y, Saborío S, et al. Trends in patterns of dengue transmission over 4 years in a pediatric cohort study in Nicaragua. J Infect Dis. 2010;201:5–14. http://dx.doi.org/10.1086/648592
23. Endy TP, Anderson KB, Nisalak A, Yoon I-K, Green S, Rothman AL, et al. Determinants of inapparent and symptomatic

dengue infection in a prospective study of primary school children in Kamphaeng Phet, Thailand. PLoS Negl Trop Dis. 2011;5:e975. http://dx.doi.org/10.1371/journal.pntd.0000975
24. Anders KL, Nga H, Thuy NTV, Ngoc TV, Tam CT, Tai LTH, et al. Households as foci for dengue transmission in highly urban Vietnam. PLoS Negl Trop Dis. 2015;9:e0003528. http://dx.doi.org/10.1371/journal.pntd.0003528
25. Limkittikul K, Brett J, L'Azou M. Epidemiological trends of dengue disease in Thailand (2000–2011): a systematic literature review. PLoS Negl Trop Dis. 2014;8:e3241. http://dx.doi.org/10.1371/journal.pntd.0003241
26. Hadinegoro SR, Arredondo-García JL, Capeding MR, Deseda C, Chotpitayasunondh T, Dietze R, et al.; CYD-TDV Dengue Vaccine Working Group. Efficacy and long-term safety of a dengue vaccine in regions of endemic disease. N Engl J Med. 2015;373:1195–206. http://dx.doi.org/10.1056/NEJMoa1506223
27. Dengue vaccine: WHO position paper, September 2018—recommendations. Vaccine. 2018;S0264-410X(18)31339-2.
28. Mulligan K, Dixon J, Joanna Sinn C-L, Elliott SJ. Is dengue a disease of poverty? A systematic review. Pathog Glob Health. 2015; 109:10–8. http://dx.doi.org/10.1179/2047773214Y.0000000168
29. Khun S, Manderson LH. Abate distribution and dengue control in rural Cambodia. Acta Trop. 2007;101:139–46. http://dx.doi.org/10.1016/j.actatropica.2007.01.002
30. Polson KA, Curtis C, Seng CM, Olson JG, Chantha N, Rawlins SC. Susceptibility of two Cambodian populations of *Aedes aegypti* mosquito larvae to temephos during 2001. Dengue Bull. 2001; 25:79–83.
31. Martínez-Vega RA, Danis-Lozano R, Díaz-Quijano FA, Velasco-Hernández J, Santos-Luna R, Román-Pérez S, et al. Peridomestic infection as a determining factor of dengue transmission. PLoS Negl Trop Dis. 2015;9:e0004296. http://dx.doi.org/10.1371/journal.pntd.0004296
32. Yoon I-K, Getis A, Aldstadt J, Rothman AL, Tannitisupawong D, Koenraadt CJM, et al. Fine scale spatiotemporal clustering of dengue virus transmission in children and *Aedes aegypti* in rural Thai villages. PLoS Negl Trop Dis. 2012;6:e1730. http://dx.doi.org/10.1371/journal.pntd.0001730
33. Grange L, Simon-Loriere E, Sakuntabhai A, Gresh L, Paul R, Harris E. Epidemiological risk factors associated with high global frequency of inapparent dengue virus infections. Front Immunol. 2014;5:280. http://dx.doi.org/10.3389/fimmu.2014.00280
34. Clapham HE, Cummings DAT, Johansson MA. Immune status alters the probability of apparent illness due to dengue virus infection: evidence from a pooled analysis across multiple cohort and cluster studies. PLoS Negl Trop Dis. 2017;11:e0005926. http://dx.doi.org/10.1371/journal.pntd.0005926
35. Simon-Lorière E, Duong V, Tawfik A, Ung S, Ly S, Casadémont I, et al. Increased adaptive immune responses and proper feedback regulation protect against clinical dengue. Sci Transl Med. 2017;9:eaal5088. http://dx.doi.org/10.1126/scitranslmed.aal5088
36. Li D, Lott WB, Lowry K, Jones A, Thu HM, Aaskov J. Defective interfering viral particles in acute dengue infections. PLoS One. 2011;6:e0019447. https://doi.org/10.1371/journal.pone.0019447
37. Yung C-F, Lee K-S, Thein T-L, Tan L-K, Gan VC, Wong JGX, et al. Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, Singapore. Am J Trop Med Hyg. 2015;92:999–1005. http://dx.doi.org/10.4269/ajtmh.14-0628

Address for correspondence: Arnaud Tarantola, Institut Pasteur du Cambodge, Epidemiology and Public Health Unit, PO Box 983, Phnom Penh, Cambodia; email: arnaud.tarantola@pasteur.fr

Facility-Associated Release of Polioviruses into Communities—Risks for the Posteradication Era

Ananda S. Bandyopadhyay, Harpal Singh, Jacqueline Fournier-Caruana, John F. Modlin, Jay Wenger, Jeffrey Partridge, Roland W. Sutter, Michel J. Zaffran

The Global Polio Eradication Initiative continues to make progress toward the eradication target. Indigenous wild poliovirus (WPV) type 2 was last detected in 1999, WPV type 3 was last detected in 2012, and over the past 2 years WPV type 1 has been detected only in parts of 2 countries (Afghanistan and Pakistan). Once the eradication of poliomyelitis is achieved, infectious and potentially infectious poliovirus materials retained in laboratories, vaccine production sites, and other storage facilities will continue to pose a risk for poliovirus reintroduction into communities. The recent breach in containment of WPV type 2 in an inactivated poliovirus vaccine manufacturing site in the Netherlands prompted this review, which summarizes information on facility-associated release of polioviruses into communities reported over >8 decades. Successful polio eradication requires the management of poliovirus containment posteradication to prevent the consequences of the reestablishment of poliovirus transmission.

In 1988, the World Health Assembly resolved to eradicate poliomyelitis by the year 2000 (*1*). Much progress has been made toward this goal. Two of the 3 wild poliovirus (WPV) serotypes are either certified as eradicated (WPV type 2 [WPV2]) or have not been detected globally since 2012 (WPV type 3 [WPV3]). WPV type 1 (WPV1) continues to circulate only in parts of Afghanistan and Pakistan (*2*). The eradication goal appears within reach.

Once the eradication of poliomyelitis is achieved, apart from rare cases of poliovirus excretors among immunodeficient persons (*3*,*4*), polioviruses will only exist as virus stocks, cultures, and reagents in laboratories, vaccine production sites, and other facilities where live poliovirus stocks are maintained. An essential consideration for the certification of eradication of poliomyelitis as described in the Polio Eradication and Endgame Strategic Plan 2013–2018 (*5*) and the Global Action Plan for Poliovirus Containment (GAPIII) (*6*) is the safe and secure containment of poliovirus within facilities designated by their governments for the posteradication retention of poliovirus materials. The declaration of the certification of WPV2 eradication in September 2015 (*7*) accelerated the implementation of containment work as described in GAPIII. The withdrawal of Sabin poliovirus type 2 from the oral poliovirus vaccine (OPV) in April 2016 highlighted the importance of containment, and annual meetings of the Global Certification Commission (GCC) for the Eradication of Poliomyelitis beginning in September 2015, and especially in October 2017, clarified the GCC oversight responsibilities for containment (*8*,*9*).

A laboratory accident involving the release of WPV2 from an inactivated poliovirus vaccine (IPV) manufacturing site in the Netherlands in April 2017 (*10*–*12*) motivated this historical review to describe the frequency and consequences of similar breaches. Our objective is to remind management and workers at all laboratory and manufacturing facilities of their responsibility to assess the risks of stored materials (*13*).

Methods

We performed a literature search by using PubMed with no date restrictions, applying the following search terms in various combinations to identify episodes of facility-associated strains infecting humans or being isolated from environmental samples in nonendemic and nonoutbreak reporting areas to complement the information known to us: poliovirus or polio, contamination, accidental environmental contamination, accidental release, and laboratory accidents. This search found a total of 29 references, all of which are described in this article.

Author affiliations: Bill & Melinda Gates Foundation, Seattle, Washington, USA (A.S. Bandyopadhyay, J.F. Modlin, J. Wenger, J. Partridge); World Health Organization, Geneva, Switzerland (H. Singh, J. Fournier-Caruana, R.W. Sutter, M.J. Zaffran); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (R.W. Sutter)

DOI: https://doi.org/10.3201/eid2507.181703

Results

Major Documented Events

Facility-associated release of polioviruses resulting from either laboratory or vaccine production sources was not uncommon in the period before the development and widespread use of poliovirus vaccines (*14–20*) (Tables 1, 2; Figure). In 1933, a 29-year-old physician conducting experimental work on poliomyelitis was bitten by a macaque monkey. Although the exposure to poliovirus could not be confirmed, the physician later experienced paralysis and died (*20*,*21*). The first case of known exposure to poliovirus in a laboratory setting was reported in 1941 in a technician handling infected tissues in preparation for inoculation into monkeys (*19*,*20*,*23*). Six additional laboratory-associated releases of poliovirus through an infected worker occurred during the same decade: 3 in the United States, of which 2 involved a worker infected with Lansing (Armstrong) strain virus (*18–20*,*24*,*36*); and 1 each in Zimbabwe (formerly Rhodesia) (*20*,*37*), Canada (*20*,*25*), and the United Kingdom (*20*,*26*). Cases of poliomyelitis attributable to clinical trial use of vaccines or faulty production have also been reported. In 1935, twelve cases of paralytic poliomyelitis, of which 6 were fatal, were reported among those receiving trial vaccinations against poliomyelitis (*22*). In 1955, distribution of 120,000 doses of IPV that had been inadequately inactivated during the production step resulted in the paralysis of 51 children, 5 of whom died; secondary transmission was reported among 113 contacts who experienced

Table 1. Reported incidents of poliovirus release from laboratories and vaccine production facilities in the pre–polio vaccine era*

Year	Location (reference)	Source	Poliovirus type	No. cases*	Exposure			Description
					Primary	Secondary	Tertiary	
1933	United States (*20*,*21*)	Lab	Not indicated	1	Physician	NA	NA	Bitten (cutaneous disruption) by a normal macaque while doing work on poliomyelitis (paralysis); filterable virus capable of reproducing the disease in rabbits was isolated from the case; case was fatal
1935	United States (*22*)	Vaccine production facility	Not indicated	12	Vaccine trial patients, age 5 mo to 20 y	NA	NA	12 cases of paralytic poliomyelitis in patients receiving trial vaccination against poliomyelitis; natural infections ruled out as cause; 6 deaths
1941	United States (*19*,*20*,*23*)	Lab	Not indicated	1	Lab staff	NA	NA	Lab staff member experienced paralysis after preparation of infected tissues for inoculation into monkeys; cutaneous inoculation; no polio outbreaks reported in place of residence or areas of travel
1945	United States (*20*,*23*)	Lab	Not indicated	1	Lab staff	NA	NA	Lab staff member scratched on hand by noninoculated monkey during transport; subsequent virus contamination of hands might have occurred while feeding or inoculating monkeys; patient experienced paralysis and later died
1946	Zimbabwe (formerly Rhodesia) (*20*,*25*)	Lab	Not indicated	1	Lab staff	NA	NA	Infection acquired during inoculation of monkeys with polio virus; paralysis occurred, case was fatal
1949	United States (*20*,*24*)	Lab	WPV2 (mouse-adapted Lansing strain)	2	Lab staff	NA	NA	Two lab technicians were infected in the eyes and nose with Lansing (Armstrong) strain while inoculating mice during polio experiments; both experienced paralysis
1950	Canada (*20*,*25*)	Lab	Not indicated	1	Physician	Na	NA	Doctor acquired poliomyelitis while performing autopsy on poliomyelitis patient; intracutaneous inoculation; residual weakness; case was not fatal
1954	United Kingdom (*20*,*26*)	Lab	WPV2 (MEF-1) strain	1	Lab staff	NA	NA	Lab technicians infected by cutaneous inoculation while performing necropsy on animals infected with wild type-2 (MEF-1) strain; subsequent paralysis; cases were not fatal

*Cases defined as laboratory positive (with or without paralysis) for poliovirus by standard methods of virus isolation or known exposure to poliovirus. Lab, laboratory; MEF-1, wild poliovirus type 2 laboratory reference strain; NA, not applicable; WPV2, wild poliovirus type 2.

paralysis, 5 of whom died (*27*,*28*). Although the 1955 incident was inherently distinct from all other examples discussed in this review (with the root cause being faulty production procedure instead of accidental release or exposure), we include it in this report for completeness. The number of subclinical infections with poliovirus during this period is unknown, so the total number of persons affected might have been many times higher (*29*).

In the 3 decades since the WHA resolution to eradicate poliomyelitis and the formation of the Global Polio Eradication Initiative (GPEI) in 1988, seven documented incidents underscore the potential for facility-associated release of polioviruses into the community in the modern era. In 1991, a WPV3 (Saukett strain), probably from a laboratory source, was isolated in France from a woman from Algeria. A year later, a worker in a vaccine manufacturing facility in the Netherlands transmitted a WPV1 (Mahoney strain) used for IPV production to his son (*30*). In another incident in the Netherlands in 1993, a child with a travel history to France was reported to have been infected with a strain of WPV3 (Saukett strain) almost identical to that used for IPV production in France. The possibility of laboratory contamination was ruled out, and environmental samples collected from around the child's home and among his family contacts were negative for poliovirus in cell culture. The source of this infection was not determined (*30*). In India, 2 incidents were reported during 2000–2003 after the interruption of WPV2 transmission in 1999. A WPV2 laboratory reference strain (MEF-1) was recovered from 3 poliomyelitis patients in September 2000 and 7 patients during November 2002–February 2003. The sources of these infections were not identified (*31*–*33*).

Table 2. Reported incidents of poliovirus release from laboratories and vaccine production facilities in the post–polio vaccine era*

Year	Location (reference)	Source	Poliovirus type	No. cases	Exposure			Description
					Primary	Secondary	Tertiary	
1955	United States (*27*,*28*)	Vaccine production facility	Not indicated	164	Vaccine recipients (≈40,000 children)	113 contacts of the children	NA	"Cutter incident"; inadequate formaldehyde virus inactivation during poliovirus vaccine production (≈120,000 doses); ≈40,000 children experienced muscle weakness, of whom 51 experienced paralysis; 5 deaths; 113 contacts of the children were also paralyzed, of whom 5 died
1991	France (*29*)	Lab and vaccine production facility	WPV3 (Saukett) strain	1	No definitive information on exposure of case			Saukett strain isolated in France from a woman from Algeria; source of this lab strain could not be confirmed
1992	Netherlands (*30*)	Vaccine production facility	WPV1 (Mahoney) strain	1	Father (worker at facility)	Son	NA	Boy (age 19 mo) with respiratory symptoms (no paralysis); father with history of accidental exposure to Mahoney strains while working in a poliovirus vaccine production facility
1993	Netherlands (*30*)	Vaccine production facility	WPV3 (Saukett) strain	1	No definitive information on exposure of case			Child with gastroenteritis (no paralysis); had travel history to France; no epidemiology established to trace lab exposure; Saukett strains typical for IPV production in France isolated from the stool samples
2000	India (*31*–*33*)	Lab and vaccine production facility	WPV2 (MEF-1) strain	3	No definitive information on exposure of case			WPV2 isolates found in Sep 2000 and Nov 2002–Feb 2003 from 10 children with AFP, 1 healthy contact, and 1 environmental sample; isolates unrelated to all previous WPV2 strains found in India; because this was a lab reference strain and not a community-derived wild strain, lab source was suspected
2002–2003	India (*31*–*33*)	Lab and vaccine production facility	WPV2 (MEF-1) strain	8	No definitive information on exposure of case			
2014	Belgium (*34*,*35*)	Vaccine production facility	WPV3 (Saukett) strain	0	NA	NA	NA	$\approx 10^{13}$ infectious WPV3 particles accidentally released into sewage system from production plant in Belgium; no poliovirus detected in environmental or human samples
2017	Netherlands (*12*)	Vaccine production facility	WPV2 (MEF-1) strain	1	Worker	None	None	Accidental leakage in vaccine production room; 1 of 2 exposed staff members tested positive by RT-PCR

*Cases are defined as laboratory positive (with or without paralysis) for poliovirus by standard methods of virus isolation or known exposure to poliovirus. AFP, acute flaccid paralysis; IPV, inactivated poliovirus vaccine; lab, laboratory; MEF-1, wild poliovirus type 2 laboratory reference strain; NA, not applicable; RT-PCR, reverse transcription PCR; WPV, wild poliovirus; WPV1, wild poliovirus type 1; WPV2, wild poliovirus type 3; WPV3, wild poliovirus type 2.

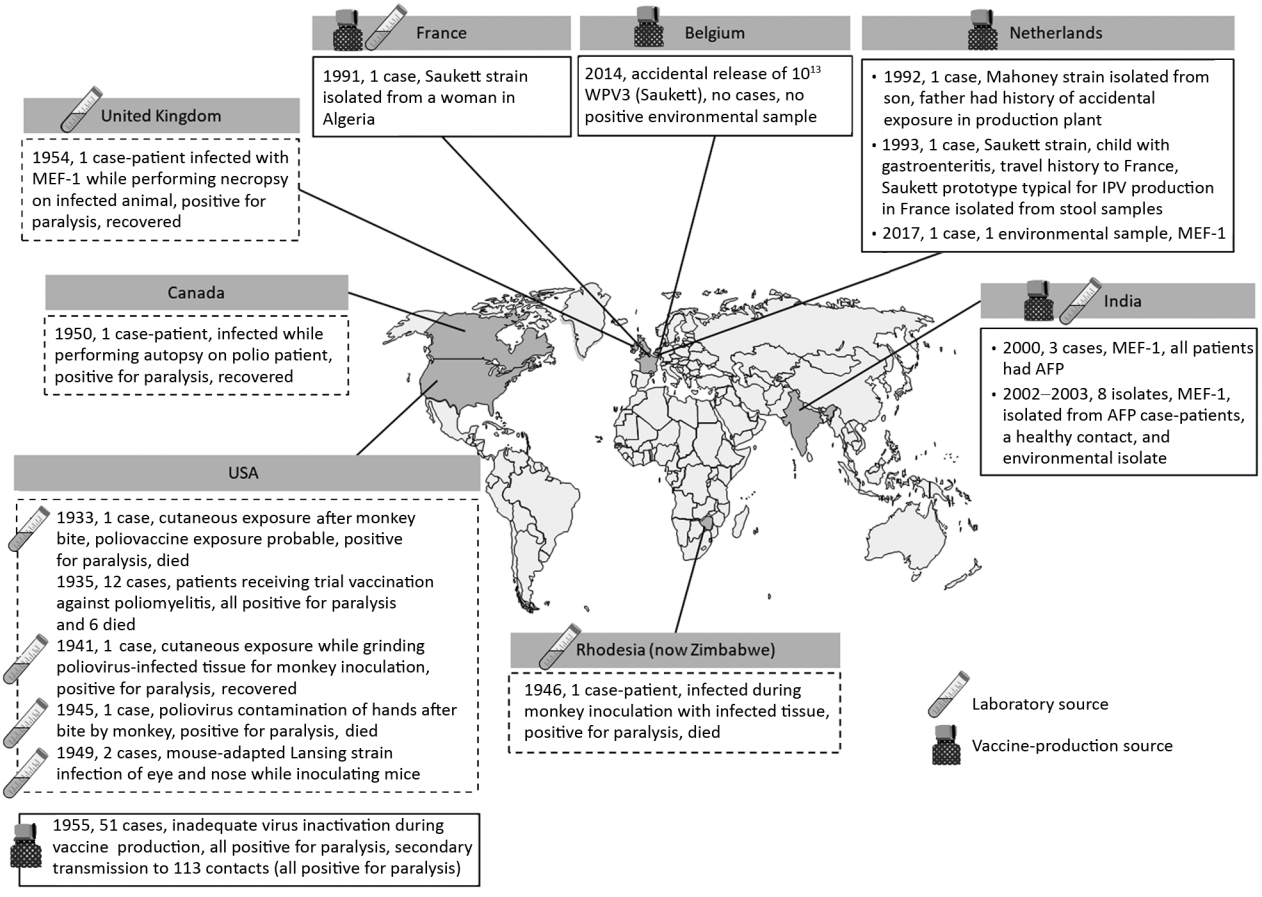


Figure. Reported incidents of facility-associated poliovirus release from laboratories and manufacturing sites in the pre–polio vaccine era (shown inside dashed-line frames) and the time of poliovirus vaccine introduction to the present (shown inside solid-line frames). AFP, acute flaccid paralysis; IPV, inactivated poliovirus vaccine; MEF-1, wild poliovirus type 2 laboratory reference strain; WPV, wild poliovirus; WPV3, wild poliovirus type 3.

In September 2014 in Belgium, ≈10^{13} infectious WPV3 particles were released into the sewage system from a vaccine production plant (*34*). Subsequent investigations revealed no evidence of WPV3 in samples from a range of environmental samples (*35*). More recently, in the Netherlands, WPV2 (MEF-1 strain) was accidentally released as an aerosolized high-titer spill when tubing became disconnected in a vaccine production room. One exposed staff member became infected and shed the wild virus strain for ≈4 weeks before testing negative by fecal culture, whereas a second staff member who was also present at the time of the spill did not test positive for poliovirus in throat swabs or stool samples (*12*). No acute flaccid paralysis (AFP) cases or secondary spread to household contacts was detected.

Discussion

The laboratory accident in April 2017 involving the exposure of a worker who subsequently excreted WPV2 into the sewage system (*12*) serves as a stark reminder that the achievements of the GPEI are fragile and can be reversed if remaining sources of polioviruses are not contained to reduce the likelihood and consequences of virus reintroduction after eradication. Even more recently, a suspected contamination of ≈150,000 bivalent OPV vials with type 2 Sabin virus strains has been reported in India (*38*), highlighting the importance of completeness of containment-related activities for type 2 OPV at all stages of vaccine manufacturing and release. Our review demonstrates that the known risk for poliovirus release from a facility into the community appears to be small, based on only a handful of reported incidents, primarily from vaccine manufacturing sites. However, these reported incidents might represent the proverbial tip of the iceberg, given that the reporting requirements in the past were not very stringent.

The release of polioviruses into the community in the posteradication era is a major public health concern for GPEI as it implements the planned, sequential cessation of OPV use during the polio endgame. With the global

discontinuation of type 2 OPV for routine and supplemental immunization in April 2016 and the planned cessation of all OPV use in the next 4–5 years, such release of polioviruses from facilities into the community will become a greater public health concern because population immunity wanes in settings of high population density, poor hygiene, and suboptimal immunity (e.g., tropical developing countries) after OPV withdrawal, increasing the potential for transmission (*39*).

We should note that in the prevaccine era most of the reported incidents occurred in research settings and the exposed persons did not have vaccine-induced immunity for protection against paralysis or virus transmission. In sharp contrast, the incidents reported in the past 3 decades were related to containment failures at vaccine production plants where the staff are expected to be fully vaccinated. Incidents in the more recent period are likely to be more representative of the public health impact of such containment failures for the current phase of the polio endgame and the near future.

The paucity of reports of laboratory-associated poliomyelitis during the past 3 decades testifies to the effectiveness of vaccines and to improved laboratory facilities and biorisk management. However, laboratory breaches in the past might not have been recognized in the absence of clinical cases, and environmental surveillance was less extensive than it is today. Environmental surveillance for polio, for example, appears to have played a major role in such incidents primarily in the past 2 decades, and more so in recent times, with the expansion and enhancement of scope and methodologies that were introduced as a component of the polio endgame. Furthermore, reporting requirements were less stringent in the past, and we assume that not all facility incidents were recorded. The lack of documented incidents of laboratory accidents during 1955–1991 is difficult to explain but might also have been affected by these factors, in addition to the fact that the formation of GPEI in 1988 led to a more concerted, globally synchronized effort to track and report polio cases and outbreaks.

As seen in the Netherlands incident in 2017, previously vaccinated persons, although probably protected from paralysis, can excrete poliovirus after accidental exposure from containment failures and put the community at risk for virus transmission. This risk underscores the importance of stringent containment measures at the vaccine production sites and preparedness for deployment or enhancement of surveillance activities, such as environmental monitoring, as a public health response strategy.

The smallpox experience illustrates the importance of containment for an eradicated pathogen. Within 1 year of detection of the last known natural case of smallpox in 1977, a case linked to laboratory transmission was reported (*40*). We have a historic opportunity to benefit from an additional 40 years of experience in risk management and disease-control measures to ensure containment measures are designed, implemented, and maintained to provide a world free from all risks for polio-related paralysis. The first step is a uniform, global awareness of the importance of implementing the GAPIII guidelines to minimize facility-associated risks for poliovirus reintroduction. The risk for accidental release can be minimized by retaining poliovirus in a limited number of poliovirus-essential facilities. GAPIII also proposes further risk reduction by establishing international standards of biorisk management for facility containment (i.e., primary safeguards), population immunity (i.e., secondary safeguards), and facility location (i.e., tertiary safeguards) with assurance by national (National Authority for Containment) and international (GCC) oversight that such standards are met. Timeliness and completeness in implementing these measures through well-defined risk management systems and an effective National Authority for Containment are key for success in sustaining a polio-free world.

Equally important is the identification of materials that are potentially infectious for polioviruses in all facilities that store human stool specimens, respiratory samples, or environmental sewage for any purpose. Depending on the place and time of collection, such materials might harbor infectious polioviruses that have been eradicated (WPV2) or are nearly eradicated (WPV1 and WPV3) in the wild. Identifying the risk, eliminating the risk through destruction, or mitigating the risk of handling such materials is essential (*13*).

Finally, the risk management approach to containment might have to be optimized and balanced to enable other risk mitigation efforts for the endgame and beyond, such as novel vaccine and drug development to further reduce any risk for vaccine-derived circulation. Timely availability of antiviral drugs and effective novel OPVs that have less risk for reversion to neurovirulence compared with current Sabin vaccines might strengthen outbreak response strategies and mitigate medical risks for inadvertent poliovirus exposure and improve reporting of accidental safety breaches by employees (*41–43*). The World Health Assembly resolution on poliovirus containment (WHA71.16) urges intense efforts to accelerate progress toward poliovirus containment globally (*44*).

About the Author

Dr. Bandyopadhyay is a senior program officer for polio research at the Bill & Melinda Gates Foundation. His research is focused on generating data regarding the best use of poliovirus vaccines to make them affordable and accessible to vulnerable and underserved populations.

References

1. World Health Organization. World Health Assembly resolution WHA41.28. Global eradication of poliomyelitis by the year 2000 [cited 2018 Oct 10]. http://www.who.int/ihr/polioresolution4128en.pdf
2. Global Polio Eradication Initiative. Global wild poliovirus 2012–2018 [cited 2018 Oct 10]. http://polioeradication.org/wp-content/uploads/2018/08/global-wild-poliovirus-2013-2018-20180807.pdf
3. Martín J. Vaccine-derived poliovirus from long term excretors and the end game of polio eradication. Biologicals. 2006;34:117–22. http://dx.doi.org/10.1016/j.biologicals.2006.02.005
4. de Silva R, Gunasena S, Ratnayake D, Wickremesinghe GD, Kumarasiri CD, Pushpakumara BA, et al. Prevalence of prolonged and chronic poliovirus excretion among persons with primary immune deficiency disorders in Sri Lanka. Vaccine. 2012;30:7561–5. http://dx.doi.org/10.1016/j.vaccine.2012.10.035
5. Global Polio Eradication Initiative. Polio eradication and endgame strategic plan 2013–2018 [cited 2018 Nov 2]. http://polioeradication.org/wp-content/uploads/2016/07/PEESP_EN_A4.pdf
6. World Health Organization. WHO global action plan to minimize poliovirus facility-associated risk after type-specific eradication of wild polioviruses and sequential cessation of oral polio vaccine use (GAPIII) [cited 2018 Nov 2]. http://www.polioeradication.org/wp-content/uploads/2016/12/GAPIII_2014.pdf
7. Global Polio Eradication Initiative. Global eradication of wild poliovirus type 2 declared [cited 2018 Nov 2]. http://polioeradication.org/news-post/global-eradication-of-wild-poliovirus-type-2-declared
8. Global Certification Commission for the Eradication of Poliomyelitis. Report from the sixteenth GCC meeting, Paris, France, 4–5 July 2017 [cited 2018 Nov 2]. http://polioeradication.org/wp-content/uploads/2017/09/GCC-16th-meeting-report-0405072017.pdf
9. Global Certification Commission for the Eradication of Poliomyelitis. Report from the special meeting of the GCC on poliovirus containment, Geneva, Switzerland, 23–25 October 2017 [cited 2018 Nov 2]. http://polioeradication.org/wp-content/uploads/2018/01/polio-global-certification-commission-report-2017-10-final-en.pdf
10. Rijksinstituut voor Volksgezondheid en Milieu (National Institute for Public Health and the Environment, the Netherlands). Employee vaccine manufacturer infected with polio virus [cited 2018 Nov 2]. https://www.rivm.nl/en/Documents_and_publications/Common_and_Present/Newsmessages/2017/Employee_vaccine_manufacturer_infected_with_polio_virus
11. Bilthoven Biologicals. Employee infected with polio virus [cited 2018 Nov 2]. https://www.bbio.nl/nieuws/2017/april/medewerker-besmet-met-poliovirus
12. Duizer E, Ruijs WL, van der Weijden CP, Timen A. Response to a wild poliovirus type 2 (WPV2)-shedding event following accidental exposure to WPV2, the Netherlands, April 2017. Euro Surveill. 2017;22:30542. http://dx.doi.org/10.2807/1560-7917.ES.2017.22.21.30542
13. World Health Organization. Guidance to minimize risks for facilities collecting, handling, or storing materials potentially infectious for polioviruses. WHO/POLIO/18.03 [cited 2019 Jan 27]. http://polioeradication.org/wp-content/uploads/2018/06/polio-containment-guidance-for-non-poliovirus-facilities-20180614-en.pdf
14. Dowdle WR, Birmingham ME. The biologic principles of poliovirus eradication. J Infect Dis. 1997;175(Suppl 1):S286–92. http://dx.doi.org/10.1093/infdis/175.Supplement_1.S286
15. Van Rooyen CE, Rhodes A. Virus diseases of man. New York: Thomas Nelson & Sons; 1948.
16. Byers K, Harding A. Viral agents of human diseases: biosafety concerns. In: Wooley D, Byers K, editors. Biological safety: principles and practices. 5th edition. Washington: ASM Press; 2017. p. 187–220.
17. Pike RM. Laboratory-associated infections: summary and analysis of 3921 cases. Health Lab Sci. 1976;13:105–14.
18. Pike RM. Laboratory-associated infections: incidence, fatalities, causes, and prevention. Annu Rev Microbiol. 1979;33:41–66. http://dx.doi.org/10.1146/annurev.mi.33.100179.000353
19. Sulkin SE, Pike RM. Viral infections contracted in the laboratory. N Engl J Med. 1949;241:205–13. http://dx.doi.org/10.1056/NEJM194908042410505
20. Pike RM, Sulkin SE, Schulze ML. Continuing importance of laboratory acquired infections. Am J Public Health Nations Health. 1965;55:190–9. http://dx.doi.org/10.2105/AJPH.55.2.190
21. Sabin AB, Wright AM. Acute ascending myelitis following a monkey bite, with the isolation of a virus capable of reproducing the disease. J Exp Med. 1934;59:115–36. http://dx.doi.org/10.1084/jem.59.2.115
22. Leake JP. Poliomyelitis following vaccination against this disease. Cal West Med. 1936;44:141–2.
23. Sabin AB, Ward R. Poliomyelitis in a laboratory worker exposed to the virus. Science. 1941;94:113–4. http://dx.doi.org/10.1126/science.94.2431.113-a
24. Beller K. Laboratory infection with the Lansing virus [in German]. Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene 1949;153:269–73.
25. Burns JS. Poliomyelitis: a case possibly due to intracutaneous inoculation. Can Med Assoc J. 1950;63:498–9.
26. McMath WFT. Accidental inoculation with poliomyelitis virus. Lancet. 1955;268:702–3. http://dx.doi.org/10.1016/S0140-6736(55)91069-2
27. Nathanson N, Langmuir AD. The Cutter incident: poliomyelitis following formaldehyde-inactivated poliovirus vaccination in the United States during the spring of 1955. II. Relationship of poliomyelitis to Cutter vaccine. Am J Hyg. 1963;78:29–60.
28. Offit PA. The Cutter incident, 50 years later. N Engl J Med. 2005;352:1411–2. http://dx.doi.org/10.1056/NEJMp048180
29. Dowdle WR, Wolff C, Sanders R, Lambert S, Best M. Will containment of wild poliovirus in laboratories and inactivated poliovirus vaccine production sites be effective for global certification? Bull World Health Organ. 2004;82:59–62.
30. Mulders MN, Reimerink JHJ, Koopmans MPG, van Loon AM, van der Avoort HG. Genetic analysis of wild-type poliovirus importation into the Netherlands (1979–1995). J Infect Dis. 1997;176:617–24. http://dx.doi.org/10.1086/514081
31. Deshpande JM, Nadkarni SS, Siddiqui ZA. Detection of MEF-1 laboratory reference strain of poliovirus type 2 in children with poliomyelitis in India in 2002 & 2003. Indian J Med Res. 2003;118:217–23.
32. World Health Organization. Wild poliovirus type 2—reference strains isolated in India. Wkly Epidemiol Rec. 2003;78:88 [cited 2018 Oct 10]. https://apps.who.int/iris/bitstream/handle/10665/232120/WER7812_88-88.PDF
33. World Health Organization. Update on actions taken following the isolation of MEF-1 reference poliovirus associated with acute flaccid paralysis cases in India in late 2002 and early 2003. Wkly Epidemiol Rec. 2003;78:277–84 [cited 2018 Oct 10]. https://www.who.int/wer/2003/wer7832
34. Duizer E, Rutjes S, de Roda Husman AM, Schijven J. Risk assessment, risk management and risk-based monitoring following a reported accidental release of poliovirus in Belgium, September to November 2014. Euro Surveill. 2016;21:30169. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.11.30169
35. Duizer E. Laboratory analysis of environmental samples taken following the reported release of live poliovirus. RIVM letter report 2015–0032 [cited 2018 Oct 10]. http://www.rivm.nl/bibliotheek/rapporten/2015-0032.pdf

36. Wenner HA, Paul JR. Fatal infection with poliomyelitis virus in a laboratory technician; isolation of virus from lymph nodes. Am J Med Sci. 1947;213:9–18. http://dx.doi.org/10.1097/00000441-194701000-00002
37. Gear JH, Rodger LM. Studies in poliomyelitis; poliomyelitis in Northern Rhodesia, with special reference to an outbreak occurring on the Roan Antelope Copper Mine, Luanshya, in 1946. S Afr Med J. 1946;20:670–3.
38. Sushmi Dey. Contaminated vaccines put India's "polio free" status at risk. The Times of India. October 1, 2018 [cited 2018 Nov 2]. http://timesofindia.indiatimes.com/articleshow/66021197.cms
39. Upfill-Brown A, Taniuchi M, Platts-Mills JA, Kirkpatrick B, Burgess SL, Oberste MS, et al. Nonspecific effects of oral polio vaccine on diarrheal burden and etiology among Bangladeshi infants. Clin Infect Dis. 2017;65:414–9. http://dx.doi.org/10.1093/cid/cix354
40. Her Majesty's Stationery Office. Report on an investigation into the cause of the 1978 Birmingham smallpox occurrence. London: Her Majesty's Stationery Office; 1980 [cited 2018 Nov 2]. https://biotech.law.lsu.edu/blaw/bt/smallpox/refs/report_1978_london.pdf
41. Bandyopadhyay AS, Garon J, Seib K, Orenstein WA. Polio vaccination: past, present and future. Future Microbiol. 2015;10:791–808. http://dx.doi.org/10.2217/fmb.15.19
42. Sutter RW. Unraveling the mucosal Immunity of inactivated poliovirus vaccine. J Infect Dis. 2018;217:344–6. http://dx.doi.org/10.1093/infdis/jix557
43. McKinlay MA, Collett MS, Hincks JR, Oberste MS, Pallansch MA, Okayasu H, et al. Progress in the development of poliovirus antiviral agents and their essential role in reducing risks that threaten eradication. J Infect Dis. 2014;210(Suppl 1):S447–53. http://dx.doi.org/10.1093/infdis/jiu043
44. World Health Organization. World Health Assembly resolution WHA71.16. Poliomyelitis—containment of polioviruses. In: Seventy-first World Health Assembly, Geneva, Switzerland; May 20–26, 2018 [cited 2018 Nov 2]. http://apps.who.int/gb/ebwha/pdf_files/WHA71/A71_R16-en.pdf

Address for correspondence: Ananda S. Bandyopadhyay, Bill & Melinda Gates Foundation, 1432 Elliott Ave W, Seattle, WA 98119, USA; email: ananda.bandyopadhyay@gatesfoundation.org

Diagnosis of Chagasic Encephalitis by Sequencing of 28S rRNA Gene

Ashrit Multani, Aabed Meer, Darvin S. Smith, Malika N. Kheraj, Edward D. Plowey, Brian G. Blackburn

We report a case of chagasic encephalitis diagnosed by 28S rRNA sequencing. The diagnosis of chagasic encephalitis is challenging, given the broad differential diagnosis for central nervous system lesions in immunocompromised patients and low sensitivity of traditional diagnostics. Sequencing should be part of the diagnostic armamentarium for potential chagasic encephalitis.

Chagasic encephalitis is a rare disease in the United States. We report a case of chagasic encephalitis in an HIV-infected man. This case was diagnosed by sequencing of the parasite 28S rRNA gene.

The Study

The patient was a 31-year-old HIV-infected man who had fevers, headaches, and ataxia for 3 weeks. He had lived in El Salvador until moving to the United States 6 years earlier. His neurologic symptoms persisted, and he was hospitalized after cranial computed tomography (CT) showed a 6-cm, heterogeneous, centrally necrotic mass in the corpus callosum. At admission, he was afebrile, oriented only to self, and had slow movements.

Testing showed a leukocyte count of 3,500 cells/μL, hemoglobin level of 12.4 g/dL, CD4 cell count of 60 cells/μL, HIV viral load of 409,302 copies/mL, and a positive result for serum *Toxoplasma gondii* IgG. Chest radiograph results were unremarkable. Magnetic resonance imaging (MRI) of the brain (Figure 1, panel A) showed an 8.1 × 7.3-cm heterogeneous mass centered within the corpus callosum and parietal–occipital subcortical white matter. Diffusion restriction was identified mostly within peripheral portions of the lesion. Administration of gadolinium showed heterogeneous peripheral enhancement and central necrotic change. Additional foci of abnormal fluid-attenuated inversion recovery signal and enhancement were noted within posterior fossa and supratentorial parenchyma. On the basis of these findings, radiologically favored diagnoses included lymphoma, glioblastoma, or tumefactive multiple sclerosis. Infection was believed less likely, given the absence of prominent central diffusion restriction.

Urgent MRI-directed stereotactic biopsy of the brain was performed. Cytologic smear preparations showed an intraoperative pathological impression of toxoplasmosis on the basis of identification of protozoal organisms. The patient was given trimethoprim/sulfamethoxazole for possible toxoplasmic encephalitis while we awaited procurement of pyrimethamine/sulfadiazine.

Subsequent review of permanent pathologic sections showed necrotizing encephalitis and abundant amastigotes with prominent kinetoplasts in astrocytes and macrophages (Figure 2). Immunostaining for *Toxoplasma* spp. was negative. Sequencing of the internal transcribed spacer 2 and D2 regions of the 28S rRNA gene in paraffin-embedded tissue identified the organism as *Trypanosoma cruzi* (Figure 3, https://wwwnc.cdc.gov/EID/article/25/7/18-0285-F3.htm) (*1*,*2*). A *T. cruzi* IgG test result was subsequently positive; results of peripheral blood smear examination were negative for circulating trypomastigotes.

Trimethoprim/sulfamethoxazole was decreased to prophylactic dosing, and benznidazole (2.5 mg/kg 2×/d) was given after receipt of this drug from the Centers for Disease Control and Prevention (Atlanta, GA, USA) Drug Service 6 days after admission. Treatment with lamivudine, zidovudine, and nevirapine was begun 1 week later.

The patient's course was complicated by leukopenia requiring benznidazole treatment interruption and replacement of zidovudine with abacavir. He completed 60 days of benznidazole therapy over a 3-month period but did not receive secondary prophylaxis for *T. cruzi*. Three months after initial presentation, his HIV viral load was suppressed, his CD4 count had increased sustainably to >200 cells/μL, he was symptomatically and radiologically (Figure 1, panel B) improved, and he returned to work.

Conclusions

T. cruzi, the causative agent of Chagas disease, is endemic to much of Latin America (*3*). Most patients infected with *T. cruzi* remain asymptomatic for years to decades, after which cardiac or gastrointestinal complications develop in some patients. In immunocompromised patients, when

Author affiliations: Stanford University School of Medicine, Stanford, California, USA (A. Multani, A. Meer, E.D. Plowey, B.G. Blackburn); Kaiser Permanente Northern California, Redwood City, California, USA (D.S. Smith, M.N. Kheraj)

DOI: https://doi.org/10.3201/eid2507.180285

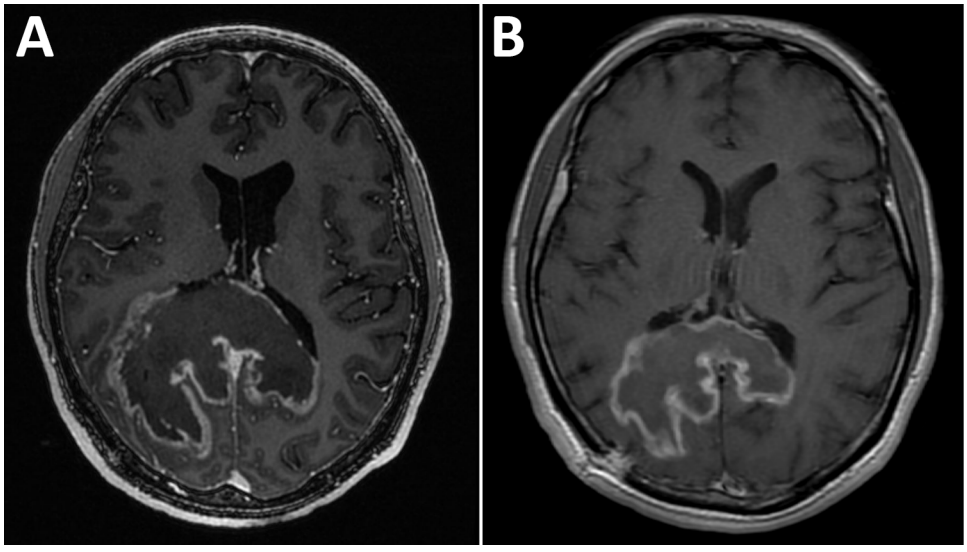


Figure 1. Images obtained during diagnosis of chagasic encephalitis in 31-year-old man in the United States. A) Contrast-enhanced T1-weighted magnetic resonance imaging of the brain showing a cerebral tumor-like chagoma in the axial plane. B) Follow-up contrast-enhanced T1-weighted magnetic resonance imaging obtained ≈8 weeks later showing improvement of the chagoma.

Chagas disease manifests clinically, it occurs most commonly because of reactivation of latent *T. cruzi* infection (*4*–*7*). Although rare in other cohorts, central nervous system (CNS) involvement is the most common manifestation of Chagas disease in patients with AIDS; chagasic encephalitis is also found in patients with other forms of immunosuppression (*4*–*8*). Chagasic encephalitis usually manifests as an abscess but can also manifest as meningoencephalitis; signs and symptoms include headache, focal neurologic deficits, fever, meningismus, seizures, and altered mentation (*4*–*8*).

Differential diagnosis of a CNS mass lesion in a patient with AIDS is broad. Brain MRI usually shows a single, large, tumor-like ring-enhancing lesion in patients with chagasic encephalitis (*4*–*8*). Imaging studies cannot distinguish chagasic encephalitis in this cohort from other common CNS conditions, including toxoplasmosis, lymphoma, progressive multifocal leukoencephalopathy, tuberculoma, cryptococcoma, nocardiosis, and pyogenic abscess (*4*–*9*). For this patient, imaging findings favored malignancy (lack of central diffusion restriction was particularly atypical of an infection). The diagnosis of toxoplasmosis was considered before chagasic encephalitis, given the relative frequency of these diagnoses in the United States, *T. gondii* seropositivity, and the preliminary pathological interpretation. These factors highlight the difficulty of accurate diagnosis of chagasic encephalitis and that more common confounders are likely to be considered before chagasic encephalitis, a rare diagnosis in the United States.

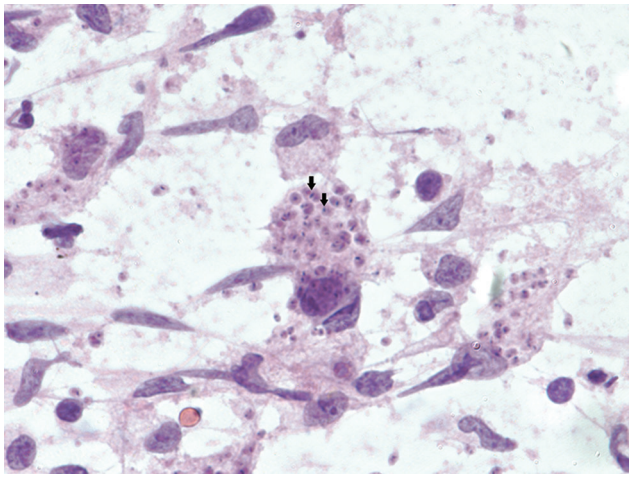


Figure 2. Tissues obtained during diagnosis of chagasic encephalitis in 31-year-old man in the United States. Touch preparation of brain tissue showing necrotizing encephalitis and abundant *Trypanosoma cruzi* amastigotes with prominent kinetoplasts (arrows) in astrocytes and macrophages (hematoxylin and eosin stain, original magnification ×600).

Because most cases of chagasic encephalitis in AIDS patients occur because of reactivation of chronic infection, patients are usually positive for *T. cruzi* IgG at the time of diagnosis; a negative result argues against this possibility (akin to the role of serology in assessing the likelihood of toxoplasmic encephalitis in AIDS patients). Conversely, although a negative result for *T. cruzi* IgG carries a high negative predictive value for chagasic encephalitis, the predictive value of a positive result for IgG is lower because patients might be seropositive from past infection unrelated to their current CNS process. *T. cruzi* parasitemia can also be detected (by microscopic examination of peripheral blood) in some patients with AIDS who have chagasic encephalitis, obviating the need for a brain biopsy, although the predictive value is not high enough to exclude chagasic encephalitis in patients with a negative result.

Conventional PCR performed on blood is not useful for diagnosing *T. cruzi* reactivation because the result can be positive for patients with chronic *T. cruzi* infection without reactivation (although quantitative PCRs performed on serial blood specimens that show increasing parasite copy numbers over

time can indicate reactivation) (*10*,*11*). Given these issues, confirmation of chagasic encephalitis often requires direct microscopic visualization of the organism (*5*–*8*). Unfortunately, *T. cruzi* is difficult to identify microscopically because polymorphism is common, resulting in confusion with other organisms and leading to the need for better confirmatory tests (*12*).

One report described use of molecular testing of cerebrospinal fluid or brain tissue to establish the diagnosis of chagasic encephalitis (*13*). Although some laboratories use real-time PCRs for molecular detection of *T. cruzi* DNA, only this pathogen can be detected in this way, and a negative PCR result for *T. cruzi* in a tissue sample does not reliably exclude the diagnosis because of low sensitivity (*14*). Because multiple infectious diseases in this scenario can be indistinguishable clinically and radiologically, a high index of suspicion and a battery of organism-specific tests are required for comprehensive evaluation. In contrast, because the D2 primers used in sequencing react with multiple protozoa and fungi, it can detect not only *T. cruzi* but also other pathogens that share the D2 subunit, such as *T. gondii*, *Cryptococcus* spp., and *Histoplasma* spp. (and *Leishmania* spp. in other clinical settings) (*2*). The ability to identify one of many potential pathogens with a single test is advantageous for timely institution of appropriate treatment and patient outcome.

As a result of increasing urbanization and globalization, migration continues from areas with high prevalence of *T. cruzi* to nonendemic areas (*3*). Also, increasing use of immunomodulatory therapies, cancer chemotherapeutics, and solid organ and hematopoietic cell transplantation places increasing numbers of patients chronically infected with *T. cruzi* at risk for reactivation. Diagnosing CNS processes in these patients is challenging because of nonspecific clinical and radiologic findings and a broad differential diagnosis, in addition to inherent limitations of traditional diagnostic tests. Chagasic encephalitis is a life-threatening condition that should be included in the differential diagnosis for immunocompromised patients from disease-endemic areas who have cerebral mass lesions or meningoencephalitis. Newer diagnostic methods, such as rRNA gene sequencing, can enable rapid diagnosis and should be considered as part of the diagnostic armamentarium.

Acknowledgments

We thank Niaz Banaei and Indre Budvytiene for providing expertise in developing and performing 28S rRNA sequencing and their contributions to this manuscript.

About the Author

Dr. Multani is an infectious diseases fellow at Stanford University School of Medicine, Stanford, CA. His primary interests are caring for immunocompromised patients and successful diagnosis, treatment, and prevention of their infectious disease–related complications.

References

1. Moncada PA, Budvytiene I, Ho DY, Deresinski SC, Montoya JG, Banaei N. Utility of DNA sequencing for direct identification of invasive fungi from fresh and formalin-fixed specimens. Am J Clin Pathol. 2013;140:203–8. http://dx.doi.org/10.1309/AJCPNSU2SDZD9WPW
2. Gomez CA, Budvytiene I, Zemek AJ, Banaei N. Performance of targeted fungal sequencing for culture-independent diagnosis of invasive fungal disease. Clin Infect Dis. 2017;65:2035–41. http://dx.doi.org/10.1093/cid/cix728
3. Bern C. Chagas' disease. N Engl J Med. 2015;373:456–66. http://dx.doi.org/10.1056/NEJMra1410150
4. Sartori AM, Ibrahim KY, Nunes Westphalen EV, Braz LMA, Oliveira OC Jr, Gakiya E, et al. Manifestations of Chagas disease (American trypanosomiasis) in patients with HIV/AIDS. Ann Trop Med Parasitol. 2007;101:31–50. http://dx.doi.org/10.1179/136485907X154629
5. Cordova E, Boschi A, Ambrosioni J, Cudos C, Corti M. Reactivation of Chagas disease with central nervous system involvement in HIV-infected patients in Argentina, 1992–2007. Int J Infect Dis. 2008;12:587–92. http://dx.doi.org/10.1016/j.ijid.2007.12.007
6. Diazgranados CA, Saavedra-Trujillo CH, Mantilla M, Valderrama SL, Alquichire C, Franco-Paredes C. Chagasic encephalitis in HIV patients: common presentation of an evolving epidemiological and clinical association. Lancet Infect Dis. 2009;9:324–30. http://dx.doi.org/10.1016/S1473-3099(09)70088-X
7. Bern C. Chagas disease in the immunosuppressed host. Curr Opin Infect Dis. 2012;25:450–7. http://dx.doi.org/10.1097/QCO.0b013e328354f179
8. Pagano MA, Segura MJ, Di Lorenzo GA, Garau ML, Molina HA, Cahn P, et al. Cerebral tumor-like American trypanosomiasis in acquired immunodeficiency syndrome. Ann Neurol. 1999;45:403–6. http://dx.doi.org/10.1002/1531-8249(199903)45:3<403::AID-ANA20>3.0.CO;2-K
9. Corti M. AIDS and Chagas' disease. AIDS Patient Care STDS. 2000;14:581–8. http://dx.doi.org/10.1089/10872910050193752
10. Sartori AM, Neto JE, Nunes EV, Braz LM, Caiaffa-Filho HH, Oliveira OC Jr, et al. *Trypanosoma cruzi* parasitemia in chronic Chagas disease: comparison between human immunodeficiency virus (HIV)-positive and HIV-negative patients. J Infect Dis. 2002;186:872–5. http://dx.doi.org/10.1086/342510
11. de Freitas VL, da Silva SC, Sartori AM, Bezerra RC, Westphalen EV, Molina TD, et al. Real-time PCR in HIV/*Trypanosoma cruzi* coinfection with and without Chagas disease reactivation: association with HIV viral load and CD4 level. PLoS Negl Trop Dis. 2011;5:e1277. http://dx.doi.org/10.1371/journal.pntd.0001277
12. Chimelli L. A morphological approach to the diagnosis of protozoal infections of the central nervous system. Patholog Res Int. 2011;2011:e290853. http://dx.doi/10.4061/2011/290853.
13. Rossi Spadafora MS, Céspedes G, Romero S, Fuentes I, Boada-Sucre AA, Cañavate C, et al. *Trypanosoma cruzi* necrotizing meningoencephalitis in a Venezuelan HIV^{+}-AIDS patient: pathological diagnosis confirmed by PCR using formalin fixed- and paraffin-embedded tissues. Anal Cell Pathol (Amst). 2014;2014:124795. http://dx.doi/10.1155/2014/124795.
14. Maldonado C, Albano S, Vettorazzi L, Salomone O, Zlocowski JC, Abiega C, et al. Using polymerase chain reaction in early diagnosis of re-activated *Trypanosoma cruzi* infection after heart transplantation. J Heart Lung Transplant. 2004;23:1345–8. http://dx.doi.org/10.1016/j.healun.2003.09.027

Address for correspondence: Ashrit Multani, Division of Infectious Diseases and Geographic Medicine, Department of Medicine, Stanford University School of Medicine, 300 Pasteur Dr, Lane Bldg L-134, Stanford CA 94305, USA; email: amultani@stanford.edu

Oropharyngeal Gonorrhea in Absence of Urogenital Gonorrhea in Sexual Network of Male and Female Participants, Australia, 2018

Vincent J. Cornelisse, Catriona S. Bradshaw, Eric P.F. Chow, Deborah A. Williamson, Christopher K. Fairley

We describe a sexual network consisting of 1 nonbinary-gendered participant and 2 male and 4 female participants in Australia, 2018. Six of 7 participants had oropharyngeal gonorrhea in the absence of urogenital gonorrhea. This observation supports a new paradigm of gonorrhea transmission in which oropharyngeal gonorrhea can be transmitted through tongue kissing.

Oropharyngeal gonorrhea is considered to be acquired primarily from an infected penis during oral sex (*1*). However, male urethral gonorrhea is usually symptomatic (*2–4*), prompting men to seek treatment soon after symptoms appear (*5*), resulting in short duration of infectivity and low point prevalence. Thus, infected penises are unlikely to be the source to explain the observed high prevalence of oropharyngeal gonorrhea (*6*,*7*). To address this epidemiologic conundrum, we previously described a paradigm of gonorrhea transmission in which oropharyngeal gonorrhea can be acquired from a partner's oropharynx during tongue kissing (*8*), as originally proposed in the 1970s and 1980s (*9*,*10*). However, investigating whether kissing can lead to gonorrhea transmission has been difficult because kissing often occurs concurrently with other sexual acts (*11*). We describe a sexual network of 1 nonbinary, 2 male, and 4 female participants who were tested for gonorrhea at genital and oropharyngeal sites in early 2018 to explore gonorrhea transmission dynamics.

The Study

Ethics approval was obtained from the Alfred Hospital Ethics Committee, Melbourne, Australia (project no. 462/18). The index case was identified during routine patient care at Melbourne Sexual Health Centre (Carlton, Victoria, Australia). After patients consented to take part in our study, they contacted their sexual partners, who then each consented and were interviewed. Participants independently provided accounts of their sexual activity to permit inter-participant verification. We describe the timing of events with respect to day 0, the day of a music festival during which most sexual activity occurred.

We tested for *Neisseria gonorrhoeae* infection by nucleic acid amplification with the Aptima Combo 2 assay and confirmed by the Aptima GC assay (Gen-Probe, https://www.hologic.com). We performed whole-genome sequencing and bioinformatic analyses on available samples (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1561-App1.pdf).

Recalled accounts of sexual activity were consistent between participants. No participant reported symptoms of gonorrhea, and none used antimicrobial drugs during the relevant period.

On day 10, the index patient (participant 1 [P1], nonbinary gender, assigned female sex at birth) sought screening for sexually transmitted infections at Melbourne Sexual Health Centre. Though asymptomatic, P1 tested positive for oropharyngeal gonorrhea and negative for urogenital gonorrhea. P1's most recent negative test for gonorrhea was 5 months prior. Between the previous negative test and day 0, P1 had sex with 4 men besides their primary male sexual partner (P2) (Figure). These 4 other male sexual partners subsequently tested negative for gonorrhea; however, we were not able to confirm what anatomic sites were tested. On day 0, P1 had tongue kissed P3 (female) and had tongue kissed and had reciprocal orogenital sex (without condoms) and penovaginal sex (without condoms) with P2.

On day 16, P3 tested positive for oropharyngeal gonorrhea and negative for urogenital gonorrhea. She had not been tested for gonorrhea in the past 4 years because, until a recent break-up, she had been in a long-term monogamous relationship. She had no other sexual contacts (including kissing) with other men or women during this time. P3's expartner was later contacted and tested and was negative for oropharyngeal and urogenital gonorrhea.

Author affiliations: Melbourne Sexual Health Centre, Carlton, Victoria, Australia (V.J. Cornelisse, C.S. Bradshaw, E.P.F. Chow, C.K. Fairley); Monash University, Melbourne, Victoria, Australia (V.J. Cornelisse, C.S. Bradshaw, E.P.F. Chow, C.K. Fairley); The University of Melbourne, Parkville, Victoria, Australia (D.A. Williamson)

DOI: https://doi.org/10.3201/eid2507.181561

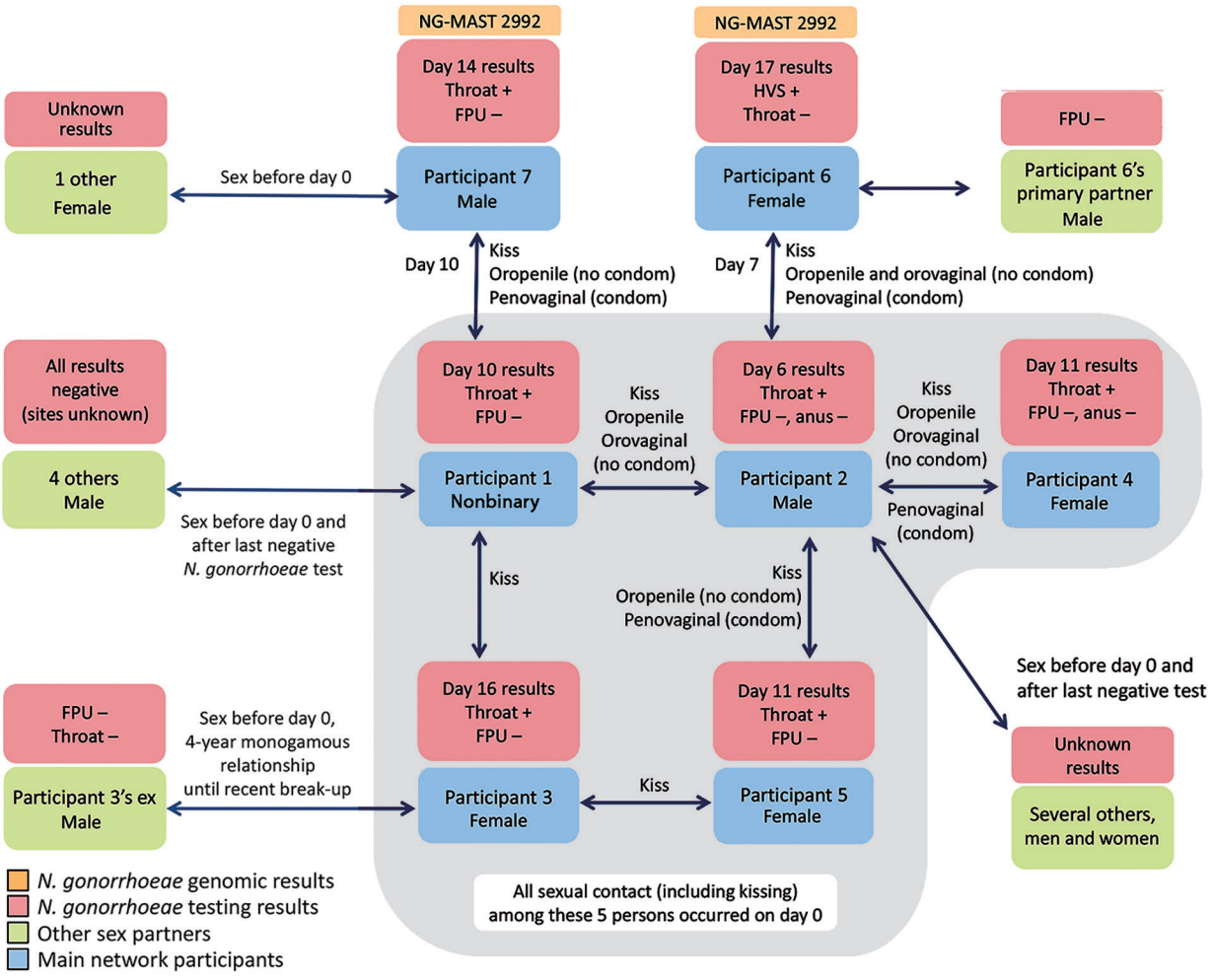


Figure. *Neisseria gonorrhoeae* diagnoses among participants of a sexual network, Australia, 2018. FPU, first-pass urine; HVS, high vaginal swab; NG-MAST, *N. gonorrhoeae* multiantigen sequence type.

On day 6, before P1 underwent testing, P2 sought routine asymptomatic screening for sexually transmitted infections at Melbourne Sexual Health Centre and tested positive for oropharyngeal gonorrhea and negative for urogenital and anal gonorrhea. P2's most recent test was 4 months earlier, when he tested negative for oropharyngeal, anal, and urogenital gonorrhea. P2 had sex with several men and women besides his primary partner between his last test and day 0; test results are not known for many of these sex partners.

On day 0, P2 had sex with P4 (female), consisting of tongue kissing, reciprocal orogenital sex without condoms, and penovaginal sex with condoms. On day 11, P4 tested positive for oropharyngeal gonorrhea and negative for urogenital and anal gonorrhea. Eleven days before day 0, P4 had tested negative for oropharyngeal and urogenital gonorrhea. P2 and P4 had had sex weekly for 5 months before day 0.

On day 0, P2 also had sex with P5 (female), consisting of tongue kissing, oropenile sex without condoms, and penovaginal sex with condoms. On day 11, P5 tested positive for oropharyngeal and negative for urogenital gonorrhea. P5's previous gonorrhea test (negative results) was 1–2 years earlier. P5 also tongue kissed P3 but had no other sexual contact with her. P5 had no other sexual contacts, including tongue kissing, the 3 months before day 0.

On day 7, P2 had sex with P6 (female), consisting of tongue kissing, reciprocal orogenital sex without condoms, and penovaginal sex with condoms. On day 17, P6 tested positive for urogenital gonorrhea but negative for oropharyngeal gonorrhea. P6 had tested negative for urogenital gonorrhea ≈3 weeks before her contact with P2. The only other person (male) P6 had sex with during the time between her negative and positive test results subsequently tested negative for urogenital gonorrhea.

On day 10, P1 had sex with P7 (male), consisting of penovaginal sex with condoms, tongue kissing, and oropenile sex without condoms. P1 and P7 had sex weekly for 2 months before day 0. On day 14, P7 tested positive for oropharyngeal and negative for urogenital gonorrhea. His previous test was 4 years prior. P7 had 1 other sexual partner (female) in the months before day 0, and she was unable to be contacted.

Two *N. gonorrhoeae* isolates (from P6 and P7) were available for whole-genome sequencing. Both were *N. gonorrhoeae* multiantigen sequence type 2992, and no single-nucleotide polymorphism differences were found between the isolates (BioProject no. PRJNA449254).

This report describes a sexual network consisting of 1 nonbinary participant and 2 male and 4 female participants, of which 6 participants had oropharyngeal gonorrhea in the absence of urogenital gonorrhea. Although it is possible that some of the oropharyngeal infections were caused by undisclosed sexual contacts or inaccurate testing information, an additional explanation is that gonorrhea was transmitted by tongue kissing.

Two gonorrhea samples were available for genomic analysis and were highly related genomically. These participants were separated in this network by 2 other participants, corroborating the epidemiologic observation that these infections were the result of within-network transmission rather than a result of sexual contact with persons external to the network. Also, given the low prevalence of gonorrhea among the general population in Melbourne (https://kirby.unsw.edu.au/sites/default/files/kirby/report/SERP_Annual-Surveillance-Report-2017_compressed.pdf), the probability that all participants acquired gonorrhea from external partners is low.

No men in this network had urethral gonorrhea, suggesting that the oropharynx-to-penis route has a lower transmission probability than tongue kissing. This finding is consistent with an existing mathematical model that included transmission by kissing, which calculated a per-act transmission of 1% for oral sex and 17% for kissing (*12*). Few observational studies have examined transmission by kissing, but 1 study of male couples found 26% concordance of oropharyngeal gonorrhea between partners (*13*).

Because this report describes sexual contacts that occurred at a music festival, participants' recall might have been affected by alcohol or drugs. Also, awareness of being part of a study involving sexual partners could have affected participants' willingness to disclose information. However, recall was consistent between participants, suggesting that their recall was accurate.

Conclusions

Accumulating evidence suggests that tongue kissing might be a common mode of gonorrhea transmission (*12–14*). The observation that expectorated saliva from persons with oropharyngeal gonorrhea contains high loads of *N. gonorrhoeae* DNA suggests a plausible mechanism for transmission (*15*). The sexual network described here adds to this evidence. We also highlight the need for routine screening for oropharyngeal gonorrhea for all persons with multiple sexual partners.

Acknowledgments

We acknowledge the extraordinary generosity of the participants included in this report for giving permission and taking the time to share this sensitive information.

V.J.C. was supported by a scholarship from the Research Training Scheme of the Australian Government's Department of Education and Training. E.P.F.C. and D.A.W. are supported by Early Career Fellowships from the Australian National Health (no. 1091226) and Medical Research Council (no. 1123854).

About the Author

Dr. Cornelisse is a sexual health physician at Melbourne Sexual Health Centre, Carlton, Victoria, Australia. His main research interests are the epidemiology and prevention of gonorrhea and HIV prevention.

References

1. Wiesner PJ, Tronca E, Bonin P, Pedersen AH, Holmes KK. Clinical spectrum of pharyngeal gonococcal infection. N Engl J Med. 1973; 288:181–5. http://dx.doi.org/10.1056/NEJM197301252880404
2. Harrison WO, Hooper RR, Wiesner PJ, Campbell AF, Karney WW, Reynolds GH, et al. A trial of minocycline given after exposure to prevent gonorrhea. N Engl J Med. 1979;300:1074–8. http://dx.doi.org/10.1056/NEJM197905103001903
3. Ong JJ, Fethers K, Howden BP, Fairley CK, Chow EPF, Williamson DA, et al. Asymptomatic and symptomatic urethral gonorrhoea in men who have sex with men attending a sexual health service. Clin Microbiol Infect. 2017;23:555–9. http://dx.doi.org/10.1016/j.cmi.2017.02.020
4. Ryder N, Lockart IG, Bourne C. Is screening asymptomatic men who have sex with men for urethral gonorrhoea worthwhile? Sex Health. 2010;7:90–1. http://dx.doi.org/10.1071/SH09100
5. Fairley CK, Chow EP, Hocking JS. Early presentation of symptomatic individuals is critical in controlling sexually transmissible infections. Sex Health. 2015;12:181–2. http://dx.doi.org/10.1071/SH15036
6. Cornelisse VJ, Chow EP, Huffam S, Fairley CK, Bissessor M, De Petra V, et al. Increased detection of pharyngeal and rectal gonorrhea in men who have sex with men after transition from culture to nucleic acid amplification testing. Sex Transm Dis. 2017;44:114–7. http://dx.doi.org/10.1097/OLQ.0000000000000553
7. Barbee LA, Dombrowski JC, Kerani R, Golden MR. Effect of nucleic acid amplification testing on detection of extragenital gonorrhea and chlamydial infections in men who have sex with men sexually transmitted disease clinic patients. Sex Transm Dis. 2014;41:168–72. http://dx.doi.org/10.1097/OLQ.0000000000000093
8. Fairley CK, Hocking JS, Zhang L, Chow EP. Frequent transmission of gonorrhea in men who have sex with men. Emerg Infect Dis. 2017;23:102–4. http://dx.doi.org/10.3201/eid2301.161205

9. Hutt DM, Judson FN. Epidemiology and treatment of oropharyngeal gonorrhea. Ann Intern Med. 1986;104:655–8. http://dx.doi.org/10.7326/0003-4819-104-5-655
10. Willmott FE. Transfer of gonococcal pharyngitis by kissing? Br J Vener Dis. 1974;50:317–8.
11. Cornelisse VJ, Walker S, Phillips T, Hocking JS, Bradshaw CS, Lewis DA, et al. Risk factors for oropharyngeal gonorrhoea in men who have sex with men: an age-matched case-control study. Sex Transm Infect. 2018;94:359–64. http://dx.doi.org/10.1136/sextrans-2017-053381
12. Zhang L, Regan DG, Chow EPF, Gambhir M, Cornelisse V, Grulich A, et al. *Neisseria gonorrhoeae* transmission among men who have sex with men: an anatomical site-specific mathematical model evaluating the potential preventive impact of mouthwash. Sex Transm Dis. 2017;44:586–92. http://dx.doi.org/10.1097/OLQ.0000000000000661
13. Cornelisse VJ, Williamson D, Zhang L, Chen MY, Bradshaw C, Hocking JS, et al. Evidence for a new paradigm of gonorrhoea transmission: cross-sectional analysis of *Neisseria gonorrhoeae* infections by anatomical site in both partners in 60 male couples. Sex Transm Infect. 2019;sextrans-2018-053803. http://dx.doi.org/10.1136/sextrans-2018-053803
14. Cornelisse VJ, Zhang L, Law M, Chen MY, Bradshaw CS, Bellhouse C, et al. Concordance of gonorrhoea of the rectum, pharynx, and urethra in same-sex male partnerships attending a sexual health service in Melbourne, Australia. BMC Infect Dis. 2018;18:95. http://dx.doi.org/10.1186/s12879-018-3003-2
15. Chow EP, Tabrizi SN, Phillips S, Lee D, Bradshaw CS, Chen MY, et al. *Neisseria gonorrhoeae* bacterial DNA load in the pharynges and saliva of men who have sex with men. J Clin Microbiol. 2016;54:2485–90. http://dx.doi.org/10.1128/JCM.01186-16

Address for correspondence: Vincent J. Cornelisse, Melbourne Sexual Health Centre, Alfred Health, 580 Swanston St, Carlton, VIC 3053, Australia; email: vcornelisse@mshc.org.au

Salmonella enterica I 4,[5],12:i:- Associated with Lesions Typical of Swine Enteric Salmonellosis

Bailey L. Arruda, Eric R. Burrough, Kent J. Schwartz

Salmonella enterica serotype I 4,[5],12:i:- has been increasingly isolated from swine. However, its pathogenic potential is not well characterized. Analysis of swine cases confirmed a strong positive association between isolation of I 4,[5],12:i:- and lesions of enteric salmonellosis and suggested a similar pathogenic potential as that for *Salmonella* Typhimurium.

Over the past decade, *Salmonella enterica* serotype I 4,[5],12:i:- has emerged as a major public health threat in Europe (*1*) and the United States (*2*). As a monophasic variant of *Salmonella* Typhimurium, *Salmonella* I 4,[5],12:i:- was rarely identified before the mid-1990s (*3*). However, it has now been detected in cattle (*4*), poultry (*5*), and swine (*4–6*), and several human disease outbreaks associated with contaminated pork products have occurred (*7–10*).

Salmonellosis is also a major disease concern in swine. *Salmonella* Typhimurium has been considered the most common cause of enteric salmonellosis in swine (*11*). Recent reports from 2 of the largest veterinary diagnostic laboratories in the United States showed that there was a noted increase in the percentage of isolates of *Salmonella* I 4,[5],12:i:- (*4,6*).

Despite the apparent increase in the isolation of *Salmonella* I 4,[5],12:i:- from swine and pork products, there is currently limited information regarding the pathogenicity of this serotype in swine. Accordingly, this study assessed the pathogenic potential of *Salmonella* I 4,[5],12:i:- through the evaluation of microscopic intestinal lesions in swine enteric cases from which *Salmonella* I 4,[5],12:i:- was isolated compared with similar cases from which *Salmonella* I 4,[5],12:i:- was not isolated.

The Study

The Iowa State University–Veterinary Diagnostic Laboratory (ISU-VDL) is a National Animal Health Laboratory Network–accredited laboratory that receives >75,000 case submissions annually, of which most are derived from swine production systems located throughout the United States. Histopathologic analysis is performed by veterinary diagnostic pathologists on ≈10,000 cases from swine per year. The ISU-VDL laboratory information management system provided the initial dataset for this analysis.

To determine whether isolation of *Salmonella* I 4,[5],12:i:- from samples submitted from pigs was associated with microscopic lesions consistent with enteric salmonellosis, we compared cases from which *Salmonella* I 4,[5],12:i:- was isolated with cases from which neither *Salmonella* I 4,[5],12:i:- nor *Salmonella* Typhimurium were isolated; these samples were collected during July 2016–December 2017. We also reviewed cases from which *Salmonella* Typhimurium or 1 of 3 *Salmonella* serogroup B serovars (*Salmonella* Derby, *Salmonella* Agona, and *Salmonella* Heidelberg) were isolated to determine the potential comparative pathogenicity of Salmonella I 4,[5],12:i:-. All of these cases met the following qualifying criteria: animals were 3–13 weeks of age, a *Salmonella* serovar as outlined above was isolated by direct culture performed on enteric tissues, and histopathologic analysis was performed on the large intestine. To determine the association between the presence of *Salmonella* I 4,[5],12:i:- and lesions consistent with enteric salmonellosis, we also reviewed 40 additional cases that met the previously stated inclusion criteria but from which neither *Salmonella* I 4,[5],12:i:- nor *Salmonella* Typhimurium were isolated; we randomly selected these cases from an Excel (Microsoft, https://www.microsoft.com) data file by using the RAND() function.

Enteric samples submitted for isolation of *Salmonella* were routinely processed and reported (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1453-App1.pdf). Microscopic lesions deemed compatible with salmonellosis in sections of the large intestine included erosion; ulceration; neutrophilic infiltration; crypt ectasia, crypt elongation, or both with associated neutrophilic exudation; goblet cell loss; luminal accumulation of neutrophils and fibrin; and submucosal accumulation of lymphocytes and macrophages. We used JMP Pro 14 (SAS Institute, https://www.sas.com) to perform all analyses. We used the Pearson χ^2 test and odds

Author affiliation: Iowa State University, Ames, Iowa, USA

DOI: https://doi.org/10.3201/eid2507.181453

ratios to determine the association between isolation of *Salmonella* I 4,[5],12:i:- and pathologic diagnosis of enteric salmonellosis. We considered a p value ≤0.05 significant.

We isolated *Salmonella* I 4,[5],12:i:- from 138 porcine cases that met all of the qualifying criteria. We isolated *Salmonella* Typhimurium from 18 cases, and other potentially lesser pathogenic *Salmonella* serogroup B serovars, including *Salmonella* Derby, Agona, and Heidelberg, from 35 cases.

A review of case data for clinical submissions to the ISU-VDL confirmed a statistically significant positive association between histologic lesions consistent with enteric salmonellosis and isolation of *Salmonella* I 4,[5],12:i:- (odds ratio 10.53, 95% CI 4.45–24.88; p<0.0001) (Table). We confirmed compatible histologic lesions of salmonellosis (Figure) for 100 (72%) of 138 cases from which *Salmonella* I 4,[5],12:i:- were isolated (Appendix Table 1). Histologic lesions consistent with enteric salmonellosis from which neither *Salmonella* I 4,[5],12:i:- nor *Salmonella* Typhimurium were isolated were observed for 8 (20%) of 40 cases (Appendix Table 2).

Salmonella was isolated from 6 of the 40 cases, 3 of which had lesions consistent with enteric salmonellosis. We confirmed compatible histologic lesions of salmonellosis in 17 (94%) of 18 cases in which *Salmonella* Typhimurium was isolated and 11 (31%) of 35 cases in which another serogroup B *Salmonella* was isolated. Other agents of enteric disease that were concurrently detected in some cases included rotaviruses, coronaviruses, coccidians, and hemolytic *Escherichia coli*. However, none of these agents caused lesions consistent with those used to define salmonellosis in this report (Appendix Tables 1, 2).

Conclusions

During an 18-month period and using the same qualifying criteria for cases, we identified *Salmonella* I 4,[5],12:i:- from 138 swine cases. However, *Salmonella* Typhimurium was isolated from only 18 cases and another serogroup B *Salmonella* as specified above was isolated from only 35 cases. This finding represents nearly an 8-fold increase in isolation of *Salmonella* I 4,[5],12:i:- compared with *Salmonella* Typhimurium and is in concordance with findings of others who have identified an increase in isolation of *Salmonella* I 4,[5],12:i:- from swine samples submitted to veterinary diagnostic laboratories (*4,6*).

A major aspect of *Salmonella* epidemiology is the variation in prevalence of serotypes or phage types over time in human and animal populations. The catalysts of such changes remain elusive (*3*). Isolation of *Salmonella* I 4,[5],12:i:- was rarely reported before 1993 (*3*), but this serotype has now become predominant in human clinical cases and has been isolated from food products, including pork, on different continents (*3*).

Table. Diagnostic cases with and without colitis in swine and isolation of *Salmonella enterica* I 4,[5],12:i:-*

	Colitis lesion	
I 4,[5],12:i:- isolated	No	Yes
No	32	8
Yes	38	100

*Odds ratio 10.5 (95% CI 4.45–24.88); p<0.0001 by Pearson χ^2 test.

Although increased isolation of *Salmonella* I 4,[5],12:i:- from swine samples has been documented, the pathogenic potential of this serovar in pigs had not been reported. In this study, we demonstrate a strong positive association between histologic lesions consistent with enteric salmonellosis and isolation of *Salmonella* I 4,[5],12:i:-. In most cases from which *Salmonella* I 4,[5],12:i:- or *Salmonella* Typhimurium were isolated, the severity of histologic lesions was similar. However, the percentage of cases in which histologic lesions consistent with enteric salmonellosis were present was lower for cases from which *Salmonella* I 4,[5],12:i:- (72%) was isolated than for cases from which *Salmonella* Typhimurium (94%) was isolated. Based on these data, we believe that *Salmonella* I 4,[5],12:i:- is a likely cause of enteric salmonellosis that has a similar or perhaps slightly lower pathogenic potential in swine than *Salmonella* Typhimurium. Pathogenicity studies are needed to further characterize the pathogenic potential and

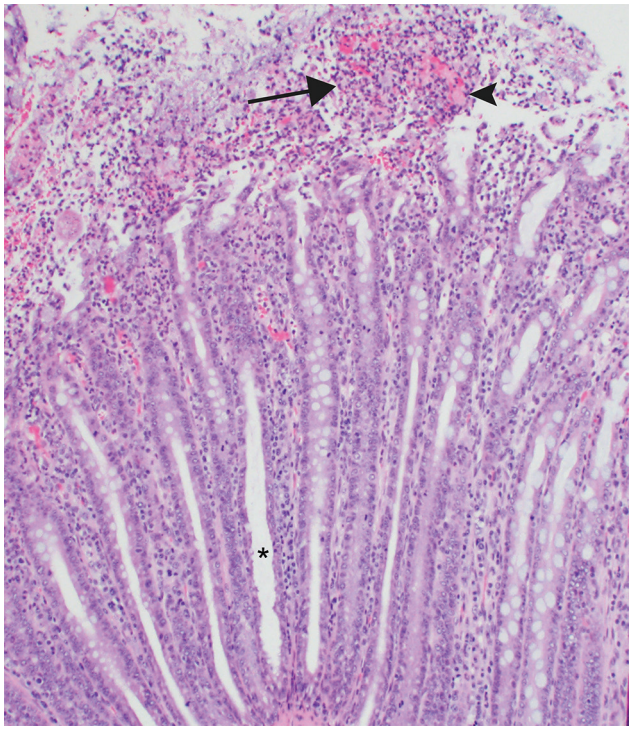


Figure. Section of large intestine from a pig infected with *Salmonella enterica* subsp. *enterica* serotype I 4,[5],12:i:-. Asterisk indicates crypt elongation and goblet cell loss, arrow indicates abundant degenerate neutrophils in the lumen, and arrowhead indicates abundant fibrin in the lumen. Hematoxylin and eosin stain; original magnification ×100.

fitness of *Salmonella* I 4,[5],12:i:- compared with that of *Salmonella* Typhimurium in swine.

We suspect that *Salmonella* I 4,[5],12:i:- has evolutionary advantages that have resulted in its predominance as one of the most common *Salmonella* serotypes responsible for swine enteric salmonellosis. Accordingly, it is essential to determine the putative attributes that facilitate its rapid spread and ecologic success. Specifically, antimicrobial drug resistance genes and genes that encode resistance to heavy metal micronutrients should be evaluated, given their current and common use in US swine production.

About the Author

Dr. Arruda is an assistant professor and diagnostic pathologist at the Iowa State University Veterinary Diagnostic Laboratory, Ames, IA. Her primary research interest is infectious diseases of swine.

References

1. European Food Safety Authority. The European Union summary report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2012. EFSA J. 2014;12:3547 [cited 2019 Mar 11]. https://www.efsa.europa.eu/en/efsajournal/pub/3547
2. Centers for Disease Control and Prevention. Outbreak of multidrug-resistant *Salmonella* I 4,[5],12:i:- infections linked to pork; 2015 [cited 2018 Jul 30]. http://www.cdc.gov/salmonella/pork-08-15/index.html
3. Switt AI, Soyer Y, Warnick LD, Wiedmann M. Emergence, distribution, and molecular and phenotypic characteristics of *Salmonella enterica* serotype 4,5,12:i-. Foodborne Pathog Dis. 2009;6:407–15. http://dx.doi.org/10.1089/fpd.2008.0213
4. Hong S, Rovira A, Davies P, Ahlstrom C, Muellner P, Rendahl A, et al. Serotypes and antimicrobial resistance in *Salmonella enterica* recovered from clinical samples from cattle and swine in Minnesota, 2006 to 2015. PLoS One. 2016;11:e0168016. http://dx.doi.org/10.1371/journal.pone.0168016
5. Centers for Disease Control and Prevention. National Antimicrobial Resistance Monitoring System: Retail Meat Interim Report; 2016 [cited 2018 Jul 30]. https://www.fda.gov/downloads/AnimalVeterinary/SafetyHealth/AntimicrobialResistance/NationalAntimicrobialResistanceMonitoringSystem/UCM498134.pdf
6. Yuan C, Krull A, Wang C, Erdman M, Fedorka-Cray PJ, Logue CM, et al. Changes in the prevalence of *Salmonella* serovars associated swine production and correlations of avian, bovine and swine-associated serovars with human-associated serovars in the United States (1997–2015). Zoonoses Public Health. 2018;65:648–61. http://dx.doi.org/10.1111/zph.12473
7. Bone A, Noel H, Le Hello S, Pihier N, Danan C, Raguenaud ME, et al. Nationwide outbreak of *Salmonella enterica* serotype 4,12:i:0- infections in France, linked to dried pork sausage, March–May 2010. Euro Surveill. 2010;15:19592.
8. Mossong J, Marques P, Ragimbeau C, Huberty-Krau P, Losch S, Meyer G, et al. Outbreaks of monophasic *Salmonella enterica* serovar 4,[5],12:i:- in Luxembourg, 2006. Euro Surveill. 2007;12:E11–2. http://dx.doi.org/10.2807/esm.12.06.00719-en
9. Hauser E, Tietze E, Helmuth R, Junker E, Blank K, Prager R, et al. Pork contaminated with *Salmonella enterica* serovar 4,[5],12:i:-, an emerging health risk for humans. Appl Environ Microbiol. 2010;76:4601–10. http://dx.doi.org/10.1128/AEM.02991-09
10. Imanishi M, Anderson TC, Routh J, Brown C, Conidi G, Glenn L, et al. Salmonellosis and meat purchased at live-bird and animal-slaughter markets, United States, 2007–2012. Emerg Infect Dis. 2014;20:167–9. http://dx.doi.org/10.3201/eid2001.131179
11. Carlson SA, Barnhill AE, Griffith RW. Salmonellosis. In: Zimmerman JJ, Karriker LA, Ramirez A, Schwartz KJ, Stevenson GW, editors. Disease of swine. Oxford: John Wiley & Sons, Inc; 2012. p. 821–31.

Address for correspondence: Bailey L. Arruda, Department of Veterinary Diagnostic and Production Animal Medicine, Iowa State University, 1850 Christensen Dr, Ames, IA 50011, USA; email: wilberts@iastate.edu

Microbiome and Antimicrobial Resistance Gene Dynamics in International Travelers

Charles Langelier, Michael Graves,[1] Katrina Kalantar,[1] Saharai Caldera, Robert Durrant, Mark Fisher, Richard Backman, Windy Tanner, Joseph L. DeRisi, Daniel T. Leung

We used metagenomic next-generation sequencing to longitudinally assess the gut microbiota and antimicrobial resistomes of international travelers to clarify global exchange of resistant organisms. Travel resulted in an increase in antimicrobial resistance genes and a greater proportion of *Escherichia* species within gut microbial communities without impacting diversity.

International travel is a known contributor to the emergence of organisms with antimicrobial resistance (AMR) (*1*–*4*). Colonization with resistant microbes acquired during travel can persist asymptomatically for extended periods and result in transmission into the environment and susceptible populations (*5*). The mechanisms underlying acquisition of AMR bacteria during travel are incompletely understood, although changes in the intestinal microbiota are hypothesized to play a role (*6*). To clarify AMR exchange during global travel, we used metagenomic next-generation sequencing (mNGS) to assess gut microbiota composition and the antimicrobial resistome.

The Study

During March 2016–2018, we recruited adults with planned travel to Asia or Africa for healthcare-related work. Participants introduced 1 tablespoon of stool into vials with either RNAprotect (QIAGEN, http://www.qiagen.com) or Cary-Blair (CB) media and then submitted samples and surveys pretravel, posttravel, 30 days posttravel, and 6 months posttravel. Upon receipt, we stored RNAprotect samples at −80°C and CB samples at 4°C until inoculation onto chromogenic agar plates selective for extended-spectrum β-lactamase (ESBL)–producing bacteria (CHROMagar ESBL) and incubation overnight at 37°C. We then inoculated single colonies into LB broth and incubated overnight at 37°C. If multiple morphotypes were identified, we conducted separate subcultures. We performed speciation by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

DNA and RNA extracted using the QIAGEN Powerfecal kit underwent metagenomic sequencing as previously described (*7*). Raw data are available publicly (Bioproject PRJNA509512). We detected enteric microbiota using a recently developed bioinformatics pipeline (*7*). We aggregated microbial alignments at the genus level before downstream analyses. To control for background environmental and reagent contaminants, we incorporated no-template water control samples alongside extracted nucleic acid and carried them forward throughout library preparation and sequencing. We then performed direct subtraction of total reads aligning to each microbial genus present in controls from each study sample before downstream analyses.

We used the SRST2 computational tool and ARGannot_r2 database (https://github.com/katholt/srst2) to identify AMR genes with allele coverage of >20% (*8*). Although a precise definition of ESBL has not been established, we used a working definition of Ambler class A–D β-lactamases with known or predicted ability to confer resistance to first- through third-generation cephalosporins (*9,10*). We required detection of chromosomally encoded Ambler class C β-lactamases (i.e., AmpC) by both DNA-Seq and RNA-Seq to capture actively expressed genes.

Nine of 10 participants were culture-positive for ESBL-producing *Escherichia coli* (ESBL-PE) upon return, including 8 persons who traveled to Nepal and 1 who went to Nigeria. One traveler was found to be colonized before departure (traveler 3 [T3]); 3 travelers had persistent carriage at the 30-day visit (T2, T3, and T5) and 2 at 6 months (T3 and T5) (Table 1). Although 4 participants experienced diarrheal symptoms during travel, only 1 (T5) had persistent diarrheal symptoms at 6 months. Diarrheal symptoms were not associated with persistent ESBL-PE colonization at any point, and no travelers reported antibiotic use or receipt of healthcare in an inpatient setting while traveling. All travelers were exposed to inpatient healthcare facilities, and 3 travelers reported street food consumption.

Author affiliations: University of California, San Francisco, California, USA (C. Langelier, K. Kalantar, S. Caldera, J.L. DeRisi); Chan Zuckerberg Biohub, San Francisco (C. Langelier, S. Caldera, J.L. DeRisi); University of Utah School of Medicine, Salt Lake City, Utah, USA (M. Graves, R. Durrant, M. Fisher, R. Backman, W. Tanner, D.T. Leung); ARUP Laboratories, Salt Lake City (R. Durrant, M. Fisher)

DOI: https://doi.org/10.3201/eid2507.181492

[1]These authors contributed equally to this article.

Table 1. Selected characteristics of 10 travelers and assessment of ESBL-producing *Enterobacteraciae**

Traveler	Destination	Duration, d	Diarrhea	Pretravel	Posttravel	30 d posttravel	6 mo posttravel
T1	Nepal	30	N	–	*AmpC*	*OXA-209*	*OXA-209*
T2	Nepal	30	N	–	*AmpC, CTX-M1*	*AmpC*	–
T3	Nepal	30	N	*OXA-209*	*AmpC, CTX-M1, OXA-209*	*OXA-209*	*OXA-209*
T4	Nepal	16	N	–	*AmpC*	–	NR
T5	Nepal	30	Y	–	*AmpC, SHV-12*	*AmpC*	*AmpC, CTX-M-1*
T6	Nepal	15	Y	–	*AmpC, CTX-M-1*	–	–
T7	Nepal	18	N	–	*AmpC, CTX-M-1*	NR	NR
T8	Uganda	14	N	–	– (*AmpC*)†	–	–
T9	Nigeria	60	Y	–	*AmpC*	–	–
T10	Nepal	30	Y	–	*AmpC, CTX-M-1*	–	NR

*Participants submitted samples pretravel (within 1 week before departure), posttravel (within 1 week after return), 30 days posttravel, and 6 months posttravel. ESBL, extended-spectrum β-lactamase; N, no; NR, no sample received; Y, yes; –, ESBL-negative by culture.
†T8 was the only participant who was phenotypically ESBL-negative after travel.

We first examined changes in gut microbiome α diversity after international travel (Figure, panel A) and found that the Shannon diversity index (SDI) did not significantly change upon return ($p = 0.674$ by Wilcoxon rank-sum test) or at day 30 posttravel ($p = 0.250$ by Wilcoxon rank-sum test). We then assessed whether microbial community composition differed across all participants at their posttravel versus pretravel visit but found no difference (Bray Curtis Index $p = 0.23$ by permutational multivariate analysis of variance). Although global composition and diversity of gut microbiota did not significantly change after travel, we observed significant differences in the abundance of discrete genera. Across all participants, *Enterobacteriaceae* demonstrated the greatest fold change in abundance posttravel; *Escherichia* was the genus most differentially increased ($p<0.001$ by Wilcoxon rank-sum test) (Figure, panel B; Appendix Figures 1, 2, https://wwwnc.cdc.gov/EID/article/25/7/18-1492-App1.pdf).

Analysis of the antimicrobial resistome revealed an increase in AMR genes and transcripts after return from travel ($p<0.01$ for DNA and $p = 0.03$ for RNA sequencing, both by Wilcoxon rank-sum test) (Table 2; Figure, panels C–D; Appendix Figure 3). ESBL-encoding genes, *AmpC*-encoding genes, or both were identified in 100%

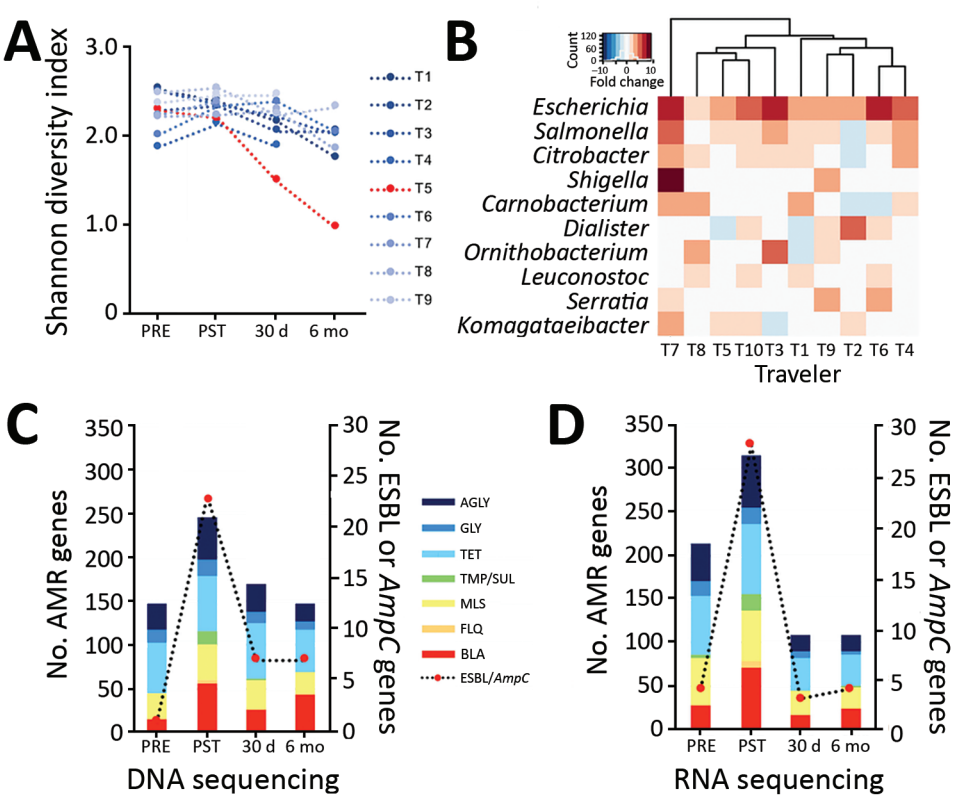


Figure. Microbiome and AMR gene dynamics in international travelers. A) Longitudinal profile of traveler gut microbiome diversity measured by Shannon diversity Index. Traveler 5 (T5) had a Shannon diversity index >3 SDs below the mean when measured at 30 days and 6 months posttravel. B) Microbes, by genus, demonstrating the greatest fold change in abundance after travel on the basis of DNA sequencing nucleotide alignments. C) Total number of AMR genes identified with ≥20% allele coverage by DNA sequencing. D) Total number of AMR genes identified with ≥20% allele coverage by RNA sequencing. AGLY, aminoglycoside; AMR, antimicrobial resistance; BLA, β-lactamase; FLQ, fluoroquinolone; MLS, macrolide, lincosamide, streptogramin; PRE, pretravel (within 1 week before departure); PST, posttravel (within 1 week after return); TMP/SUL, trimethoprim/sulfamethoxazole; TET, tetracycline; ESBL, extended-spectrum β-lactamase.

Table 2. Fold change in abundance of AMR genes found in *Enterobacteraciae* with ≥20% allele coverage compared with pretravel, by resistance gene or drug class*

	Fold change compared with pretravel					
	Posttravel		30 d		6 mo	
Resistance gene or drug class	DNA	RNA	DNA	RNA	DNA	RNA
β-lactam AMR genes						
AmpC	>100	>100	>100	1	>100	2
AmpH	>100	>100	>100	2	>100	1
CTX	>100	>100	>100	>100	>100	>100
MrdA	50	>100	0	>100	5	>100
OXA	2	1	1	0	1	0
SHV	>100	>100	>100	>100	>100	>100
TEM	>100	64	54	0	15	1
Other antibiotic classes						
Aminoglycoside	2	3	1	0	0	0
Fluoroquinolone	>100	>100	>100	>100	>100	>100
Glycopeptide	0	1	0	0	0	0
Macrolide, lincosamide, streptogramin	2	3	1	3	1	1
Sulfa	22	29	1	0	0	0
Tetracycline	1	1	1	0	1	0
Trimethoprim	>100	88	>100	0	>100	1

*Among 10 participants who submitted samples pretravel (within 1 week before departure), posttravel (within 1 week after return), 30 days posttravel, and 6 month posttravel. AMR, antimicrobial resistance.

of samples with an ESBL culture-determined phenotype and in 14% of samples without, including 1 participant (T8) who was phenotypically ESBL-negative after travel (Table 1). β-lactam–resistance genes increased posttravel, including *AmpC*, *CTX-M*, *OXA*, and *SHV* gene families known or predicted to confer ESBL production, as well as diverse additional β- lactamase genes (Appendix Figure 3). Travel also resulted in an increase in *qnr* plasmid–mediated quinolone-resistance genes, as well as trimethoprim (*dfr*)-, sulfa-, macrolide-, and aminoglycoside-resistance genes (Table 2). Genes conferring resistance to tetracyclines and aminoglycosides were most abundant in travelers at baseline and remained stable or decreased during travel (Table 2).

We found no significant differences in SDI between persistent carriers and those who lost carriage at 30 days (n = 3; p = 0.56 by *t*-test) or at 6 months (n = 2; p = 0.27 by *t*-test) posttravel. T5, who was colonized at both time points and who was the only traveler with persistent diarrheal symptoms, had an SDI >3 SDs below the mean at 6 months (Figure, panel A). Bray-Curtis distance measured pretravel or posttravel did not differ between travelers who were ESBL-PE positive by culture at 30 days or 6 months posttravel (p = 0.32 by permutational multivariate analysis of variance). No individual taxa were associated with posttravel ESBL positivity on the basis of an adjusted p value <0.05 (*t*-test) at 30 days or 6 months posttravel.

Conclusions

International travel is a well-recognized contributor to the global spread of emerging infectious diseases, including AMR bacteria (*1*,*4*). We analyzed the enteric microbiota and resistomes of returned travelers and found a marked increase in AMR genes that was associated with an increased proportion of *Escherichia* bacteria. At baseline, few participants had evidence of ESBL colonization; however, after travel, ESBL and actively transcribed *AmpC* genes were notably increased, consistent with previous reports (*4*,*11*). Both mNGS and culture-based methods found evidence of persistent ESBL colonization after 6 months, suggesting that travel can induce long-term changes in the antimicrobial resistome (*5*). In addition to β-lactamase genes, mNGS identified a diversity of other AMR gene classes that increased in abundance after travel. For example, 80% of participants acquired horizontally transferable *qnr* genes after travel, reflecting the limited utility of quinolones for treatment of traveler's diarrhea (*12*). Although we did not detect genes known to encode carbapenemases, participants might have harbored carbapenem-resistant *Enterobacteraciae*, given that a combination of an ESBL or *AmpC* gene with a porin mutation or efflux pump can lead to carbapenem resistance (*13*).

Changes in microbiome diversity were not associated with ESBL positivity at 30 days or 6 months posttravel, suggesting that disruption of the antimicrobial resistome can occur in the setting of a preserved microbial community structure. We observed a high rate of ESBL-PE acquisition in this cohort, most of whom traveled to the Indian subcontinent, consistent with previous studies of travelers returning from this region (*1*). Notably, none of the travelers in this cohort reported antibiotic use, suggesting that substantial ESBL-PE colonization can occur even in the absence of antibiotic-related disruption of commensal gut microbiota.

Because this study is limited by small sample size, relevant associations might have been missed. In addition, we could not assess the presence of carbapenem-resistant *Enterobacteriaceae* by using culture-based methods. mNGS

and phenotypic antimicrobial resistance need assessment in larger cohorts traveling to more destinations. Nonetheless, our findings highlight the pervasiveness of AMR microbe exchange during international travel and the promise of mNGS for assessing the global exchange of antimicrobial resistance.

Acknowledgment

We thank Theresa Sofarelli for inspiration and support for this study.

About the Author

Dr. Langelier is an assistant professor in the Division of Infectious Diseases at the University of California, San Francisco. His research interests involve using metagenomics and transcriptional profiling to investigate host–pathogen interactions and understand the causes of diagnostically challenging diseases.

References

1. Arcilla MS, van Hattem JM, Haverkate MR, Bootsma MCJ, van Genderen PJJ, Goorhuis A, et al. Import and spread of extended-spectrum β-lactamase-producing *Enterobacteriaceae* by international travellers (COMBAT study): a prospective, multicentre cohort study. Lancet Infect Dis. 2017;17:78–85. http://dx.doi.org/10.1016/S1473-3099(16)30319-X
2. Kuenzli E, Jaeger VK, Frei R, Neumayr A, DeCrom S, Haller S, et al. High colonization rates of extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* in Swiss travellers to South Asia—a prospective observational multicentre cohort study looking at epidemiology, microbiology and risk factors. BMC Infect Dis. 2014;14:528. http://dx.doi.org/10.1186/1471-2334-14-528
3. Hassing RJ, Alsma J, Arcilla MS, van Genderen PJ, Stricker BH, Verbon A. International travel and acquisition of multidrug-resistant *Enterobacteriaceae*: a systematic review. Euro Surveill. 2015;20:20.
4. von Wintersdorff CJH, Penders J, Stobberingh EE, Oude Lashof AML, Hoebe CJPA, Savelkoul PHM, et al. High rates of antimicrobial drug resistance gene acquisition after international travel, the Netherlands. Emerg Infect Dis. 2014;20:649–57. http://dx.doi.org/10.3201/eid2004.131718
5. van Duijkeren E, Wielders CCH, Dierikx CM, van Hoek AHAM, Hengeveld P, Veenman C, et al. Long-term carriage of extended-spectrum β-lactamase–producing *Escherichia coli* and *Klebsiella pneumoniae* in the general population in the Netherlands. Clin Infect Dis. 2018;66:1368–76. http://dx.doi.org/10.1093/cid/cix1015
6. David LA, Materna AC, Friedman J, Campos-Baptista MI, Blackburn MC, Perrotta A, et al. Host lifestyle affects human microbiota on daily timescales. Genome Biol. 2014;15:R89. http://dx.doi.org/10.1186/gb-2014-15-7-r89
7. Langelier C, Kalantar KL, Moazed F, Wilson MR, Crawford ED, Deiss T, et al. Integrating host response and unbiased microbe detection for lower respiratory tract infection diagnosis in critically ill adults. Proc Natl Acad Sci U S A. 2018;115:E12353–62. http://dx.doi.org/10.1073/pnas.1809700115
8. Inouye M, Dashnow H, Raven L-A, Schultz MB, Pope BJ, Tomita T, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. 2014;6:90. http://dx.doi.org/10.1186/s13073-014-0090-6
9. Bush K, Jacoby GA. Updated functional classification of β-lactamases. Antimicrob Agents Chemother. 2010;54:969–76. http://dx.doi.org/10.1128/AAC.01009-09
10. Giske CG, Sundsfjord AS, Kahlmeter G, Woodford N, Nordmann P, Paterson DL, et al. Redefining extended-spectrum β-lactamases: balancing science and clinical need. J Antimicrob Chemother. 2009;63:1–4. http://dx.doi.org/10.1093/jac/dkn444
11. Decker BK, Lau AF, Dekker JP, Spalding CD, Sinaii N, Conlan S, et al. Healthcare personnel intestinal colonization with multidrug-resistant organisms. Clin Microbiol Infect. 2018;24:82.e1–4. http://dx.doi.org/10.1016/j.cmi.2017.05.010
12. Riddle MS, Connor BA, Beeching NJ, DuPont HL, Hamer DH, Kozarsky P, et al. Guidelines for the prevention and treatment of travelers' diarrhea: a graded expert panel report. J Travel Med. 2017;24(Suppl_1):S57–74. https://doi.org/10.1093/jtm/tax026
13. Logan LK, Weinstein RA. The epidemiology of carbapenem-resistant *Enterobacteriaceae*: the impact and evolution of a global menace. J Infect Dis. 2017;215(Suppl_1):S28–36.

Address for correspondence: Charles Langelier, University of California San Francisco, Infectious Diseases, 513 Parnassus Ave, Room S-380, San Francisco, CA 94143, USA; email: chaz.langelier@ucsf.edu

Epidemiology of Human Parechovirus Type 3 Upsurge in 2 Hospitals, Freiburg, Germany, 2018

Roland Elling,[1] Sindy Böttcher,[1] Florian du Bois, Alexandra Müller, Christiane Prifert, Benedikt Weissbrich, Jörg Hofmann, Klaus Korn, Anna-Maria Eis-Hübinger, Markus Hufnagel, Marcus Panning

In 2018, a cluster of pediatric human parechovirus (HPeV) infections in 2 neighboring German hospitals was detected. Viral protein 1 sequence analysis demonstrated co-circulation of different HPeV-3 sublineages and of HPeV-1 and -5 strains, thereby excluding a nosocomial outbreak. Our findings underline the need for HPeV diagnostics and sequence analysis for outbreak investigations.

Most human parechovirus (HPeV) infections cause mild upper respiratory tract symptoms or unspecific febrile illnesses. Severe clinical manifestations, such as meningitis/encephalitis, myocarditis, and newborn sepsis, are caused by HPeV type 3 (HPeV-3) and have been described in children <3 months of age (*1*). Surveillance data show endemic circulation in several countries, such as the Netherlands (*2*) and the United States (*3*), but studies have discussed the epidemic potential of HPeV-3 in other countries, including Japan (*4*), Australia (*5*), and the United Kingdom (*6*). Nosocomial transmission has been documented (*7*).

However, in most outbreak investigations, determination of HPeV types was performed retrospectively (*7*,*8*). We report on our investigations on a cluster of HPeV infections in 2 neighboring hospitals in Freiburg, Germany. We provide evidence that rapid phylogenetic analysis can assist in outbreak investigations.

The Study

During routine diagnostic testing of clinical samples from infants and young children in July 2018, we detected an increase in HPeV cases (Figure 1). We collected ≥1 clinical specimens from most patients (Table 1). During July 9–August 25, 2018, we documented 19 cases, compared with 4 (2016) and 2 (2017) from this same time span, all using the same assays and diagnostic testing algorithm. In September 2018, only 2 patients tested HPeV-positive; no additional cases were identified during October and November 2018. HPeV diagnostic procedures were performed upon the request of the treating physician. For the detection of HPeV, we used commercial multiplex PCR panels: FTD respiratory pathogens 21 (Fast Track Diagnostics [FTD]; Siemens Healthineers, https://www.siemens-healthineers.com) for respiratory specimens and FTD EPA for cerebrospinal fluid (CSF), plasma, and fecal samples. Patients were hospitalized on 1 ward in hospital A and 4 wards in hospital B (Appendix Figure, http://wwwnc.cdc.gov/EID/article/25/7/19-0257-App1.pdf). We retrieved medical data on HPeV-positive patients from the hospital-based information system. We obtained written informed consent from parents or guardians.

The age of the 2018 HPeV-positive patients ranged from 10 days to 19 months (median 1 month), with 88% of patients being <4 months of age (Table 1). Plasma samples (n = 14) had a diagnostic yield of 100%. The median duration of hospitalization was 4 days (range 3–23 days). The main clinical symptoms of HPeV-3 cases were fever (n = 21; 100%) and poor feeding (n = 16; 76%) (Table 2). None of our patients required admission to a pediatric intensive care unit. All of our patients were discharged from the hospital without complications.

After we detected the first cases in July 2018, we performed molecular typing of HPeV by amplifying and sequencing the complete viral protein 1 (VP1) genomic region (*9*). Of the 25 HPeV strains detected in Freiburg in 2018, 21 were typed as HPeV-3, 2 were assigned to HPeV-1, and 2 to HPeV-5 (Table 1). This compares with 7 HPeV-1 and 6 HPeV-3 types in Freiburg in 2016, and 3 HPeV-1, 3 HPeV-3, and 5 strains not typed in 2017 (Figure 1).

For phylogenetic analyses, we included HPeV strains detected during January 2016–September 2018 at another 4 university hospitals: Würzburg (n = 56)

Author affiliations: Medical Center–University of Freiburg Faculty of Medicine, Freiburg, Germany (R. Elling, F. du Bois, A. Müller, M. Hufnagel, M. Panning); Robert Koch-Institute, Berlin, Germany (S. Böttcher); University of Würzburg, Würzburg, Germany (C. Prifert, B. Weissbrich); Charité – Universitätsmedizin Berlin, Berlin (J. Hofmann); Friedrich Alexander University Erlangen-Nuremberg, Erlangen, Germany (K. Korn); University of Bonn Medical Centre, Bonn, Germany (A.-M. Eis-Hübinger)

DOI: https://doi.org/10.3201/eid2507.190257

[1]These authors contributed equally to this article.

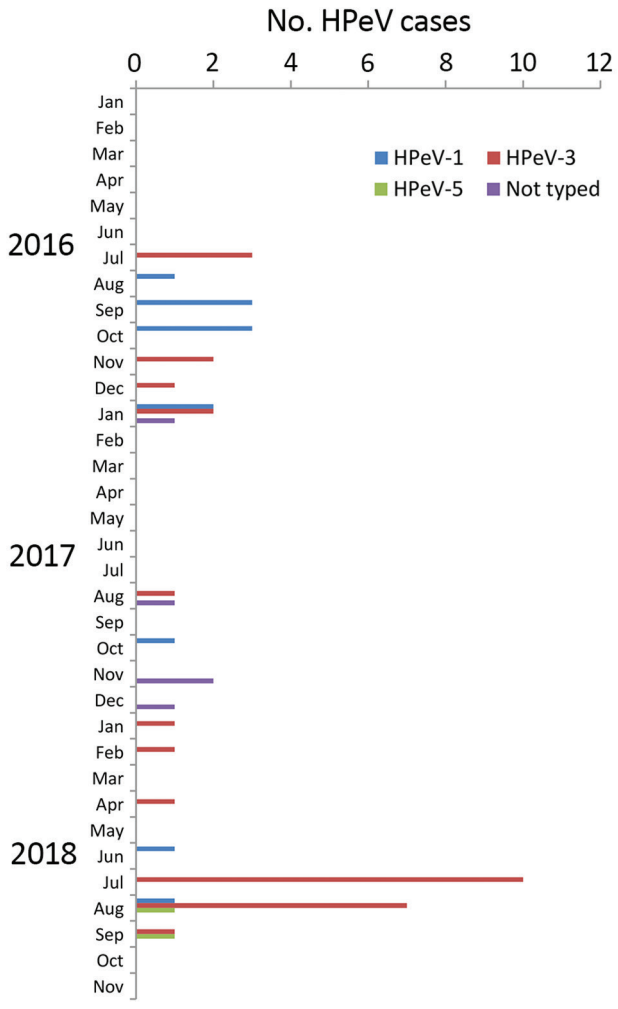


Figure 1. Number of human parechovirus (HPeV) cases in infants and young children by month, Freiburg, Germany, 2016–2018.

and Erlangen (n = 10) in southern Germany, Bonn (n = 10) in western Germany, and Charité Berlin (n = 14) in northeastern Germany. We detected 134 HPeV strains in respiratory, fecal, CSF, and serum samples. These were typed based on the VP1 genomic region (*9*). We detected HPeV types 1, 3, 4, 5, and 6. We deposited all sequences in GenBank under accession numbers MK204942–MK204985 and MK291273–MK291362 (Appendix Tables 1, 2). For HPeV-3 phylogenetic analysis, we included 74 strains identified during 2016–2018 and compared them with representative reference strains available from GenBank (Figure 2). Because of high nucleotide variability in the 3′ end of the VP1 coding region, we included only complete VP1 sequences.

As recently described, 2 HPeV-3 lineages have been identified (*10*). Widespread clustering proved co-circulation of the 2016–2018 HPeV strains from Germany (Figure 2). One cluster comprising German strains was most closely related to HPeV-3 identified in Japan (98.82% nt identity), Australia (98.82%), and the United Kingdom (99.12%).

Among the 21 Freiburg 2018 HPeV-3 strains, 3 groups of completely identical VP1 sequences (10, 3, and 2 sequences) were observed. However, no separate clustering could be detected among these strains because HPeV sequences from other regions in Germany also were assigned to these groups. A direct epidemiologic link could be drawn between 2 cases (cases 12 and 14, with completely identical VP1 sequences, were in twins; Figure 2). Another 2 cases (cases 15 and 18) shared time on the same ward and also displayed 100% identical sequences. However, no hospital ward–specific clustering was observed, suggesting community-acquired transmission.

Conclusions

Routine diagnostics showed an unexpectedly high number of HPeV cases during a 6-week period in 2 neighboring hospitals in Freiburg, Germany. This raised concern about the possibility of a nosocomial outbreak. Recently, healthcare-associated transmission of HPeV-3 has been described. This makes timely identification of outbreaks essential from a hospital hygiene, as well as a public health, perspective (*7*).

Several patients showed signs of sepsis-like illness, including the clinical triad of fever, poor feeding, and irritability. This is similar to a UK case series reporting a cluster of HPeV infections among infants in 2016 (*6*). In our study, HPeV-3 was detected exclusively in CSF samples, indicating a more severe clinical phenotype compared with HPeV-1 and -5 infections, supporting previous data (*11*). Studies have shown that rapid detection of HPeV reduced length of hospital stay and antimicrobial drug use. This emphasizes the usefulness of HPeV diagnostics (*1*). We showed that HPeV diagnostics, including molecular typing, helped to exclude a nosocomial outbreak. Diagnostically, plasma, respiratory swab, and fecal samples all showed high detection rates, and most patients were positive in ≥1 area. Testing of blood samples for enterovirus detection was recently proposed for infants and should be considered for HPeV accordingly (*12*).

We demonstrated different HPeV types and sublineages, including 2 rare HPeV-5 infections. By conducting phylogenetic analysis in combination with reviewing epidemiologic data, we could exclude a nosocomial outbreak. However, based on this information, transmissions could not be ruled out in 2 independent events with 2 cases each. Although a cluster of HPeV-3 infections has been described (*6*), retrospective sequence analysis showed different clustering of the identified strains (*13*). Because of low nucleotide variability, sequence-based differentiation between HPeV-3 strains remains

Table 1. Epidemiologic data for HPeV cases in Freiburg, Germany, January–September 2018*

Case no.	Patient age, mo/sex	Specimen type				HPeV type
		Cerebrospinal fluid	Upper respiratory tract	Plasma	Feces	
1	2/M	Negative	Positive	NA	Negative	3
2	3/M	Positive	Positive	Positive	Positive	3
3	0/F	Positive	Positive	NA	NA	3
4	19/M	NA	NA	NA	Positive	1
5	1/M	Positive	Positive	Positive	Positive	3
6	1/M	NA	NA	Positive	Positive	3
7	1/F	NA	NA	NA	Positive	3
8	7/M	Negative	NA	NA	Positive	3
9	2/M	Negative	NA	NA	Positive	3
10	2/M	NA	NA	Positive	NA	3
11	1/M	NA	Positive	NA	Positive	3
12	0/M	Positive	Positive	Positive	Positive	3
13	1/M	Positive	Positive	NA	NA	3
14	0/F	Positive	Positive	Positive	Positive	3
15	0/F	NA	NA	Positive	Positive	3
16	2/M	NA	NA	Positive	Positive	3
17	2/M	NA	NA	Positive	Positive	3
18	1/F	NA	NA	Positive	Positive	3
19	17/F	NA	Positive	NA	NA	1
20	1/F	NA	NA	Positive	Positive	3
21	4/F	NA	Positive	NA	Positive	3
22	2/M	Positive	NA	NA	Positive	3
23	0/M	NA	NA	Positive	Positive	5
24	0/M	Positive	NA	NA	Positive	3
25	1/M	NA	NA	Positive	Positive	5

*HPeV, human parechovirus; NA, not applicable (no specimen).

Table 2. Clinical signs and symptoms of HPeV cases in Freiburg, Germany, January–September 2018*

Clinical signs and symptoms	No. (%) positive patients		
	HPeV-1, n = 2	HPeV-3, n = 21	HPeV-5, n = 2
Fever	1 (50)	21 (100)	2 (100)
Poor feeding	1 (50)	16 (76)	1 (50)
Irritability	0	13 (62)	1 (50)
Rash	1 (50)	6 (29)	1 (50)
Diarrhea	1 (50)	5 (24)	0
Respiratory distress	0	5 (24)	0
Vomiting	1 (50)	0	0

*HPeV, human parechovirus

ambiguous, a circumstance that impedes molecular outbreak investigation (*14*).

Our study has limitations. There is a lack of available sequence data from pediatric patients in Germany. In contrast to reports from the Netherlands and the UK, a biannual cycle of HPeV infections has not been demonstrated in Germany; however, our data suggest a biannual cycle. From a public health perspective, a central repository for HPeV sequences, together with key anonymized clinical data from human cases, would improve our understanding of HPeV epidemiology and virus evolution. Institutionalized surveillance similar to the enterovirus surveillance and typing systems already in place across Europe could serve as a blueprint (*8*,*15*).

Our report underscores the usefulness of HPeV diagnostics in infants. It illustrates the power of VP1 sequence–guided phylogenetic HPeV analysis, which helped, in combination with epidemiologic data, to rapidly investigate an HPeV outbreak.

About the Authors

Dr. Elling is a pediatric infectious diseases specialist at the Centers for Pediatrics and Adolescent Medicine and Chronic Immunodeficiency in Freiburg, Germany. His research interests are pediatric infectious diseases and innate immunity. He is supported by the Berta-Ottenstein Clinician Scientist program of the Medical Faculty, University of Freiburg. Dr. Böttcher is a virologist at the German National Reference Center for Poliomyelitis and Enteroviruses at the Robert Koch-Institute in Berlin, Germany. Her research focuses on molecular virology and epidemiology of enteroviruses and parechoviruses.

References

1. Kadambari S, Harvala H, Simmonds P, Pollard AJ, Sadarangani M. Strategies to improve detection and management of human parechovirus infection in young infants. Lancet Infect Dis. 2019;19:e51–8. http://dx.doi.org/10.1016/S1473-3099(18)30288-3
2. van der Sanden S, de Bruin E, Vennema H, Swanink C, Koopmans M, van der Avoort H. Prevalence of human

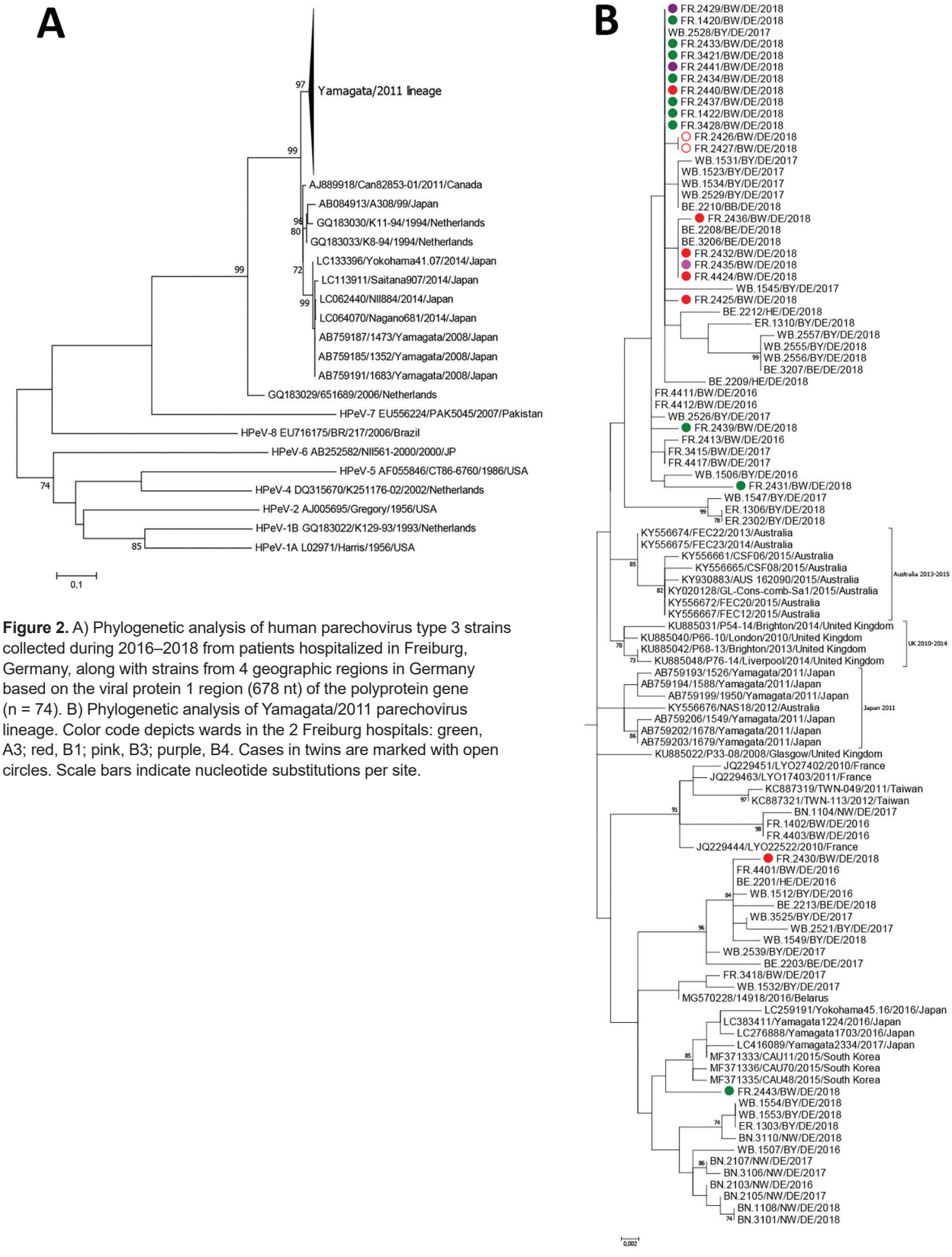


Figure 2. A) Phylogenetic analysis of human parechovirus type 3 strains collected during 2016–2018 from patients hospitalized in Freiburg, Germany, along with strains from 4 geographic regions in Germany based on the viral protein 1 region (678 nt) of the polyprotein gene (n = 74). B) Phylogenetic analysis of Yamagata/2011 parechovirus lineage. Color code depicts wards in the 2 Freiburg hospitals: green, A3; red, B1; pink, B3; purple, B4. Cases in twins are marked with open circles. Scale bars indicate nucleotide substitutions per site.

parechovirus in the Netherlands in 2000 to 2007. J Clin Microbiol. 2008;46:2884–9. http://dx.doi.org/10.1128/JCM.00168-08
3. Abedi GR, Watson JT, Pham H, Nix WA, Oberste MS, Gerber SI. Enterovirus and human parechovirus surveillance—United States, 2009–2013. MMWR Morb Mortal Wkly Rep. 2015;64:940–3. http://dx.doi.org/10.15585/mmwr.mm6434a3
4. Aizawa Y, Suzuki Y, Watanabe K, Oishi T, Saitoh A. Clinical utility of serum samples for human parechovirus type 3 infection in neonates and young infants: the 2014 epidemic in Japan. J Infect. 2016;72:223–32. http://dx.doi.org/10.1016/j.jinf.2015.10.010
5. Cumming G, Khatami A, McMullan BJ, Musto J, Leung K, Nguyen O, et al. Parechovirus genotype 3 outbreak among Infants, New South Wales, Australia, 2013–2014. Emerg Infect Dis. 2015;21:1144–52. http://dx.doi.org/10.3201/eid2107.141149
6. Tang JW, Holmes CW, Elsanousi FA, Patel A, Adam F, Speight R, et al. Cluster of human parechovirus infections as the predominant cause of sepsis in neonates and infants, Leicester, United Kingdom, 8 May to 2 August 2016. Euro Surveill. 2016;21:30326. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.34.30326
7. Strenger V, Diedrich S, Boettcher S, Richter S, Maritschnegg P, Gangl D, et al. Nosocomial outbreak of parechovirus 3 infection among newborns, Austria, 2014. Emerg Infect Dis. 2016;22:1631–4. http://dx.doi.org/10.3201/eid2209.151497
8. Ferreras Antolín L, Kadambari S, Braccio S, Tang JW, Xerry J, Allen DJ, et al.; Parechovirus Surveillance Network. Increased detection of human parechovirus infection in infants in England during 2016: epidemiology and clinical characteristics. Arch Dis Child. 2018;103:1061–6.
9. Cristanziano VD, Böttcher S, Diedrich S, Timmen-Wego M, Knops E, Lübke N, et al. Detection and characterization of enteroviruses and parechoviruses in healthy people living in the south of Côte d'Ivoire. J Clin Virol. 2015;71:40–3. http://dx.doi.org/10.1016/j.jcv.2015.08.004
10. Nelson TM, Vuillermin P, Hodge J, Druce J, Williams DT, Jasrotia R, et al. An outbreak of severe infections among Australian infants caused by a novel recombinant strain of human parechovirus type 3. Sci Rep. 2017;7:44423. http://dx.doi.org/10.1038/srep44423
11. Harvala H, Robertson I, Chieochansin T, McWilliam Leitch EC, Templeton K, Simmonds P. Specific association of human parechovirus type 3 with sepsis and fever in young infants, as identified by direct typing of cerebrospinal fluid samples. J Infect Dis. 2009;199:1753–60. http://dx.doi.org/10.1086/599094
12. Lafolie J, Labbé A, L'Honneur AS, Madhi F, Pereira B, Decobert M, et al.; Blood Enterovirus Diagnosis Infection (BLEDI) in paediatric population study team. Assessment of blood enterovirus PCR testing in paediatric populations with fever without source, sepsis-like disease, or suspected meningitis: a prospective, multicentre, observational cohort study. Lancet Infect Dis. 2018;18:1385–96. http://dx.doi.org/10.1016/S1473-3099(18)30479-1
13. Holmes CW, Rahman S, Allen DJ, Bandi S, Tang JW. Human parechovirus cluster in the UK, 8 May–2 August 2016—sequence analysis. J Clin Virol. 2017;93:37–9. http://dx.doi.org/10.1016/j.jcv.2017.05.018
14. Faria NR, de Vries M, van Hemert FJ, Benschop K, van der Hoek L. Rooting human parechovirus evolution in time. BMC Evol Biol. 2009;9:164. http://dx.doi.org/10.1186/1471-2148-9-164
15. Harvala H, Jasir A, Penttinen P, Pastore Celentano L, Greco D, Broberg E. Surveillance and laboratory detection for non-polio enteroviruses in the European Union/European Economic Area, 2016. Euro Surveill. 2017;22. http://dx.doi.org/10.2807/1560-7917.ES.2017.22.45.16-00807

Address for correspondence: Marcus Panning, University of Freiburg Institute of Virology, Medical Centre, Hermann-Herder-Str. 11, 79104 Freiburg, Germany; email: marcus.panning@uniklinik-freiburg.de

Carbapenem and Cephalosporin Resistance among *Enterobacteriaceae* in Healthcare-Associated Infections, California, USA[1]

Kyle Rizzo, Sam Horwich-Scholefield, Erin Epson

We analyzed antimicrobial susceptibility test results reported in healthcare-associated infections by California hospitals during 2014–2017. Approximately 3.2% of *Enterobacteriaceae* reported in healthcare-associated infections were resistant to carbapenems and 26.9% were resistant to cephalosporins. The proportion of cephalosporin-resistant *Escherichia coli* increased 7% (risk ratio 1.07, 95% CI 1.04–1.11) per year during 2014–2017.

The Centers for Disease Control and Prevention (CDC) identified carbapenem-resistant *Enterobacteriaceae* (CRE) as an urgent public health threat and extended-spectrum β-lactamase (ESBL)–producing *Enterobacteriaceae* as a serious public health threat (*1*). Antimicrobial-resistant pathogens, such as CRE, can spread across regions when infected or colonized patients transfer between healthcare facilities without infection control measures in place to prevent transmission (*2*). Therefore, tracking regional changes in antimicrobial resistance (AMR) is essential to inform public health prevention and containment strategies.

The Study

Healthcare-associated infection (HAI) pathogen data reported to the National Healthcare Safety Network (NHSN) can be used to estimate the prevalence of AMR among hospitals within a region (*3*–*5*). Hospitals provide pathogen and antimicrobial susceptibility test results for ≤3 microorganisms when reporting central line–associated bloodstream infections (CLABSI), surgical site infections (SSI), and catheter-associated urinary tract infections (CAUTI) to NHSN (*6*). Data on molecular mechanisms of resistance are not collected for CLABSI, SSI, or CAUTI.

We applied CDC definitions to identify antimicrobial-resistant phenotypes among *Enterobacteriaceae*, including *Escherichia coli*, *Klebsiella* species, and *Enterobacter* species, reported in CLABSI, SSI, and CAUTI by general acute-care hospitals in California (*3*). We included multiple pathogens per HAI if reported. California hospitals report HAI data for ≤28 surgical procedures; we included pathogen data from any SSI reported. We excluded HAI data reported by other hospital types, such as critical access and long-term acute-care hospitals, due to limited HAI data reported by these hospitals.

According to CDC definitions, CRE were resistant to imipenem, meropenem, doripenem, or ertapenem. Extended-spectrum cephalosporin-resistant (ESCR) *Enterobacteriaceae* were resistant to ceftriaxone, ceftazidime, cefepime, or cefotaxime. We applied modified phenotype definitions from Magiorakos et al. to identify multidrug-resistant (MDR), extensively drug-resistant (XDR), and pandrug-resistant (PDR) *Enterobacteriaceae* (*7*). Susceptibility data for 2 antimicrobial drugs (ceftaroline and fosfomycin) included in these definitions were not available in our NHSN data. Resistance was defined by an isolate's nonsusceptibility to ≥1 agent (e.g., imipenem) within a category of antimicrobial drugs (e.g., carbapenems) and the total number of antimicrobial categories (≤15) for which the isolate was nonsusceptible. MDR *Enterobacteriaceae* were nonsusceptible to ≥3 antimicrobial categories; XDR *Enterobacteriaceae* were nonsusceptible to all but 1 or 2 antimicrobial categories, and PDR *Enterobacteriaceae* were nonsusceptible to all antimicrobial categories. We also assessed the phenotype difficult-to-treat (DTR) proposed by Kadri et al. (*8*). DTR included an intermediate or resistant result to all reported agents within carbapenem, cephalosporin, and fluoroquinolone categories, as well as piperacillin-tazobactam and aztreonam when results were available.

We used log binomial regression models to estimate statewide, year-to-year change in the proportion of antimicrobial-resistant *Enterobacteriaceae* during 2014–2017. To understand regional differences in CRE and ESCR *Enterobacteriaceae*, we performed a subgroup analysis in which we aggregated HAI data in 2-year increments and measured percentage resistance by county when susceptibility test results for ≥30 *Enterobacteriaceae* were available. CDC has explored risk adjustment for regional-level comparisons using NHSN data and determined unadjusted measures are satisfactory until additional covariates are adopted in NHSN (*9*).

Author affiliation: California Department of Public Health, Richmond, California, USA

DOI: https://doi.org/10.3201/eid2507.181938

[1]Preliminary data from this analysis were presented in a poster at IDWeek 2018 in San Francisco, California, USA, October 5, 2018.

We completed data analyses in SAS version 9.4 (SAS, http://www.sas.com) and spatial analyses in ArcMap version 10.4 (Environmental Systems Research Institute, Inc., https://www.esri.com). This public health surveillance analysis met criteria for nonresearch activity and did not require an exemption determination from the California Committee for the Protection of Human Subjects.

During 2014–2017, 305 (91%) of 335 California hospitals reported ≥1 *Enterobacteriaceae* in HAI with cephalosporin susceptibility test results; 296 (88%) hospitals reported ≥1 *Enterobacteriaceae* with carbapenem susceptibility test results. The median number of *Enterobacteriaceae* reported with cephalosporin susceptibility test results by hospitals per year was 8 (interquartile range 16–3), and 6 (interquartile range 14–3) for *Enterobacteriaceae* with carbapenem susceptibility test results.

Approximately 3.2% of *Enterobacteriaceae* reported in HAI during 2014–2017 were resistant to carbapenems and 26.9% of *Enterobacteriaceae* reported in HAI were cephalosporin resistant. We observed increases in the proportions of *Enterobacteriaceae* that were ESCR and MDR during 2014–2017; these changes were driven by *E. coli* (Table 1). We observed a 7% (risk ratio [RR] 1.07; 95% CI 1.04–1.11) annual increase in the proportion of *E. coli* resistant to cephalosporins and a 4% (RR 1.04; 95% CI 1.02–1.06) annual increase in the proportion of *E. coli* with an MDR phenotype during 2014–2017 (Table 1). The proportion of *E. coli* exhibiting carbapenem resistance also increased 24% (RR 1.24; 95% CI 1.00–1.56) per year during 2014–2017.

Table 1. Carbapenem and cephalosporin resistance among *Enterobacteriaceae* reported in healthcare-associated infections by California hospitals, 2014–2017*

	2014		2015		2016		2017		Change	
Antimicrobial agent	No. (%) isolates†	% R	No. (%) isolates†	% R	No. (%) isolates†	% R	No. (%) isolates†	% R	Risk ratio (95% CI)	p value
Enterobacteriaceae										
Carbapenems	2,747 (60.2)	3.1	3,310 (64.2)	3.1	3,409 (64.2)	3.5	3,247 (65.1)	3.0	1.00 (0.92–1.09)	0.98
Cephalosporins	3,303 (74.3)	24.0	3,837 (76.2)	27.7	4,020 (77.4)	27.5	3,885 (79.6)	28.0	1.04 (1.02–1.07)	0.001
DTR	2,298 (50.0)	2.2	2,786 (53.5)	2.0	2,916 (54.5)	2.1	2,856 (56.6)	1.6	0.92 (0.81–1.04)	0.16
MDR	4,500 (98.0)	38.8	5,129 (98.5)	43.3	5,228 (97.6)	43.8	4,942 (97.9)	44.0	1.04 (1.02–1.05)	<0.001
Escherichia coli										
Carbapenems	1,623 (59.9)	0.7	1,969 (64.9)	0.7	1,969 (64.6)	1.1	1,893 (66.7)	1.2	1.24 (1.00–1.56)	0.05
Cephalosporins	1,890 (71.1)	22.9	2,158 (72.4)	28.0	2,229 (74.2)	27.1	2,147 (76.8)	29.7	1.07 (1.04–1.11)	<0.001
DTR	1,323 (48.6)	0.5	1,577 (51.7)	0.3	1,615 (52.7)	0.5	1,613 (56.3)	0.4	0.99 (0.69–1.42)	0.95
MDR	2,669 (98.0)	42.8	3,004 (98.4)	47.0	2,998 (97.8)	47.1	2,812 (98.2)	49.2	1.04 (1.02–1.06)	<0.001
Enterobacter spp.										
Carbapenems	489 (62.1)	3.7	550 (62.9)	6.9	602 (63.7)	5.2	559 (63.0)	5.4	1.06 (0.90–1.24)	0.51
Cephalosporins	701 (94.1)	29.8	786 (94.5)	30.9	855 (94.9)	33.6	811 (94.5)	30.8	1.02 (0.97–1.07)	0.47
DTR	488 (60.6)	0.2	554 (61.8)	0.5	608 (63.1)	0.5	564 (61.2)	0	0.79 (0.38–1.57)	0.50
MDR	789 (98.0)	43.5	883 (98.5)	53.0	940 (97.5)	55.6	897 (97.3)	50.3	1.04 (1.01–1.07)	0.01
Klebsiella spp.										
Carbapenems	635 (59.7)	8.8	791 (63.2)	6.6	838 (63.8)	7.9	795 (63.3)	5.7	0.90 (0.80–1.01)	0.07
Cephalosporins	712 (68.3)	21.4	893 (73.1)	24.2	936 (72.6)	22.8	927 (75.5)	21.5	0.99 (0.94–1.05)	0.76
DTR	487 (45.7)	8.8	655 (52.1)	7.2	693 (52.4)	7.2	679 (53.8)	5.7	0.88 (0.77–1.00)	0.06
MDR	1,042 (97.8)	25.1	1,242 (98.7)	27.7	1,290 (97.5)	27.2	1,233 (97.7)	27.8	1.03 (0.99–1.07)	0.21

*Carbapenem-resistant *Enterobacteriaceae* were resistant to imipenem, meropenem, doripenem, or ertapenem. *Enterobacteriaceae* resistant to ceftriaxone, ceftazidime, cefepime, or cefotaxime were cephalosporin-resistant. MDR *Enterobacteriaceae* were nonsusceptible to ≥3 antimicrobial categories; XDR *Enterobacteriaceae* were nonsusceptible to all but 1 or 2 antimicrobial categories and pandrug-resistant *Enterobacteriaceae* were nonsusceptible to all antimicrobial categories (n = 15). DTR *Enterobacteriaceae* were intermediate or resistant to all reported agents within carbapenem, cephalosporin, and fluoroquinolone categories, as well as piperacillin-tazobactam and aztreonam when results were available. DTR, difficult-to-treat; MDR, multidrug-resistant; % R, percentage resistant.
†The number and percentage of *Enterobacteriaceae* with antimicrobial susceptibility test results as a proportion of the overall number reported (i.e., with or without antimicrobial susceptibility test results).

We observed decreasing trends in carbapenem resistance (RR 0.90; 95% CI 0.80–1.01) and in the DTR phenotype (RR 0.88; 95% CI 0.77–1.00) among *Klebsiella* species reported in HAI. Among *Enterobacteriaceae* assessed for the DTR phenotype, *Klebsiella* species accounted for 86% (n = 193) of DTR isolates and comprised 23% of the overall total of *Enterobacteriaceae* analyzed among HAI. In addition, 1 XDR *Klebsiella pneumoniae* was reported in HAI during 2014–2017 and no PDR *Enterobacteriaceae* were reported.

Percentages of CRE and ESCR phenotypes varied by county and reporting years (Table 2; Figures 1, 2). Carbapenem and cephalosporin resistance was higher in California regions more densely populated with hospitals and residents, such as the greater Los Angeles region and San Francisco Bay area. Counties with hospitals reporting <30 *Enterobacteriaceae* may still have antimicrobial-resistant HAI or receive patients from healthcare facilities where antimicrobial resistance is endemic.

Several factors limit the interpretation of our results. Only 4 years of data were available for measuring AMR trends. Selective reporting of susceptibility test results may have restricted sample sizes and increased the potential for sampling bias to affect our results. Furthermore, there may be differences in how California hospitals and laboratories interpret MIC breakpoints or changes in how breakpoints are applied over time. Data on molecular mechanisms of resistance are not collected in CLABSI, SSI, or CAUTI, which limits our understanding of how transmissible elements, including ESBL and carbapenemases, may contribute to the trends we observed.

Conclusions

Increases in carbapenem, cephalosporin, and MDR *E. coli* reported in HAI by California hospitals are concerning, given that *E. coli* are common causes of both hospital and community-associated infections. ESBL-producing *E. coli* have been reported in community-associated urinary tract infections among patients in California, with estimates of resistance among *E. coli* from 5% up to 17% in complicated pyelonephritis (*10*,*11*). MDR and DTR *Enterobacteriaceae* further limit treatment options and present

Table 2. Carbapenem and cephalosporin resistance among *Enterobacteriaceae* reported in healthcare-associated infections by California hospitals, aggregated by county, 2014–2017*

	Carbapenems				Cephalosporins			
	2014–2015		2016–2017		2014–2015		2016–2017	
County	No. (%) isolates†	% R	No. (%) isolates†	% R	No. (%) isolates†	% R	No. (%) isolates†	% R
Alameda	280 (72.4)	1.8	342 (84)	2.0	215 (56.3)	27.4	258 (63.2)	29.8
Butte	40 (97.6)	0	46 (93.9)	0	40 (100)	22.5	47 (97.9)	14.9
Contra Costa	141 (50.5)	5.7	197 (57.8)	3.0	120 (43.8)	40.0	159 (46.9)	40.3
Fresno	324 (91.3)	0	379 (95.5)	0.3	293 (85.4)	18.4	351 (89.5)	21.1
Imperial	33 (100)	3.0	NS	NS	32 (97.0)	43.8	NS	NS
Kern	165 (97.6)	1.8	137 (89)	0.7	100 (61.0)	17.0	110 (71.9)	19.1
Kings	35 (100)	0	33 (100)	0	36 (100)	16.7	33 (100)	27.3
Los Angeles	1,294 (46.9)	6.6	1,477 (49.6)	7.1	2,044 (74.7)	28.7	2,263 (75.8)	32.4
Marin	38 (97.4)	0	NS	NS	NS	NS	NS	NS
Monterey	85 (95.5)	0	111 (78.7)	0	69 (76.7)	8.7	112 (80.6)	16.1
Napa	NS	NS	31 (93.9)	0	NS	NS	32 (97.0)	9.4
Orange	365 (56.6)	3.6	363 (54.7)	4.1	530 (85.8)	26.4	548 (84.8)	24.5
Placer	70 (70.0)	1.4	108 (89.3)	1.9	60 (60.0)	21.7	79 (64.8)	24.1
Riverside	233 (56.0)	3.4	249 (55.8)	2.4	315 (77.2)	23.8	371 (84.1)	22.6
Sacramento	381 (86.6)	1.0	392 (81.8)	3.3	336 (78.1)	22.9	388 (82.9)	27.3
San Bernardino	301 (67.9)	3.0	337 (72.9)	3.0	217 (61.8)	33.2	212 (67.9)	42.5
San Diego	657 (53.8)	4.0	573 (50.8)	3.1	946 (80.0)	32.7	901 (81.5)	31.7
San Francisco	405 (96.4)	1.2	432 (96.0)	2.1	374 (87.4)	21.7	381 (84.1)	22.6
San Joaquin	61 (71.8)	0	53 (54.1)	3.8	80 (94.1)	16.3	95 (97.9)	18.9
San Luis Obispo	NS	NS	NS	NS	NS	NS	30 (100)	13.3
San Mateo	137 (98.6)	0.7	107 (98.2)	2.8	92 (67.2)	28.3	82 (73.9)	25.6
Santa Barbara	113 (99.1)	1.8	106 (98.1)	0.9	108 (95.6)	11.1	107 (99.1)	11.2
Santa Clara	370 (55.2)	1.4	557 (69.2)	1.8	413 (62.5)	25.9	659 (81.1)	26.9
Shasta	NS	NS	NS	NS	67 (97.1)	11.9	59 (96.7)	10.2
Solano	91 (95.8)	6.6	121 (99.2)	0.8	55 (58.5)	32.7	80 (67.2)	35.0
Sonoma	71 (68.3)	0	99 (92.5)	1.0	81 (78.6)	17.3	77 (72.6)	14.3
Stanislaus	92 (62.2)	2.2	98 (66.2)	1.0	106 (74.6)	29.2	98 (68.1)	28.6
Tulare	NS	NS	NS	NS	36 (69.2)	22.2	41 (74.5)	14.6
Ventura	42 (29.0)	0	61 (48.4)	3.3	128 (89.5)	11.7	119 (95.2)	20.2
Yuba	NS	NS	36 (97.3)	2.8	NS	27.4	37 (97.4)	13.5

*Carbapenem-resistant *Enterobacteriaceae* were resistant to imipenem, meropenem, doripenem, or ertapenem. *Enterobacteriaceae* resistant to ceftriaxone, ceftazidime, cefepime, or cefotaxime were cephalosporin resistant. The percentage of resistant *Enterobacteriaceae* is not shown when <30 *Enterobacteriaceae* are reported within a county. NS, not shown; % R, percentage resistant.
†The number and percentage of *Enterobacteriaceae* with reported antimicrobial susceptibility test results as a proportion of the overall number reported (i.e., with or without antimicrobial susceptibility test results).

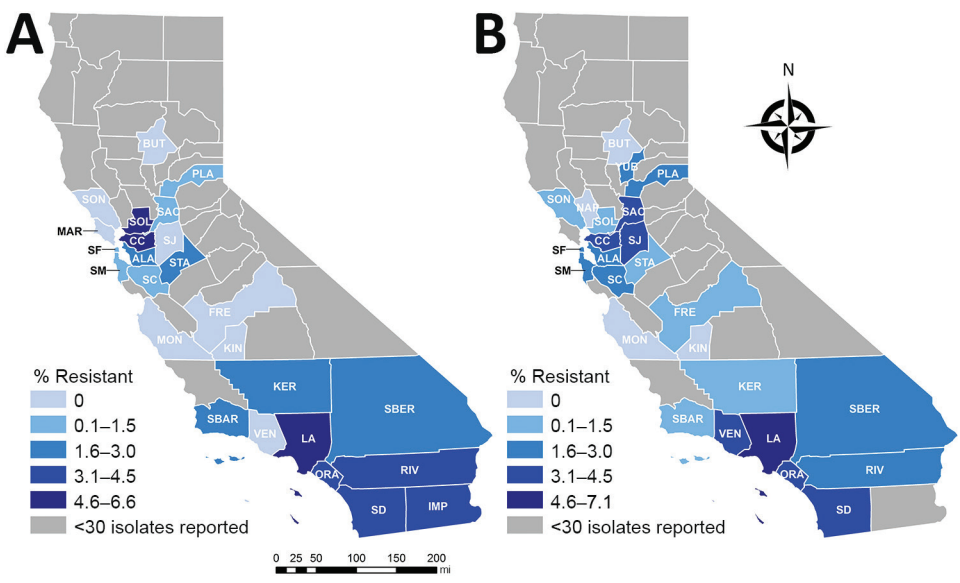


Figure 1. Geographic distribution of carbapenem resistance among *Enterobacteriaceae* reported in healthcare-associated infections by hospitals, aggregated by county, California, 2014–2015 (A) and 2016–2017 (B). ALA, Alameda; BUT, Butte; CC, Contra Costa; FRE, Fresno; IMP, Imperial; KER, Kern; KIN, Kings; LA, Los Angeles; MAR, Marin; MON, Monterey; NAP, Napa; ORA, Orange; PLA, Placer; RIV, Riverside; SAC, Sacramento; SBER, San Bernardino; SD, San Diego; SF, San Francisco; SJ, San Joaquin; SM, San Mateo; SBAR, Santa Barbara; SC, Santa Clara; SOL, Solano; SON, Sonoma; STA, Stanislaus; VEN, Ventura; YUB, Yuba.

management challenges, particularly in outpatient settings when there are no oral antimicrobial treatment options.

AMR prevention and containment strategies may depend on the local prevalence. For example, prompt detection and rapid, aggressive containment responses to individual AMR cases can be effective in low-prevalence regions. Admission screening and empiric use of transmission-based precautions for patients at high risk for AMR might be more feasible in higher-prevalence regions.

Healthcare facilities can prevent HAI and the spread of AMR by implementing best practices in infection control and antimicrobial stewardship. State and local health departments can coordinate prevention efforts across the healthcare continuum, investigate and control outbreaks in healthcare facilities, and set expectations for healthcare facilities to communicate patients' AMR infection and colonization status during all patient transfers. Decreasing trends in carbapenem resistance and in the DTR phenotype among *Klebsiella* species, often the focus of AMR containment efforts, indicate the potential effectiveness of such prevention strategies (*5*). Nonetheless, increases and regional variation in carbapenem-resistant and ESCR *E. coli* highlight the urgent need for ongoing, local infection prevention and antimicrobial stewardship efforts.

Aspects of this work were supported by Centers for Disease Control and Prevention Epidemiology and Laboratory Capacity grant funds.

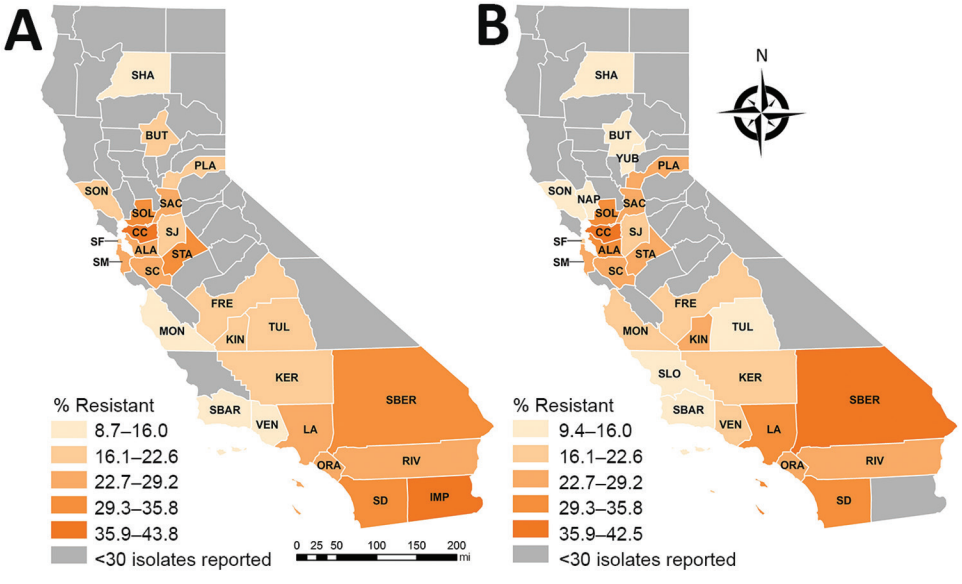


Figure 2. Geographic distribution of cephalosporin resistance among *Enterobacteriaceae* reported in healthcare-associated infections by hospitals, aggregated by county, California, 2014–2015 (A) and 2016–2017 (B). ALA, Alameda; BUT, Butte; CC, Contra Costa; FRE, Fresno; IMP, Imperial; KER, Kern; KIN, Kings; LA, Los Angeles; MON, Monterey; NAP, Napa; ORA, Orange; PLA, Placer; RIV, Riverside; SAC, Sacramento; SBER, San Bernardino; SD, San Diego; SF, San Francisco; SJ, San Joaquin; SLO, San Luis Obispo; SM, San Mateo; SBAR, Santa Barbara; SC, Santa Clara; SHA, Shasta; SOL, Solano; SON, Sonoma; STA, Stanislaus; TUL, Tulare; VEN, Ventura; YUB, Yuba.

About the Author

Mr. Rizzo is an epidemiologist with the Healthcare-Associated Infections Program at the California Department of Public Health. His work focuses on surveillance of antimicrobial-resistant healthcare-associated infections and evaluation of prevention programs.

References

1. Centers for Disease Control and Prevention. Antibiotic/antimicrobial resistance. Biggest threats and data. 2018 [cited 2019 Feb 19]. https://www.cdc.gov/drugresistance/biggest_threats.html
2. Huang SS, Avery TR, Song Y, Elkins KR, Nguyen CC, Nutter SK, et al. Quantifying interhospital patient sharing as a mechanism for infectious disease spread. Infect Control Hosp Epidemiol. 2010;31:1160–9. http://dx.doi.org/10.1086/656747
3. Centers for Disease Control and Prevention. Patient safety atlas. 2018 [cited 2018 Jul 13]. https://www.cdc.gov/hai/surveillance/ar-patient-safety-atlas.html
4. Weiner LM, Webb AK, Limbago B, Dudeck MA, Patel J, Kallen AJ, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2011–2014. Infect Control Hosp Epidemiol. 2016;37:1288–301. http://dx.doi.org/10.1017/ice.2016.174
5. Woodworth KR, Walters MS, Weiner LM, Edwards J, Brown AC, Huang JY, et al. Vital signs: containment of novel multidrug-resistant organisms and resistance mechanisms—United States, 2006–2017. MMWR Morb Mortal Wkly Rep. 2018;67:396–401. http://dx.doi.org/10.15585/mmwr.mm6713e1
6. National Healthcare Safety Network, Centers for Disease Control and Prevention. Surveillance reporting for enrolled facilities, 2015 [cited 2018 Jul 10]. https://www.cdc.gov/nhsn/enrolled-facilities/index.html
7. Magiorakos A-P, Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, et al. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. Clin Microbiol Infect. 2012;18:268–81. http://dx.doi.org/10.1111/j.1469-0691.2011.03570.x
8. Kadri SS, Adjemian J, Lai YL, Spaulding AB, Ricotta E, Prevots DR, et al.; National Institutes of Health Antimicrobial Resistance Outcomes Research Initiative (NIH–ARORI). Difficult-to-treat resistance in gram-negative bacteremia at 173 US hospitals: retrospective cohort analysis of prevalence, predictors, and outcome of resistance to all first-line agents. Clin Infect Dis. 2018;67:1803–14.
9. Soe MM, Edwards JR, Sievert DM, Ricks PM, Magill SS, Fridkin SK. Evaluating state-specific antibiotic resistance measures derived from central line-associated bloodstream infections, national healthcare safety network, 2011. Infect Control Hosp Epidemiol. 2015;36:54–64. http://dx.doi.org/10.1017/ice.2014.11
10. Talan DA, Takhar SS, Krishnadasan A, Abrahamian FM, Mower WR, Moran GJ; EMERGEncy ID Net Study Group. Fluoroquinolone-resistant and extended-spectrum β-lactamase–producing *Escherichia coli* infections in patients with pyelonephritis, United States. Emerg Infect Dis. 2016;22. http://dx.doi.org/ 10.3201/eid2209.160148
11. Frazee BW, Trivedi T, Montgomery M, Petrovic D-F, Yamaji R, Riley L. Emergency department urinary tract infections caused by extended-spectrum β-lactamase–producing *Enterobacteriaceae*: many patients have no identifiable risk factor and discordant empiric therapy is common. Ann Emerg Med. 2018;72:449–56. http://dx.doi.org/10.1016/j.annemergmed.2018.05.006

Address for correspondence: Kyle Rizzo, California Department of Public Health, Healthcare-Associated Infections Program, 850 Marina Bay Pkwy, Building E, 1st Floor, Richmond, CA 94804, USA; email: kyle.rizzo@cdph.ca.gov

etymologia

Carbapenem [kahr″bə-pen′əm]

Ronnie Henry

A class of broad-spectrum β-lactam antibiotics, structurally similar to penicillins, with the substitution of a carbon atom (*carba*-) for a sulfur atom. This substitution creates a double bond on the pentane ring, which becomes a pentene ring (*-penem*).

The first carbapenem, thienamycin (*theion* ["sulfur"] + *enamine* [an unsaturated compound that forms the backbone of the molecule] + -mycin [suffix for drugs produced by *Streptomyces* spp.]), was discovered in 1976 in culture broths of the newly recognized species *Streptomyces cattleya*. Thienamycin rapidly decomposes in the presence of water, which limits its clinical utility.

The first carbapenem approved for use in the United States was imipenem, the stable N-formimidoyl derivative of thienamycin, in 1985. Resistance to imipenem, encoded on a mobile genetic element, was first identified in *Pseudomonas aeruginosa* in Japan in 1991, and carbapenemase-producing organisms have since spread globally.

Sources

1. Kahan JS, Kahan FM, Goegelman R, Currie SA, Jackson M, Stapley EO, et al. Thienamycin, a new beta-lactam antibiotic. I. Discovery, taxonomy, isolation and physical properties. J Antibiot (Tokyo). 1979;32:1–12. http://dx.doi.org/10.7164/antibiotics.32.1
2. Kesado T, Hashizume T, Asahi Y. Antibacterial activities of a new stabilized thienamycin, N-formimidoyl thienamycin, in comparison with other antibiotics. Antimicrob Agents Chemother. 1980;17:912–7. http://dx.doi.org/10.1128/AAC.17.6.912
3. Meletis G. Carbapenem resistance: overview of the problem and future perspectives. Ther Adv Infect Dis. 2016;3:15–21. http://dx.doi.org/10.1177/2049936115621709

Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E28, Atlanta, GA 30329-4027, USA; email: boq3@cdc.gov

DOI: https://doi.org/10.3201/eid2507.ET2507

Whole-Blood Testing for Diagnosis of Acute Zika Virus Infections in Routine Diagnostic Setting

Jolanda J.C. Voermans, Suzan D. Pas,[1] Anne van der Linden, Corine GeurtsvanKessel, Marion Koopmans, Annemiek van der Eijk, Chantal B.E.M. Reusken[2]

We evaluated the benefit of whole blood versus plasma to detect acute Zika virus infections. Comparison of Zika virus quantitative reverse transcription PCR results in single timepoint whole blood–plasma pairs from 227 patients with suspected Zika virus infection resulted in confirmation of 8 additional patients with Zika virus infection.

Since its emergence in South and Central America in 2015/2016, Zika virus (genus *Flavivirus*) has become a major public health concern. Zika virus infections are linked to congenital malformations in neonates from mothers infected during pregnancy and to neurologic disorders in adults (*1*). Thus, the stakes for an accurate diagnosis are high when congenital Zika syndrome might be involved, such as in diagnosis in pregnant women and their partners, because Zika virus infections can be sexually transmitted (*1*). Diagnostics are based on Zika virus RNA detection, detection of Zika virus–specific antibodies, or both. However, a definitive diagnosis based on serology only is hampered by the existence of a high degree of cross-reactivity between Zika virus and other flaviviruses and their vaccines. In addition, populations with a high background of other flavivirus infections, such as dengue virus, might lack high-titer Zika virus–specific antibody production (also known as original antigenic sin) (*2*,*3*). Reverse transcription PCR (RT-PCR) is the most reliable method for confirming Zika virus infections. Viremia in pregnant women can be prolonged, up to 70 days, but more commonly the window of detection for Zika virus RNA in serum or plasma is much shorter (3–14 days after onset of symptoms). The window of detection can be considerably longer for urine and semen, but these specimens are not routinely collected (*4*–*7*).

Various studies have suggested that flavivirus genomic RNA might be detectable for longer periods in whole blood than in plasma, thereby expanding the timeframe for viral genome detection to up to 120 days after onset of symptoms (*4*,*5*,*8*–*11*). Therefore, molecular detection of Zika virus RNA in whole blood instead of plasma might improve Zika virus case confirmation (*12*,*13*). In a prospective study, we systematically evaluated the benefit of whole blood versus plasma as the sample of choice to detect acute Zika virus infections in a routine diagnostic setting.

The Study

We compared Zika virus quantitative reverse transcription PCR (qRT-PCR) results for 249 EDTA–whole blood and EDTA–plasma pairs submitted for laboratory testing from 227 patients with suspected Zika virus infection during July 2016–May 2017. These patients were those with a Zika virus diagnostic request in this period from whom both plasma and whole blood could be collected. In line with previous observations in our laboratory (*14*), the first day of illness was provided infrequently, in only 29 (12.8%) of the 227 patients.

Using a standard EDTA blood collection tube, we aliquoted 600 μL of whole blood before the centrifugation step (10 min at 2,400 × *g*) to collect plasma. We stored the samples at –80°C until use. For testing, we spiked the samples with an internal control and extracted total nucleic acids from a 500-μL sample in 100 μL of eluate using the MagNAPure 96 DNA and Viral NA large volume kit and Viral NA Universal LV 2.0 protocol (Roche, https://www.roche.com), according to the manufacturer's instructions. Extraction was followed by an ISO15189:2012-validated laboratory-developed Zika virus qRT-PCR, as described previously (*15*). We confirmed all Zika virus RNA–positive samples using a commercial Zika virus qRT-PCR (Altona Diagnostics, http://www.altona-diagnostics.com), as described by the manufacturer.

We detected Zika virus RNA in 31 (12.4%) of 249 whole-blood samples and in 23 (74.2%) of the 31 corresponding plasma samples. The 31 positive whole-blood samples were collected from 31 individual patients. This comparison indicated that 8 additional Zika virus–positive patients would have been identified if whole blood had been used routinely

Author affiliation: Erasmus Medical Center, Rotterdam, the Netherlands

DOI: https://doi.org/10.3201/eid2507.182000

[1]Current affiliation: Microvida, Roosendaal, the Netherlands.

[2]Current co-affiliation: Netherlands Centre for Infectious Disease Control, National Institute for Public Health and the Environment, Bilthoven, the Netherlands.

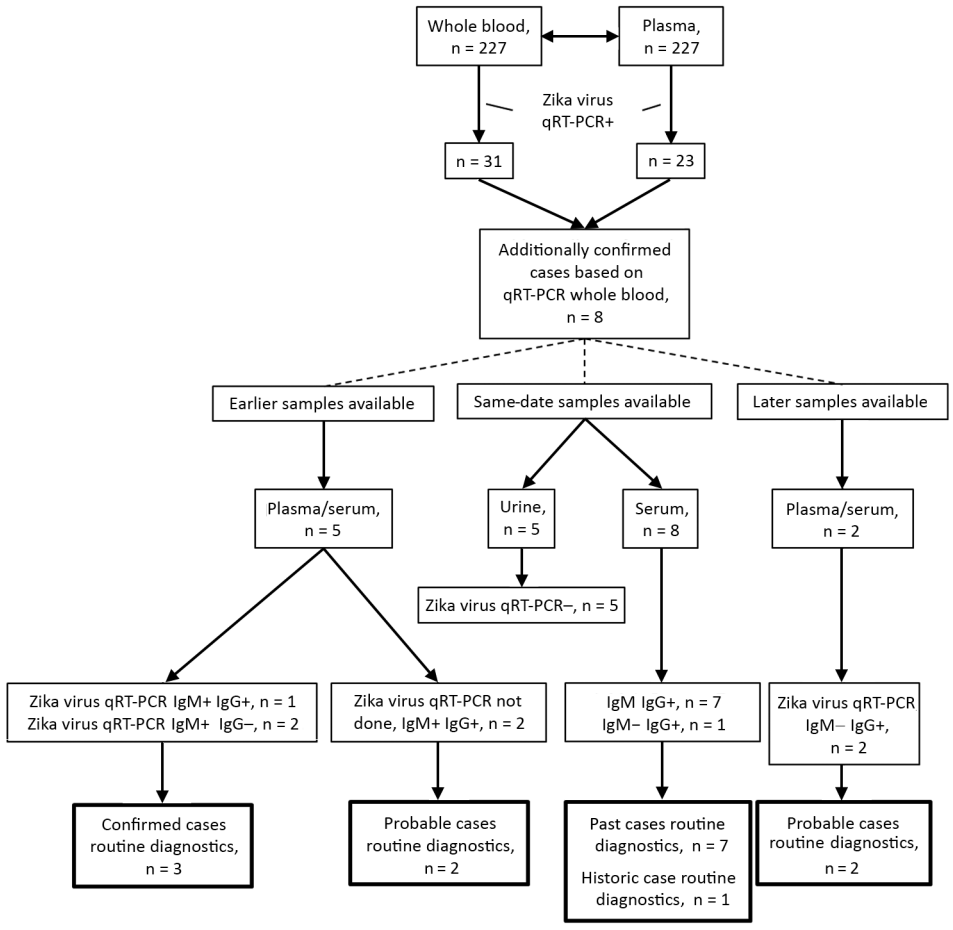


Figure. Overview of results of Zika virus diagnostic testing on total sample sets for 8 Zika patients additionally confirmed with Zika virus infection on the basis of whole-blood qRT-PCR. IgG, Zika virus IgG ELISA; IgM, Zika virus IgM ELISA; qRT-PCR, quantitative reverse transcription PCR; +, positive; –, negative.

instead of plasma (Figure). This finding represented a 34% increase in confirmed cases of Zika virus infection.

Standard practice in international guidelines on diagnostic algorithms for Zika virus is to combine molecular testing of plasma with molecular testing of urine, along with serology, to come to an accurate Zika virus diagnosis. However, confirmation of cases based on serology only is usually limited to expert Biosafety Level 3 laboratories being able to perform virus neutralization tests comparing Zika virus titers with titers of other flaviviruses (*12*,*13*). In our center, we routinely perform qRT-PCR on plasma and urine while running ELISA IgM/IgG testing in parallel on corresponding serum samples, provided these samples are submitted by treating physicians. Preferably, ELISAs are performed on paired serum samples taken at least 2 weeks apart (acute and convalescent phases) to monitor titer changes. However, these paired samples are not always submitted; for example, in our study cohort a second sample was provided for only 11 (61.1%) of 18 patients who were seropositive by ELISA and RT-PCR negative in plasma in the initial sample.

To determine whether our routine Zika virus testing algorithm, in which whole blood is not a sample of choice, would have missed the 8 additional identified patients, we evaluated the Zika virus test results of the complete sample set submitted for these patients (Figure). We tested urine and plasma by qRT-PCR as described previously and tested serum by ELISA (Euroimmun, https://www.euroimmun.com) for the presence of Zika virus–specific IgM and IgG, as described by the manufacturer. For 3 of the 8 additional patients, Zika virus infection had already been confirmed on the basis of the presence of Zika virus RNA and IgM in an earlier plasma sample. For the remaining 5 patients, only a status of probable case was achieved without the whole-blood testing (*12*). Two of these patients had a status of probable infection on the basis of the presence of Zika virus IgM and IgG in an earlier sample, but no PCR was performed. Seven patients had the status of a probable Zika virus infection on the basis of serology performed on a same-date serum sample, and 1 patient had the status of past infection because of the absence of IgM. Two patients had no evidence for a recent Zika virus infection on the basis of a later serum sample that tested negative for RNA IgM and positive for IgG. The semiquantitative Zika virus ELISA did not show significant titer changes between different collection dates (data not shown).

Conclusions

Our overall results indicate that, in our routine diagnostic algorithm in the absence of whole-blood testing, the infections of 5 of 227 patients would have been identified as probable Zika virus cases, whereas with whole-blood testing, they would have been identified as confirmed cases on the basis of positive qRT-PCR results. In cases for which only 1 sampling date would have been available, our systematic analysis showed that, of infections in 227 patients, 8 additional Zika virus cases would have been confirmed. Based on these observations, we conclude that individual patient care might benefit from whole-blood testing in a routine diagnostic laboratory setting, thereby possibly reducing the need for more specialized serology (i.e., comparative flavivirus neutralization tests) to confirm cases based on serology. Therefore, we have implemented whole-blood RT-PCR testing for Zika virus diagnostic requests in our routine diagnostic setup. Further studies in larger cohorts, including dengue and chikungunya virus testing, as well to address the often multiplex settings in endemic countries, are needed to demonstrate the general usefulness of our observations.

The work described here was funded by the European Union's Horizon 2020 research and innovation program under project ZIKALLIANCE (grant agreement no. 734548).

About the Author

Ms. Voermans works in the molecular diagnostics unit of the department of viroscience at Erasmus University Medical Centre, Rotterdam, the Netherlands. She focuses on diagnostic research for emerging viruses of public health importance.

References

1. Pierson TC, Diamond MS. The emergence of Zika virus and its new clinical syndromes. Nature. 2018;560:573–81. http://dx.doi.org/10.1038/s41586-018-0446-y
2. Charrel RN, Leparc-Goffart I, Pas S, de Lamballerie X, Koopmans M, Reusken C. Background review for diagnostic test development for Zika virus infection. Bull World Health Organ. 2016;94:574–584D. http://dx.doi.org/10.2471/BLT.16.171207
3. Lustig Y, Cotar AI, Ceianu CS, Castilletti C, Zelena H, Burdino E, et al. Lack of Zika virus antibody response in confirmed patients in non-endemic countries. J Clin Virol. 2018;99–100:31–4. http://dx.doi.org/10.1016/j.jcv.2017.12.007
4. Rossini G, Gaibani P, Vocale C, Cagarelli R, Landini MP. Comparison of Zika virus (ZIKV) RNA detection in plasma, whole blood and urine—case series of travel-associated ZIKV infection imported to Italy, 2016. J Infect. 2017;75:242–5. http://dx.doi.org/10.1016/j.jinf.2017.05.021
5. Joguet G, Mansuy JM, Matusali G, Hamdi S, Walschaerts M, Pavili L, et al. Effect of acute Zika virus infection on sperm and virus clearance in body fluids: a prospective observational study. Lancet Infect Dis. 2017;17:1200–8. http://dx.doi.org/10.1016/S1473-3099(17)30444-9
6. Suy A, Sulleiro E, Rodó C, Vázquez É, Bocanegra C, Molina I, et al. Prolonged Zika virus viremia during pregnancy. N Engl J Med. 2016;375:2611–3. http://dx.doi.org/10.1056/NEJMc1607580
7. Lozier MJ, Rosenberg ES, Doyle K, Adams L, Klein L, Muñoz-Jordan J, et al. Prolonged detection of zika virus nucleic acid among symptomatic pregnant women: a cohort study. Clin Infect Dis. 2018;67:624–7. http://dx.doi.org/10.1093/cid/ciy209
8. Lustig Y, Mendelson E, Paran N, Melamed S, Schwartz E. Detection of Zika virus RNA in whole blood of imported Zika virus disease cases up to 2 months after symptom onset, Israel, December 2015 to April 2016. Euro Surveill. 2016;21:30269. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.26.30269
9. Lustig Y, Mannasse B, Koren R, Katz-Likvornik S, Hindiyeh M, Mandelboim M, et al. Superiority of West Nile virus RNA detection in whole blood for diagnosis of acute infection. J Clin Microbiol. 2016;54:2294–7. http://dx.doi.org/10.1128/JCM.01283-16
10. Huang GKL, Tio SY, Caly L, Nicholson S, Thevarajan I, Papadakis G, et al. Prolonged detection of Japanese encephalitis virus in urine and whole blood in a returned short-term traveler. Open Forum Infect Dis. 2017;4:ofx203. http://dx.doi.org/10.1093/ofid/ofx203
11. El Sahly HM, Gorchakov R, Lai L, Natrajan MS, Patel SM, Atmar RL, et al. Clinical, virologic, and immunologic characteristics of Zika virus infection in a cohort of US patients: prolonged RNA detection in whole blood. Open Forum Infect Dis. 2018;6:ofy352.
12. World Health Organization. Zika virus disease Interim case definitions, 12 February 2016. 2016 [cited 2018 Oct 8]. http://apps.who.int/iris/bitstream/handle/10665/204381/WHO_ZIKV_SUR_16.1_eng.pdf?sequence=1
13. European Commission. Commission implementing decision (EU) 2018/945 of 22 June 2018 on the communicable diseases and related special health issues to be covered by epidemiological surveillance as well as relevant case definitions 2018 [cited 2018 Oct 9]. https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32018D0945&from=EN#page=53
14. Cleton N, Reusken C, Murk JL, de Jong M, Reimerink J, van der Eijk A, et al. Using routine diagnostic data as a method of surveillance of arboviral infection in travellers: a comparative analysis with a focus on dengue. Travel Med Infect Dis. 2014;12:159–66. http://dx.doi.org/10.1016/j.tmaid.2013.10.015
15. van der Eijk AA, van Genderen PJ, Verdijk RM, Reusken CB, Mögling R, van Kampen JJ, et al. miscarriage associated with Zika virus infection. N Engl J Med. 2016;375:1002–4. http://dx.doi.org/10.1056/NEJMc1605898

Address for correspondence: Chantal B.E.M. Reusken, Erasmus MC, Viroscience, PO Box 2040, 3000CA Rotterdam, the Netherlands; email: c.reusken@erasmusmc.nl

Dengue Outbreak during Ongoing Civil War, Taiz, Yemen

KhairAlah A. Alghazali, Boon-Teong Teoh, Shih-Keng Loong, Sing-Sin Sam, Nurul-Asma-Anati Che-Mat-Seri, Nur-Izyan Samsudin, Che-Norainon Yaacob, Noor-Syahida Azizan, Adrian Oo, Nur-Adilah Baharudin, Kim-Kee Tan, Juraina Abd-Jamil, Siti-Sarah Nor'e, Chee-Sieng Khor, Jefree Johari, Mohammed A.K. Mahdy, Sazaly AbuBakar

We identified dengue in ≈51% of patients given a clinical diagnosis of suspected dengue in Taiz, Yemen, during 2016. The cosmopolitan genotype of dengue virus type 2 was most common; viruses appeared to have originated in Saudi Arabia. Damage to public health infrastructure during the ongoing civil war might enable dengue to become endemic to Yemen.

The association between wars and dengue virus (DENV) transmission has been well-recognized. During World War II (1939–1945), extensive ecologic disruption and demographic changes created an abundance of ideal breeding sites for *Aedes aegypti* mosquitoes, as well as pools of susceptible military personnel and displaced populations to support the spread of dengue (*1*). After World War II, unprecedented population growth and rapid unplanned urbanization exacerbated the epidemic spread of dengue to the major cities of Southeast Asia and the Pacific regions (*2*).

The current civil war in Yemen, which started in March 2015 (*3*), has caused widespread destruction of the infrastructure of this country and displaced >2.2 million persons into living in cramped shelters with inadequate healthcare support. The sum of human displacement, damaged infrastructure, and poor hygiene conditions (*3*–*5*) has created an ideal environment for the spread of infectious diseases, including mosquitoborne diseases. In particular, the war has created numerous potential mosquito-breeding sites, such as open water storage containers, areas with inadequate drainage, discarded plastic containers in which water accumulates, and puddles of water (*6*).

In 2015, a total of ≥6,777 suspected dengue cases were recorded in Yemen, which suggested that the country was experiencing an unprecedented increase in the number of dengue cases (*7*). Taiz, a governorate in southwestern Yemen, experienced fierce fighting related to the civil war (*5*). An extreme spike in dengue cases was recorded in this governorate beginning in August 2015, soon after the start of the war. A total of 1,178 suspected dengue cases were reported during weeks 32–36 (*7*) in comparison to only 54 suspected dengue cases during the same period in 2013 (*8*). We report the prevalence, detection, and isolation of DENV from febrile patients seen at the few operating healthcare facilities in Taiz during July–October 2016, at the height of the war.

The Study

The study was approved by the University of Malaya Science and Technology Medical Ethical Committee (approval no. 2016/24). A total of 436 serum specimens were obtained from patients with clinically suspected dengue (age range 1–70 years) who sought healthcare within 2–7 days after the onset of fever (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/25/7/18-0046-App1.pdf). These patients were seen at the main hospitals and medical centers that were still in operation (Figure 1) in Taiz during the study period. Samples were kept at –20°C until they were transported to the World Health Organization Collaborating Centre for Arbovirus Reference and Research (Dengue/Severe Dengue) at the Tropical Infectious Diseases Research and Education Centre, University of Malaya (Kuala Lumpur, Malaysia).

For detection of DENV, we extracted total RNA from serum specimens by using the KingFisher Pure Viral NA Kit (Thermo Fisher Scientific, https://www.thermofisher.com). We used a DENV-specific reverse transcription–recombinase polymerase amplification (RT-RPA) to screen for DENV RNA (*9*). We subjected all RT-RPA–reactive specimens to molecular DENV serotyping by using DengueDetect, an in-house multiplex reverse transcription PCR (RT-PCR) kit (http://www.tidrec.com/services). We tested all RT-RPA–nonreactive specimens for IgM against DENV by using the SD Dengue IgM-Capture ELISA Kit (Standard Diagnostics Inc., https://www.devex.com). All

Author affiliations: University of Malaya, Kuala Lumpur, Malaysia (K.A. Alghazali, B.-T. Teoh, S.-K. Loong, S.-S. Sam, N.-A.-A. Che-Mat-Seri, N.-I. Samsudin, C.-N. Yaacob, N.-S. Azizan, A. Oo, N.-A. Baharudin, K.-K. Tan, J. Abd-Jamil, S.-S. Nor'e, C.-S. Khor, J. Johari, S. AbuBakar); University of Science and Technology, Sana'a, Yemen (M.A.K. Mahdy)

DOI: https://doi.org/10.3201/eid2506.180046

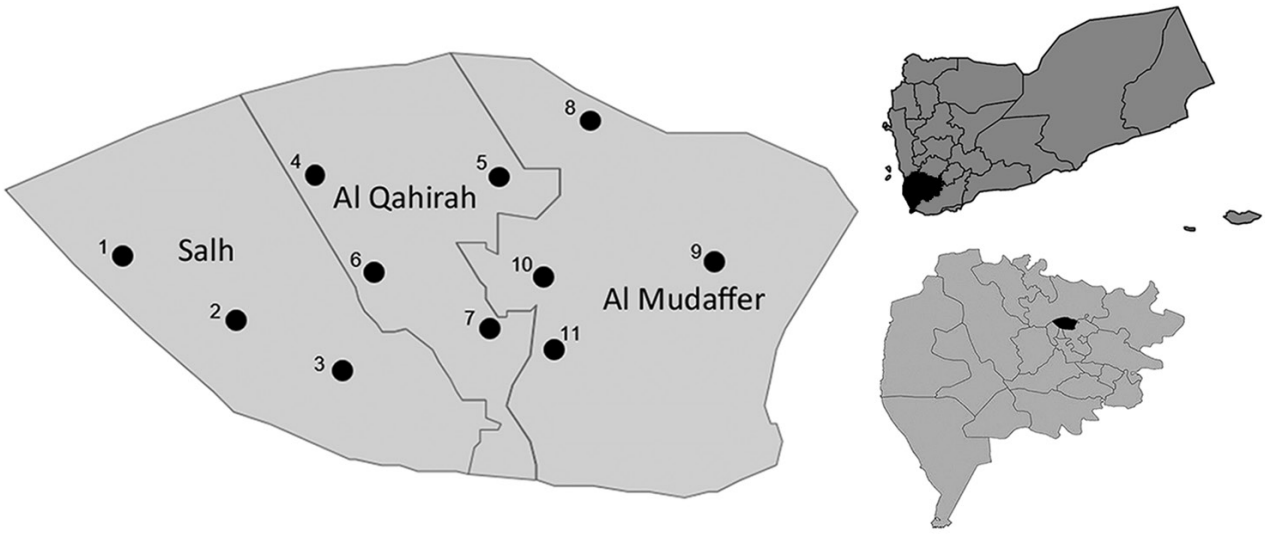


Figure 1. Locations of hospitals and medical centers in Taiz, Yemen, where dengue virus samples were obtained during 2016: 1, Aljawharah Medical Center; 2, Gulf Hospital; 3, Alrefaee Hospital; 4, Althawrah Hospital; 5, Altawn Hospital; 6, Alrawdhah Hospital; 7, Alsawidy Hospital; 8, Palastein Hospital; 9, Alboraihy Hospital; 10, Alhekmah Hospital; 11, Dr. Sadek Shogaa Center. Top inset shows location of Taiz in Yemen (black shading), and bottom inset shows location of collection area in Taiz (black shading).

DENV IgM–nonreactive specimens were tested for DENV IgG by using the SD Dengue IgG-Capture ELISA Kit (Standard Diagnostics Inc.). All diagnostic tests were performed in an MS ISO/IEC 17025–accredited laboratory at the Tropical Infectious Diseases Research and Education Centre, University of Malaya.

Of 436 patients with clinically suspected dengue, 119 (≈27%) were reactive by RT-RPA; among those patients, 83 (≈70%) were serotyped by RT-PCR as DENV-2. The remaining 36 RT-RPA–reactive specimens were nonreactive by RT-PCR. However, the RT-RPA is known to be more sensitive than the RT-PCR; thus, this result could be caused by low viral loads in the specimens (*9*). All RT-PCR–reactive specimens were further subjected to virus isolation by using the C6/36 mosquito cell line. Complete envelope (E) genes of DENV-2 were successfully amplified by using primers specific for the DENV-2 E gene (Appendix Table) and sequenced from 9 of 83 specimens after a third passage in cell culture. A phylogenetic tree constructed by using E genes suggested that the DENV-2 isolates from Yemen clustered within the DENV-2 Cosmopolitan genotype (Figure 2). The E gene sequences generated in this study are available from the European Nucleotide Archive (accession no. PRJEB27739).

Of the 317 RT-RPA–nonreactive specimens, 102 (≈32%) were positive for DENV IgM. Of 215 dengue IgM–nonreactive samples, 74 (≈34%) were positive for DENV IgG. Those samples positive for only DENV IgG were likely caused by previous exposure to DENV infection. We identified acute DENV infection in ≈51% (221 of 436) of febrile patients with suspected dengue either by RT-RPA or DENV IgM ELISA. In addition, ≈34% (74 of 215) of febrile patients who did not have dengue had previous exposure to dengue.

Conclusions

In this study, the prevalence of dengue among patients with suspected dengue (≈51%) in Taiz was higher than that (29%) during the previous dengue outbreak in Hodeidah in 2012 (*10*). The percentage of previous exposure to DENV among febrile patients was higher in Hodeidah (≈75%) than that seen in Taiz (≈34%) (*10*). It is possible that dengue is a relatively new disease in Taiz, but its prevalence has increased markedly, possibly because of the ongoing civil war. The febrile patients who did not have dengue seen in Taiz could have had other infections, including chikungunya and malaria, because these 2 mosquitoborne infectious diseases are also present in Yemen (chikungunya prevalence 12% and malaria prevalence 15.3%) (*10,11*). Nonetheless, in a previous study in Al-Mukalla, 222 patients with clinically suspected viral hemorrhagic fever were nonreactive to chikungunya virus, Alkhurma virus, Rift Valley fever virus, and yellow fever virus by RT-PCR (*12*), suggesting that other studies are needed to identify the possible causative agents.

Before the current civil war began, all 4 DENV serotypes were present in Yemen, but only DENV-2 (*10*), DENV-3 (*12,13*), and DENV-4 (*14*) have been documented to cause outbreaks. Using partial nonstructural protein 1 gene sequences, Ciccozzi et al. identified the DENV-2 Cosmopolitan genotype as the virus that caused the outbreak in Hodeidah during 2012 (*15*). We report that this virus type was also found as the dominant virus causing the outbreak in Taiz during 2016. However, the 2012 and 2016 viruses grouped in different clades within the DENV-2 Cosmopolitan

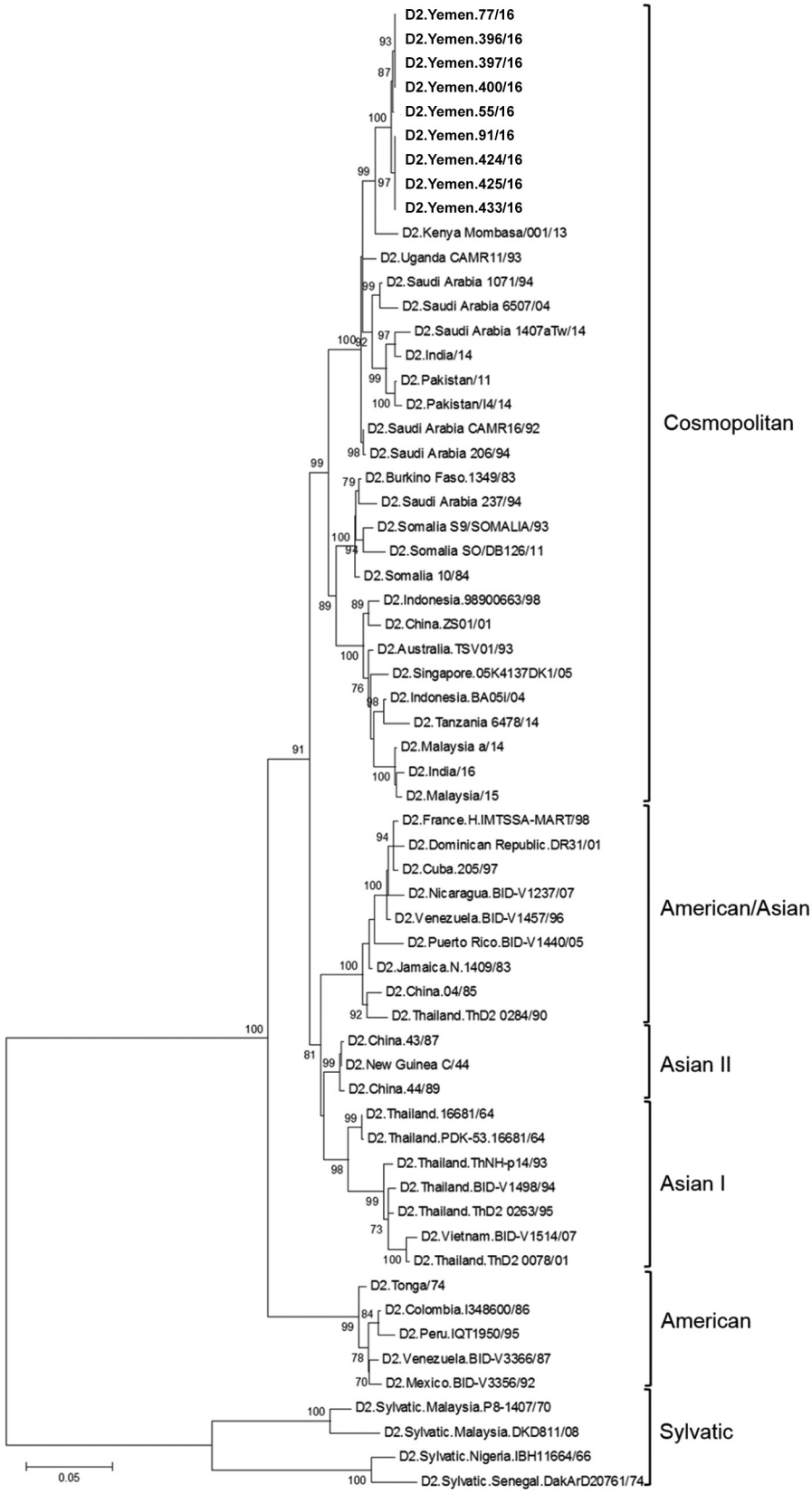


Figure 2. Maximum-likelihood phylogenetic tree of dengue virus type 2 isolates from Taiz, Yemen, 2016 (top branch), and reference isolates. The tree was constructed by using envelope gene sequences. Numbers on nodes indicate bootstrap values (%) for 1,000 replicates. Only bootstrap values ≥70% are indicated. Scale bar indicates nucleotide substitutions per site.

genotype (Appendix Figure 2). The origin of the viruses that caused the outbreak during 2012 is uncertain, whereas the viruses that caused the outbreak during 2016 grouped in a clade composed mainly of DENV-2 isolates (1992–2014) from Saudi Arabia. This finding suggests that the DENV-2 that caused the outbreak in Yemen during 2016 was most likely introduced from Saudi Arabia and that the ongoing civil war might ensure its lasting presence.

Dengue is emerging to be a serious mosquitoborne disease in war-torn Yemen. Its presence among 51% of febrile patients suspected to have dengue is almost comparable to the percentage reported in dengue-endemic countries of Southeast Asia. It is expected that the public health problems associated with dengue will worsen during the continuing civil war in Yemen.

This study was supported in part by the Ministry of Science, Technology and Innovation Malaysia (Project FP0514D0025-2 [Work Package 2 of the Dasar Sains Teknologi dan Inovasi Negara DSTIN Flagship Program]), the Ministry of Higher Education Malaysia (Fundamental Research Grant Scheme grant FP013-2017A), the University of Malaya (grants RU008-2018 and PG207-2016A), and the University of Malaya Centre of Excellence Top 100 Research Grants (grant UM.00000188/HGA.GV).

About the Author

Mr. Alghazali is a PhD candidate at the University of Malaya, Kuala Lumpur, Malaysia. His primary research interest is dengue epidemiology in Yemen.

References

1. Sabin AB. Research on dengue during World War II. Am J Trop Med Hyg. 1952;1:30–50. http://dx.doi.org/10.4269/ajtmh.1952.1.30
2. Gubler DJ. Dengue and dengue hemorrhagic fever. Clin Microbiol Rev. 1998;11:480–96. http://dx.doi.org/10.1128/CMR.11.3.480
3. Burki T. Yemen health situation "moving from a crisis to a disaster". Lancet. 2015;385:1609. http://dx.doi.org/10.1016/S0140-6736(15)60779-6
4. Cholera in Yemen: war, hunger, disease…and heroics. Lancet Infect Dis. 2017;17:781. http://dx.doi.org/10.1016/S1473-3099(17)30406-1
5. Baron A, Ugolini M, Marini L, Goeminne P, Davies G. Mapping the Yemen conflict. European Council of Foreign Relations [cited 2017 Aug 24]. http://www.ecfr.eu/mena/yemen
6. Mosquitoes winning Yemen's war. Integrated Regional Information Networks, 2015 [cited 2017 Sep 17]. http://www.irinnews.org/feature/2015/09/10/mosquitoes-winning-yemens-war
7. World Health Organization. Yemen conflict. Situation report number 14–15. 2015 [cited 2019 Mar 23]. https://www.who.int/hac/crises/yem/sitreps/en
8. World Health Organization. Yemen. Wkly Epidemiol Bulletin. 2013;1:22–6.
9. Teoh BT, Sam SS, Tan KK, Danlami MB, Shu MH, Johari J, et al. Early detection of dengue virus by use of reverse transcription-recombinase polymerase amplification. J Clin Microbiol. 2015;53:830–7. http://dx.doi.org/10.1128/JCM.02648-14
10. Rezza G, El-Sawaf G, Faggioni G, Vescio F, Al Ameri R, De Santis R, et al. Co-circulation of dengue and chikungunya viruses, Al Hudaydah, Yemen, 2012. Emerg Infect Dis. 2014;20:1351–4. http://dx.doi.org/10.3201/eid2008.131615
11. Abdulsalam MQAM, Mohamme AKM, Ahmed AA, Fong MY. Clinical situation of endemic malaria in Yemen. Trop Biomed. 2010;27:551–8.
12. Madani TA, Abuelzein TM, Al-Bar HM, Azhar EI, Kao M, Alshoeb HO, et al. Outbreak of viral hemorrhagic fever caused by dengue virus type 3 in Al-Mukalla, Yemen. BMC Infect Dis. 2013;13:136. http://dx.doi.org/10.1186/1471-2334-13-136
13. Bin Ghouth AS, Amarasinghe A, Letson GW. Dengue outbreak in Hadramout, Yemen, 2010: an epidemiological perspective. Am J Trop Med Hyg. 2012;86:1072–6. http://dx.doi.org/10.4269/ajtmh.2012.11-0723
14. Alahdal M, Al-Shabi J, Ogaili M, Abdullah QY, Alghalibi S, Jumaan AO, et al. Detection of dengue fever virus serotype-4 by using one-step real-time RT-PCR in Hodeidah, Yemen. Br Microbiol Res J. 2016;14:24380. http://dx.doi.org/10.9734/BMRJ/2016/24380
15. Ciccozzi M, Lo Presti A, Cella E, Giovanetti M, Lai A, El-Sawaf G, et al. Phylogeny of dengue and chikungunya viruses in Al Hudayda Governorate, Yemen. Infect Genet Evol. 2014;27:395–401. http://dx.doi.org/10.1016/j.meegid.2014.08.010

Address for correspondence: Sazaly AbuBakar, Tropical Infectious Diseases Research and Education Centre and Department of Medical Microbiology, Faculty of Medicine, University of Malaya, 50603 Kuala Lumpur, Malaysia; email: sazaly@um.edu.my

Nontuberculous Mycobacteria, Botswana, 2011–2014

Bontle Mbeha, Madisa Mine, Modisa Sekhamo Motswaledi, John Dewar

We documented a 6-fold increase in the frequency of nontuberculous mycobacteria isolated from clinical samples in Botswana during 2011–2014. Because antituberculosis treatment is often initiated only on the basis of acid-fast bacilli smear-positive microscopy results, some patients with nontuberculous mycobacterial infections might have received inappropriate treatment.

Nontuberculous mycobacteria (NTM) isolated from clinical samples are often classified as contaminants (*1*). However, some NTM species are pathogenic to humans (*2*) and, in some parts of the world, cause more illness than infection with *Mycobacterium tuberculosis* does (*3*).

In Botswana, as in many other developing countries, patients with acid-fast bacilli–positive sputum are presumed to be infected with *M. tuberculosis* and are treated with antituberculosis agents (*4*), even though acid-fast bacilli smear microscopy does not distinguish between *M. tuberculosis* and NTM, and most antituberculosis drugs might not be effective against NTM (*5*). This observation, along with the number of increasing reports of NTM worldwide (*1*,*4*,*6*–*9*), prompted this study.

The Study

During October 2015, we retrospectively analyzed 36,242 electronic records from 2011–2014 that were stored at the National Tuberculosis Reference Laboratory (Gaborone, Botswana) (Table). The records represented specimens referred for tuberculosis (TB) culture from 52 facilities across the country. Demographic parameters comprised age and sex. We compared proportions by calculating z values from observed frequencies, then used an online calculator to derive p values.

Author affiliations: National Tuberculosis Reference Laboratory, Gaborone, Botswana (B. Mbeha); National Health Laboratory, Gaborone (M. Mine); University of Botswana, Gaborone (M.S. Motswaledi); University of South Africa, Pretoria, South Africa (J. Dewar)

DOI: https://doi.org/10.3201/eid2507.181440

The proportions of *M. tuberculosis* and NTM cases during the study period were comparable (5.6% vs. 5.5%). This finding creates a high possibility of misdiagnosis and inappropriate treatment of *M. tuberculosis*/NTM cases. Most (74.5%) NTM samples were sputum; 22% were gastric aspirates, and the remaining 3.4% were obtained from other body sites. Of specimens from which NTM was isolated, 53.3% were from male patients and 43.4% were from female patients; for 3.1%, patient sex was not captured. The first specimen submitted per patient was analyzed. From the analysis we removed duplicate samples that might have resulted from submission of samples for follow-up from the same patient.

One third (33.5%) of samples from which NTM was isolated were from patients 0–14 years of age, followed by 27.3% from patients 35–54 years of age (Figure 1). For at least 207 (10.4%) of the 1,999 NTM samples, the age of the patient was not captured.

Overall, the proportion of isolated NTM increased 6-fold, from 0.113/100,000 population in 2011 to 0.693/100,000 in 2014 (the estimated Botswana population in 2017 was 2.292 million). Moreover, isolation of NTM significantly increased during 3 periods: 2011–2012, 2012–2013, and 2011–2014 ($p<0.0001$ for each period). We found no significant change in NTM occurrence for 2013–2014 (Figure 2).

Before 2013, NTM was not speciated in Botswana. In 2013, a total of 324 (39.4%) of 823 NTM isolates were speciated. Of these 324 samples, 161 (49.7%) were *M. intracellulare*, the NTM species most frequently isolated, followed by *M. malmoense* (17 [5.2%] of 324) and *M. gordonae* (15 [4.6%] of 324). Seventy-eight (24.0%) isolates could be identified only as *Mycobacterium* spp. because of a limitation of the assay used. Seventy-four (46.0%) patients in whom *M. intracellulare* was isolated were also HIV positive, suggesting that NTM might be an important opportunistic pathogen in HIV infection. *M. avium-intracellulare* has also been reported to cause most pulmonary infections in several studies (*8*,*10*,*11*). These reports are useful because failure to speciate NTM isolates or conduct drug susceptibility testing might be overlooking an emerging pathogen with potential to cause illness and even death in HIV-infected persons.

Most samples were from male patients, a finding consistent with findings by Moore et al. (*1*). This finding has

Table. Comparison of *Mycobacterium tuberculosis* and nontuberculous mycobacteria isolates, Botswana, 2011–2014*

Year	Total no. samples analyzed	*Mycobacterium tuberculosis*		Nontuberculous mycobacteria	
		No. (%) isolates	Incidence	No. (%) isolates	Incidence
2011	11,799	228 (1.9)	9.9	113 (0.96)	4.9
2012	6,357	526 (8.3)	22.9	370 (5.8)	16
2013	9,429	681 (7.2)	29.7	823 (8.7)	35.9
2014	8,657	594 (6.9)	25.9	693 (8.0)	30

*Per capita calculated on the basis of total country population. Incidence is per 100,000 population.

been attributed to risk factors such as increased rates of smoking and chronic obstructive pulmonary disease among men (*1*). However, investigators in the United States (*3*) observed a female predominance.

Conclusions

We observed a high occurrence of NTM in patients 35–54 years of age. We also noted an unexpected increase of >30% in reported NTM cases in children <14 years of age, which might represent a peculiar childhood vulnerability to NTM infections.

According to the Botswana National Tuberculosis Programme Manual, no information is available in Botswana about the prevalence of disseminated NTM (*12*). Our study provides basic but crucial data that may stimulate discussion toward policy change in the laboratory investigation and treatment of NTM isolates for better patient care.

The change in 2011 in the use of media from Lowenstein-Jensen to MGIT (Beckton-Dickinson, https://www.bd.com) culture might have contributed to an increase in NTM identification. A similar study by Chihota et al. (*13*) yielded more NTM after such a change. However, if the increase in this study resulted from a change in media, only the first year would have recorded an increase, followed by a stabilizing trend in subsequent years. Therefore, the increasing trend we observed reflects a convincing climb in NTM cases. Furthermore, our results suggest that patients with NTM might have received unnecessary TB treatment (*7*). This finding underscores the need to unequivocally identify mycobacterial isolates because NTM appears to be more frequently encountered (*9*).

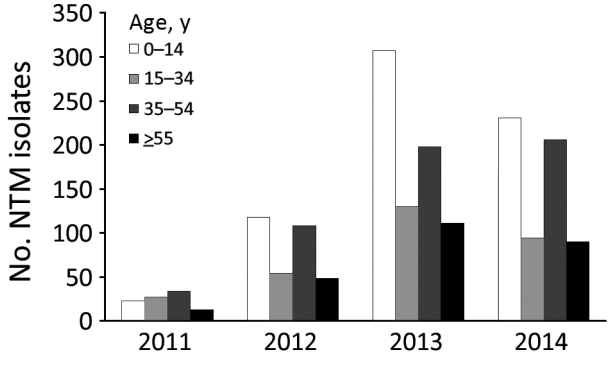


Figure 1. Age range of study population and total number of NTM isolates per year, Botswana, 2011–2014. NTM, nontuberculous mycobacteria.

Our study has limitations. Our results might not reflect the prevalence of NTM in the country because the samples analyzed comprised only persons suspected to have TB. This fact notwithstanding, the National Tuberculosis Referral Laboratory is the sole laboratory in Botswana that performs culture and thus represents a reasonable sampling of NTM countrywide among persons suspected to have TB infection. The prevalence of NTM might therefore be higher than that reported in this study.

We propose that mycobacterial isolates be routinely speciated and subjected to drug susceptibility testing. We base this proposal on our observation that NTM is isolated at nearly the same frequency as *M. tuberculosis* and is isolated predominantly in children <14 years of age. Treatment of NTM as *M. tuberculosis* underestimates the clinical significance of NTM and is likely to negatively affect treatment outcomes and inflate reports of *M. tuberculosis*.

Acknowledgments

We thank the Botswana Ministry of Health and Wellness for allowing access to the data used in this study.

About the Author

Ms. Mbeha is a Laboratory Scientific Officer at the National Tuberculosis Reference Laboratory in Gaborone, Botswana. Her primary research interests include tuberculosis pathogenesis and treatment effectiveness.

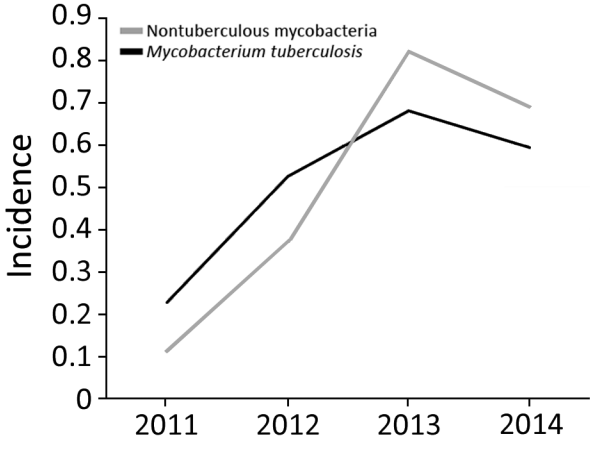


Figure 2. Incidence per 100,000 population of nontuberculous mycobacteria and *Mycobacteria tuberculosis* isolates in clinical specimens, Botswana, 2011–2014. For both, p<0.0001.

References

1. Moore JE, Kruijshaar ME, Ormerod LP, Drobniewski F, Abubakar I. Increasing reports of non-tuberculous mycobacteria in England, Wales and Northern Ireland, 1995–2006. BMC Public Health. 2010;10:612. http://dx.doi.org/10.1186/1471-2458-10-612
2. Northern Territory Department of Health. Guidelines for the control of nontuberculous mycobacteria in the Northern Territory [cited 2018 Dec 10]. https://hdl.handle.net/10137/527
3. Cassidy PM, Hedberg K, Saulson A, McNelly E, Winthrop KL. Nontuberculous mycobacterial disease prevalence and risk factors: a changing epidemiology. Clin Infect Dis. 2009;49:e124–9. http://dx.doi.org/10.1086/648443
4. Lai CC, Tan CK, Chou CH, Hsu HL, Liao CH, Huang YT, et al. Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis. 2010;16:294–6. http://dx.doi.org/10.3201/eid1602.090675
5. Zhang ZY, Sun ZQ, Wang ZL, Hu HR, Wen ZL, Song YZ, et al. Identification and pathogenicity analysis of a novel non-tuberculous mycobacterium clinical isolate with nine-antibiotic resistance. Clin Microbiol Infect. 2013;19:91–6. http://dx.doi.org/10.1111/j.1469-0691.2012.03818.x
6. Kwon YS, Koh WJ. Diagnosis of pulmonary tuberculosis and nontuberculous mycobacterial lung disease in Korea. Tuberc Respir Dis (Seoul). 2014;77:1–5. http://dx.doi.org/10.4046/trd.2014.77.1.1
7. Wu J, Zhang Y, Li J, Lin S, Wang L, Jiang Y, et al. Increase in nontuberculous mycobacteria isolated in Shanghai, China: results from a population-based study. PLoS One. 2014;9:e109736. http://dx.doi.org/10.1371/journal.pone.0109736
8. O'Brien RJ, Geiter LJ, Snider DE Jr. The epidemiology of nontuberculous mycobacterial diseases in the United States. Results from a national survey. Am Rev Respir Dis. 1987;135:1007–14.
9. Novosad S, Henkle E, Winthrop KL. The challenge of pulmonary nontuberculous mycobacterial infection. Curr Pulmonol Rep. 2015;4:152–61. http://dx.doi.org/10.1007/s13665-015-0119-3
10. Choudhri S, Manfreda J, Wolfe J, Parker S, Long R. Clinical significance of nontuberculous mycobacteria isolates in a Canadian tertiary care center. Clin Infect Dis. 1995;21:128–33. http://dx.doi.org/10.1093/clinids/21.1.128
11. Marras TK, Chedore P, Ying AM, Jamieson F. Isolation prevalence of pulmonary non-tuberculous mycobacteria in Ontario, 1997 2003. Thorax. 2007;62:661–6. http://dx.doi.org/10.1136/thx.2006.070797
12. Botswana Ministry of Health. The National Tuberculosis Programme manual. 7th ed. Gaborone (Botswana): The Ministry; 2011.
13. Chihota VN, Grant AD, Fielding K, Ndibongo B, van Zyl A, Muirhead D, et al. Liquid vs. solid culture for tuberculosis: performance and cost in a resource-constrained setting. Int J Tuberc Lung Dis. 2010;14:1024–31.

Address for correspondence: Modisa Sekhamo Motswaledi, University of Botswana, School of Allied Health Professions, Block 246/214, Gaborone, Botswana; email: Motswaledims@ub.ac.bw

Low Circulation of Subclade A1 Enterovirus D68 Strains in Senegal during 2014 North America Outbreak

Amary Fall, Mamadou Malado Jallow, Ousmane Kebe, Davy Evrard Kiori, Sara Sy, Déborah Goudiaby, Cheikh Saad Bouh Boye, Mbayame Ndiaye Niang,[1] Ndongo Dia[1]

To retrospectively investigate enterovirus D68 circulation in Senegal during the 2014 US outbreak, we retrieved specimens from 708 persons, mostly children, who had acute respiratory symptoms during September–December 2014. Enterovirus D68 was detected in 14 children (2.1%); most cases occurred in October. Phylogenetic analysis revealed that all strains clustered within subclade A1.

A 2014 outbreak of ≥1,153 cases of respiratory enterovirus (EV) D68 infection in the United States was responsible for the deaths of 13 children (*1*). This occurrence led public health officials to improve surveillance systems worldwide. Enhanced surveillance revealed a continuous spread of EV-D68 in several North and South America countries, including Canada (*2*), Chile (*3*), and Mexico (*4*), and in several countries in Europe, including Germany (*5*), Denmark (*6*), and France (*7*). However, in Africa, data on the spread of EV-D68 from the 2014 US outbreak were scarce. This retrospective study sought to confirm the circulation of EV-D68 in Senegal during the 2014 outbreak and to characterize the molecular composition of any EV-D68 strains that circulated.

The Study

For this retrospective case series, we studied records of children with acute respiratory illness (ARI) and, with consent, those of outpatients with influenza-like illness (ILI) attending sentinel sites dedicated to influenza surveillance, most recorded in Senegal during September–December 2014. For each patient, in addition to recording demographic and clinical data, healthcare personnel collected a nasopharyngeal swab specimen. As part of routine surveillance, specimens were initially tested for respiratory viruses including influenza, respiratory syncytial virus, adenoviruses, metapneumovirus, coronaviruses, parainfluenza, rhinoviruses, enteroviruses, and bocaviruses (*8*). Later, samples collected during September–December 2014 were screened for EV-D68, as described elsewhere (*9*). For the molecular studies, the viral capsid protein 1 (VP1) gene region of EV-D68 was amplified and sequenced as described elsewhere (*10*).

Specimens were collected from 708 patients, ranging in age from 1 month to 95 years, and tested for EV-D68. The median age was 9 months; 45.8% of patients were children <5 years of age. The male-to-female ratio was 0.95. EV-D68 was detected in 14 patients (2.0%): 13 of 680 with ILI and 1 of 28 with ARI. A similar rate was reported by Poelman et al. (*11*) in a Europe-wide retrospective and prospective laboratory analysis of clinical specimens during July–December 2014. A single infection was found in 5 samples; 9 samples were found to have ≥1 additional respiratory virus (Table). EV-D68 detection was associated with cough in 12 of 14 patients and rhinitis in 8 of 14. The single patient with ARI who tested positive for EV-D68 was a 7-month-old child, with no underlying disease, who had clinical signs of acute bronchitis, including dry cough, pulmonary condensation, rhinorrhea, progressive breathing difficulty, and an unusually long duration of illness of >1 month; his condition deteriorated and he was hospitalized. This extended duration, linked to EV-D68 infection, was reported elsewhere (*12*).

The highest rate of infection during 2014 (46%) was recorded in the United States, probably at the beginning of the outbreak (*1*). In Senegal, most of the EV-D68–positive cases (12/14; 86.6%) were detected in October (Figure 1). Most EV-D68–positive patients (64.3%) were children <5 years of age.

From all 14 samples testing positive for EV-D68, we successfully obtained a 900-nt fragment of the VP1 gene; we deposited these sequences into GenBank (accession nos. MH885638–51). BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) showed that all EV-D68 strains from Senegal shared >98% homology with strains detected in France (GenBank accession no. LN6813392), Canada

Author affiliations: Institute Pasteur de Dakar, Dakar, Senegal (A. Fall, M.M. Jallow, O. Kebe, D.E. Kiori, S. Sy, D. Goudiaby, M.N. Niang, N. Dia); Aristide Le Dantec Teaching Hospital, Dakar (C.S.B. Boye)

DOI: https://doi.org/10.3201/eid2507.181441

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

Table. Demographic and clinical characteristics and co-detected infections for 14 patients infected with EV-D68, Senegal, September–December 2014*

NLABID	Illness type	Patient sex	Locality	Epidemiologic week	Clinical signs/symptoms	Co-detected infections
E1462/2014	ILI	F	Tambacounda	41	Fever, cough, rhinitis	AdV, HRV
E1464/2014	ILI	M	Dakar	41	Fever, cough, rhinitis, pharyngitis	AdV, EV
E1465/2014	ILI	F	Dakar	41	Fever, cough, rhinitis, pharyngitis	AdV
E1470/2014	ILI	M	Kaolack	41	Fever, cough, pharyngitis	AdV, EV, influenza B
E1486/2014	ILI	F	Mbour	41	Fever, cough, diarrhea	AdV, RSV
E1554/2014	ILI	M	Dielmo	42	Fever, rhinitis, myalgia	EV
E0048/2014	ARI	M	Dakar	42	Fever, cough, rhinitis	None
E1560/2014	ILI	F	Dielmo	42	Fever, cough, rhinitis	Influenza B, HRV
E1583/2014	ILI	M	Dakar	43	Fever, cough	EV, influenza B
E1626/2014	ILI	F	Fatick	43	Fever, cough, vomiting	AdV, PIV, influenza B
E1704/2014	ILI	F	Ziguinchor	44	Fever, cough	None
E1709/2014	ILI	M	Mbour	44	Fever, cough	None
E1787/2014	ILI	M	Dielmo	45	Fever, rhinitis	None
E1899/2014	ILI	F	Dakar	50	Fever, cough, rhinitis	None

*AdV, adenovirus; ARI, acute respiratory infection; EV, enterovirus; HRV, human rhinovirus; ILI, influenza-like illness; NLABID, laboratory identification number; PIV, parainfluenza virus; RSV, respiratory syncytial virus.

(accession no. KP455258), and Germany (accession no. KP657740.1). Phylogenetic analysis of the VP1 fragment revealed that all 14 sequences belonged to the A1 variant of clade A (Figure 2). The strains from this study clustered with several other strains circulating during the same period in Germany, France, Philippines, Spain, Canada, the Netherlands, and Finland, with a bootstrap value of 97. However, genotype B, which had been detected in the United States, Canada, Germany, France, and other countries during the 2014 outbreak, was not found in Senegal.

The phylogenetic evidence indicates EV-D68 circulation in Senegal, approximately concurrent with, but unrelated to, the large US outbreak in 2014. Results identified EV-D68 activity from October through December 2014 in Senegal, with peak EV-D68 detection occurring in October. This aligns with findings reported in countries in Europe (*11*); in North America, peaks were mapped in August

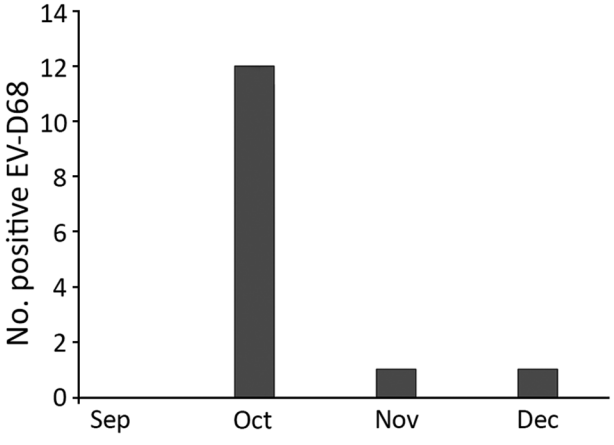


Figure 1. EV-D68 occurrence in Senegal, September–December 2014. A total of 708 nasopharyngeal samples were collected and tested for EV-68 during this period: 225 in September (0 positive), 218 in October (12 positive), 193 in November (1 positive), and 72 in December (1 positive). EV-D68, enterovirus D68.

in the United States (*13*) and in September in Canada (*14*). These results suggest circulation of the virus in Senegal 2 months after the beginning of the outbreak in the United States (*1*). This 2-month delay was also noted by Nathaniel et al. (*15*) before the introduction of EV-D68 strains in the Caribbean region. However, earlier EV-D68 circulation was reported in Latin America (*3*,*4*) and Europe (*5*–*7*).

During the outbreak period, global circulation of EV-D68 strains belonging to clade B (B1 and B2, specifically) and strains from A1 and A2 subclades were reported (*1*,*7*,*11*). All EV-D68 strains identified in Senegal for this study belonged to subclade A1, unlike in the United States, where clade B EV-D68 strains circulated during almost the same period. Phylogenetically, the US outbreak was characterized by a novel subclade, B1, which emerged rapidly and was the dominant strain. Available data indicate that the exclusive circulation of clade A in Senegal during the 2014 outbreak was reported elsewhere only in Spain. In contrast, Slovenia, Norway (*11*), the Caribbean region (*15*), Mexico (*4*), and Chile (*3*) reported only clade B over the same period.

Our study had some limitations. First, the inclusion of fever as a clinical sign for the case definition may have contributed to underestimating the number of EV-D68 infections during the North America outbreak; several studies have reported cases of afebrile EV-D68 infections (*13*). A second limitation was the small number of severe acute respiratory infection (SARI) cases in this study, because a higher proportion of detected EV-D68 in SARI patients or those with other concurrent conditions has been previously reported (*5*). At the time of data collection, the SARI sentinel surveillance system we used was not as developed as it is now. Inclusion in the screening of a larger number of hospitalized patients would probably present a more accurate picture of EV-D68 circulation. Finally, we conducted a retrospective study. The database we used contained

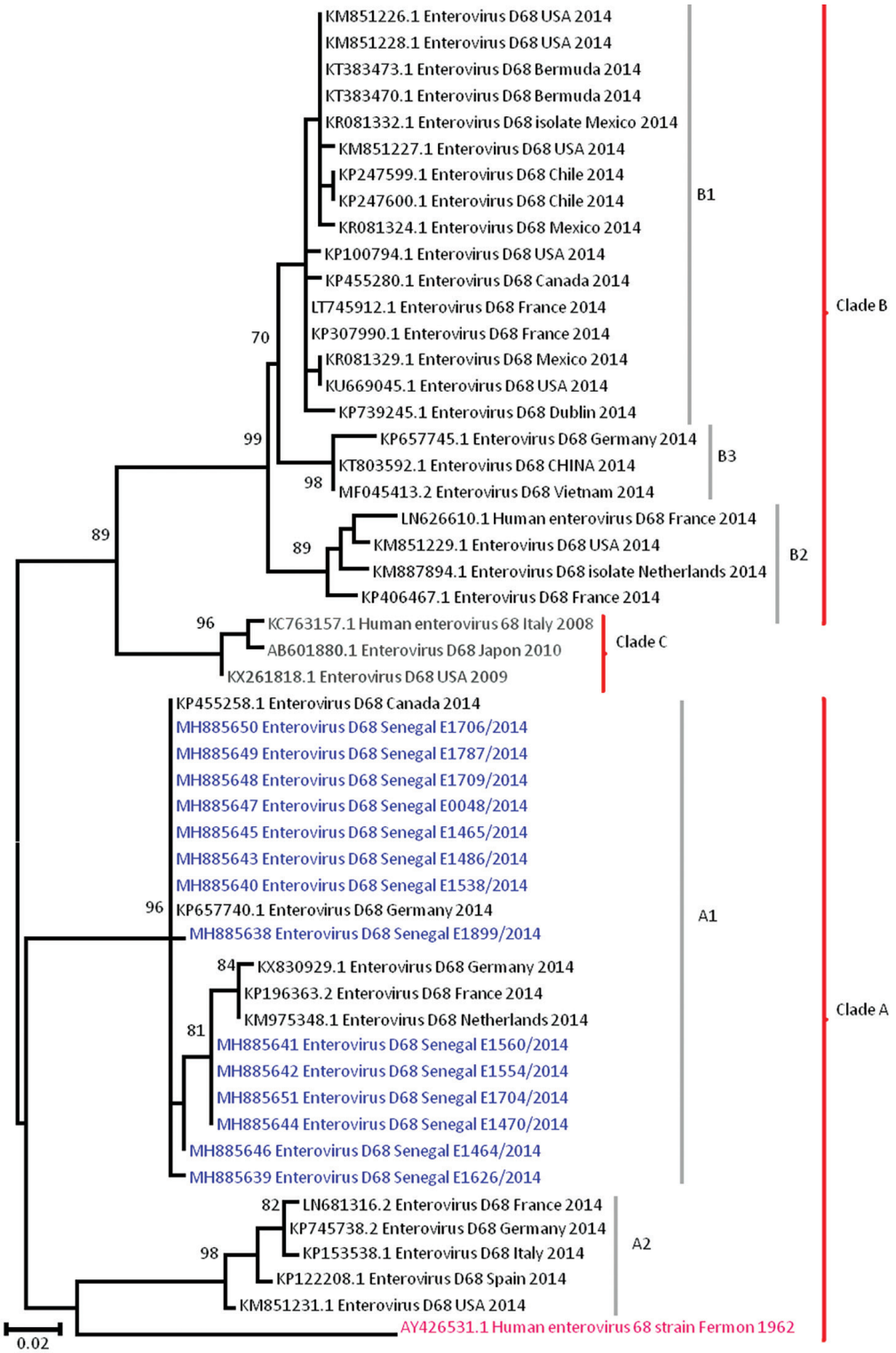


Figure 2. Phylogenetic relationships among enterovirus D68 (EV-D68) strains detected in Senegal (blue) and other countries (black) during the US outbreak period, September–December 2014. The phylogenetic tree based on nucleotide sequences of partial viral protein 1 genomic regions of EV-D68 strains was generated using the neighbor-joining method in MEGA6 (http://www.megasoftware.net). Sequences are identified by GenBank accession number, country, and period of detection. The phylogenetic tree is rooted by the oldest EV-D68 sequence in GenBank, the Fermon strain (pink), collected in 1962 in California, USA. We performed 1,000 bootstrap replicates to determine the consensus tree; support for nodes present in >70% of the trees are annotated. Scale bar indicates nucleotide substitutions per site.

limited information on disease outcome, and atypical clinical symptoms were not reported. Thus, the association between EV-D68 infections and severe clinical signs could not be established.

Conclusions

Despite the study's shortcomings, we have confirmed circulation of EV-D68, exclusively of the A1 lineage, in Senegal at the time of the outbreak in the United States. Our study also adds to the growing body of evidence that EV-D68 may cause severe respiratory disease, especially in children, even in those without underlying chronic respiratory diseases. The detection of EV-D68 in a child with a severe respiratory infection reinforces the need to include this virus in SARI sentinel surveillance, which is focused mainly on pediatric hospitalization. Through this system, data on disease outcome, underlying complications, atypical clinical signs, duration of symptoms or hospitalization, and treatment are routinely collected to better assess burden. Additional research will be necessary to investigate cases of acute flaccid paralysis to determine their relationship with EV-D68 infections.

Acknowledgments

We would like to acknowledge the Ministry of Health for its support and all the sentinel site healthcare workers. We convey special thanks to Sarah Respaut and Nicole Prada for their unwavering support.

This study was supported by the US Department of Human Health Services (grant no. IDSEP140020-01-00) via the International Division of Pasteur Institutes.

About the Author

Dr. Fall is a postdoctoral student in the Department of Virology at the Pasteur Institute of Dakar. His research interest is the burden of noninfluenza respiratory viruses (mainly enterovirus, rhinoviruses, and respiratory syncytial virus) in pediatric populations in Senegal.

References

1. Midgley CM, Watson JT, Nix WA, Curns AT, Rogers SL, Brown BA, et al.; EV-D68 Working Group. Severe respiratory illness associated with a nationwide outbreak of enterovirus D68 in the USA (2014): a descriptive epidemiological investigation. Lancet Respir Med. 2015;3:879–87. http://dx.doi.org/10.1016/S2213-2600(15)00335-5
2. Drews SJ, Simmonds K, Usman HR, Yee K, Fathima S, Tipples G, et al. Characterization of enterovirus activity, including that of enterovirus D68, in pediatric patients in Alberta, Canada, in 2014. J Clin Microbiol. 2015;53:1042–5. http://dx.doi.org/10.1128/JCM.02982-14
3. Torres JP, Farfan MJ, Izquierdo G, Piemonte P, Henriquez J, O'Ryan ML. Enterovirus D68 infection, Chile, Spring 2014. Emerg Infect Dis. 2015;21:728–9. http://dx.doi.org/10.3201/eid2104.141766
4 Vazquez-Perez JA, Ramirez-Gonzalez JE, Moreno-Valencia Y, Hernandez-Hernandez VA, Romero-Espinoza JA, Castillejos-Lopez M, et al. EV-D68 infection in children with asthma exacerbation and pneumonia in Mexico City during 2014 autumn. Influenza Other Respir Viruses. 2016;10:154–60. http://dx.doi.org/10.1111/irv.12384
5. Reiche J, Böttcher S, Diedrich S, Buchholz U, Buda S, Haas W, et al. Low-level circulation of enterovirus D68-associated acute respiratory infections, Germany, 2014. Emerg Infect Dis. 2015;21:837–41. http://dx.doi.org/10.3201/eid2105.141900
6. Midgley SE, Christiansen CB, Poulsen MW, Hansen CH, Fischer TK. Emergence of enterovirus D68 in Denmark, June 2014 to February 2015. Euro Surveill. 2015;20:21105. http://dx.doi.org/10.2807/1560-7917.ES2015.20.17.21105
7. Schuffenecker I, Mirand A, Josset L, Henquell C, Hecquet D, Pilorgé L, et al. Epidemiological and clinical characteristics of patients infected with enterovirus D68, France, July to December 2014. Euro Surveill. 2016;21:30226. http://dx.doi.org/10.2807/1560-7917.ES.2016.21.19.30226
8. Dia N, Diene Sarr F, Thiam D, Faye Sarr T, Espié E, OmarBa I, et al.; 4S Network Group. Influenza-like illnesses in Senegal: not only focus on influenza viruses. PLoS One. 2014;9:e93227. http://dx.doi.org/10.1371/journal.pone.0093227
9. Tokarz R, Firth C, Madhi SA, Howie SR, Wu W, Sall AA, et al. Worldwide emergence of multiple clades of enterovirus 68. J Gen Virol. 2012;93:1952–8. http://dx.doi.org/10.1099/vir.0.043935-0
10. Piralla A, Girello A, Premoli M, Baldanti F. A new real-time reverse transcription-PCR assay for detection of human enterovirus 68 in respiratory samples. J Clin Microbiol. 2015;53:1725–6. http://dx.doi.org/10.1128/JCM.03691-14
11. Poelman R, Schuffenecker I, Van Leer-Buter C, Josset L, Niesters HG, Lina B; ESCV-ECDC EV-D68 study group. European surveillance for enterovirus D68 during the emerging-North-American outbreak in 2014. J Clin Virol. 2015;71:1–9. http://dx.doi.org/10.1016/j.jcv.2015.07.296
12. Imamura T, Oshitani H. Global reemergence of enterovirus D68 as an important pathogen for acute respiratory infections. Rev Med Virol. 2015;25:102–14. http://dx.doi.org/10.1002/rmv.1820
13. Oermann CM, Schuster JE, Conners GP, Newland JG, Selvarangan R, Jackson MA. Enterovirus d68. A focused review and clinical highlights from the 2014 U.S. outbreak. Ann Am Thorac Soc. 2015;12:775–81. http://dx.doi.org/10.1513/AnnalsATS.201412-592FR
14. Peci A, Winter AL, Warshawsky B, Booth TF, Eshaghi A, Li A, et al. Epidemiology of enterovirus D68 in Ontario. PLoS One. 2015;10:e0142841. http://dx.doi.org/10.1371/journal.pone.0142841
15. Nathaniel S, Ahmed S, Wilson J, Gutierrez C, Chadee DD, Olowokure B, et al. First reported enterovirus D68 infection in pediatric patients from the Caribbean region: evidence of spread from the U.S. outbreak. Rev Panam Salud Publica. 2017;41:e11.

Address for correspondence: Ndongo Dia, Institute Pasteur de Dakar, Virology Department, 36 Ave Pasteur, Dakar 220, Senegal; email: ndia@pasteur.sn

Respiratory Syncytial Virus Infection in Homeless Populations, Washington, USA

Jim Boonyaratanakornkit,[1] Seda Ekici,[1] Amalia Magaret, Kathryn Gustafson, Emily Scott, Micaela Haglund, Jane Kuypers, Ronald Pergamit, John Lynch, Helen Y. Chu

Homelessness has not previously been identified as a risk factor for respiratory syncytial virus (RSV) infection. We conducted an observational study at an urban safety-net hospital in Washington, USA, during 2012–2017. Hospitalized adults with RSV were more likely to be homeless, and several clinical outcome measures were worse with RSV than with influenza.

Respiratory syncytial virus (RSV) is increasingly recognized as a major pathogen in adults and shows a disease burden comparable to that for influenza (*1*). No vaccine is currently available. However, several vaccine candidates against RSV are in clinical trials, and the elderly, those with chronic lung disease, infants, and immunocompromised persons remain priority target populations for prevention efforts (*2*,*3*).

Rates of homelessness are increasing in major urban centers because of lack of affordable housing and slower wage growth (*4*). Homeless persons experience higher rates of illness and death compared with the general population, partly because of infectious diseases from lack of access to sanitation, crowding in shelters, untreated chronic medical conditions, and higher rates of mental health issues and substance use (*5*). Studies have described local outbreaks of rhinovirus and influenza in homeless shelters (*6*). Identification of homeless persons as an at-risk population for severe RSV disease might guide prioritization strategies for RSV vaccines and therapeutics as they become available. We aimed to evaluate risk factors and clinical outcomes of adults hospitalized with RSV infections versus those with influenza in an urban medical center serving a region that had high rates of homelessness.

Author affiliations: University of Washington, Seattle, Washington, USA (J. Boonyaratanakornkit, S. Ekici, A. Magaret, K. Gustafson, E. Scott, M. Haglund, J. Kuypers, J. Lynch, H.Y. Chu); Harborview Medical Center, Seattle (J. Boonyaratanakornkit, R. Pergamit)

DOI: https://doi.org/10.3201/eid2507.181261

The Study

We conducted a retrospective case–control study of adults hospitalized with RSV and influenza at Harborview Medical Center (Seattle, WA, USA) during July 2012–June 2017. This center is an academic tertiary medical center that functions as the safety-net hospital for the Seattle metropolitan area. We identified patients on the basis of laboratory records of specimens containing influenza A/B virus and RSV by using a rapid PCR assay (Focus Diagnostics, https://www.focusdx.com) or the Xpert Xpress Flu/RSV test (Cepheid, http://www.cepheid.com) (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1261-App1.pdf).

A total of 865 patients were hospitalized with RSV infection (n = 157) or influenza A/B (n = 708) during July 2012–June 2017 (Table 1; Figure 1). We showed by multivariable analysis of risk factors for hospitalization with RSV infection versus influenza that older age, homelessness, having chronic obstructive pulmonary disease (COPD) or asthma, and drug use were associated with an increased odds ratio (OR) for RSV hospitalization compared with influenza (Table 1; Figure 2). Drug use showed a correlation with homelessness (OR 5.18, 95% CI 3.17–8.46).

Overall, a higher proportion of adults hospitalized with RSV infection were admitted to the intensive care unit (ICU), readmitted within 30 days, and received any antimicrobial drug compared with patients hospitalized with influenza (Table 2). A total of 4% (7/158) of adults given a diagnosis of RSV infection died during hospitalization, compared with 3% (21/712) of those with influenza. Having COPD/asthma was not correlated with antimicrobial drug use (OR 1.07, 95% CI 0.71–1.60). Only 10% (4/40) of patients with RSV infection who were readmitted within 30 days had a positive swab specimen for the same virus at the second admission.

We sought to determine whether increased hospital readmission after hospitalization for RSV infection had other potential explanatory factors. We found by multivariable analysis that having RSV infection (OR 2.40, 95% CI 1.54–3.76) and homelessness (OR 2.06, 95% CI 1.31–3.24) remained associated with an increased odds of hospital readmission. Because homelessness and RSV infection increased the odds of readmission, persons at highest risk were homeless persons with RSV infection (OR 2.4

[1]These authors contributed equally to this article.

Table 1. Analysis of sociodemographic characteristics of patients admitted with RSV infection or influenza, Washington, USA, 2012–2017*

Characteristic	RSV, n = 157	Influenza, n = 708	Univariable p value	RSV vs. influenza, OR (95% CI)	Multivariable p value
Age, y (range)	56.0 (18–93)	52.8 (18–100)	0.035	1.01 (1.00–1.02)	0.01
Male sex	100 (64)	433 (61)	0.590	–	–
American Indian or Alaska Native	16 (10)	31 (4)	0.006	–	–
Black or African American	26 (17)	201 (28)	0.002	–	–
Homeless	50 (32)	137 (19)	<0.001	2.00 (1.33–3.03)	0.001
Drug use	26 (17)	60 (8)	0.005	1.79 (1.06–3.03)	0.028
Asthma/COPD	32 (20)	95 (13)	0.034	1.67 (1.06–2.63)	0.027
Smoking	38 (24)	71 (10)	<0.001	–	–

*Values are no. (%) unless otherwise indicated. COPD, chronic obstructive pulmonary disease; OR, odds ratio; RSV, respiratory syncytial virus. –, variable was excluded from multivariable analysis.

× 2.06 = 4.95 relative to housed persons with influenza). Age (OR 1.00, 95% CI 0.99–1.02), having COPD/asthma (OR 0.88, 95% CI 0.47–1.58), and drug use (OR 1.15, 95% CI 0.62–2.13) were not correlated with readmission.

We found that 6.5% (24,452/374,672) of all patients discharged from Harborview Medical Center during 2012–2017 were homeless. In that same period, 32% (50/157) of those with RSV infection were homeless, compared with 19% (147/708) of those with influenza (p = 0.003), 3.4% (286/8,488) of patients with a urinary tract infection (p<0.001), and 2.0% (25/1,278) of patients with an ischemic stroke (p<0.001).

Conclusions

In this study of adults hospitalized during 5 years in an urban hospital, 32% of patients given a diagnosis of RSV infection were homeless, compared with 6.5% of all patients hospitalized. Patients hospitalized with RSV infection were more likely to be older, homeless, drug users, or have COPD/asthma compared with persons with influenza. Homelessness has reached a national public health crisis, and many homeless persons seek acute care in emergency departments (*9*). The city of Seattle has the largest concentration of homelessness per capita in the country (*10*). Outbreaks of infections with respiratory viruses have been described in homeless shelters, in which transmission might be facilitated by crowding, poor sanitation, and the ability of RSV to spread through fomites (*6*,*11*). Our findings for RSV in this homeless population might be generalizable to other urban public hospitals.

Several outcome measures were worse in patients hospitalized for RSV infection than for influenza, including 30-day readmission, admission to the ICU, and receipt of antimicrobial drugs. A previous study similarly reported higher rates of ICU admission among patients with RSV infection than for those with influenza (*12*). More severe disease might have led clinicians to preferentially use antimicrobial drugs for patients hospitalized with RSV infection compared with influenza. The higher number of patients with RSV infection than influenza admitted to the ICU supports this interpretation. These results suggest that patients hospitalized with RSV infection might benefit from closer monitoring, follow-up, and antimicrobial drug stewardship to prevent readmission and overuse of antimicrobial drugs.

Homelessness and having RSV infection were independent risk factors for hospital readmission, demonstrating that the higher risk for poor outcomes in homeless persons was not simply explained by the disproportionately higher number of diagnosis of RSV infection in this group. All-cause readmission within 30 days is a major quality metric used by the Centers for Medicare and Medicaid

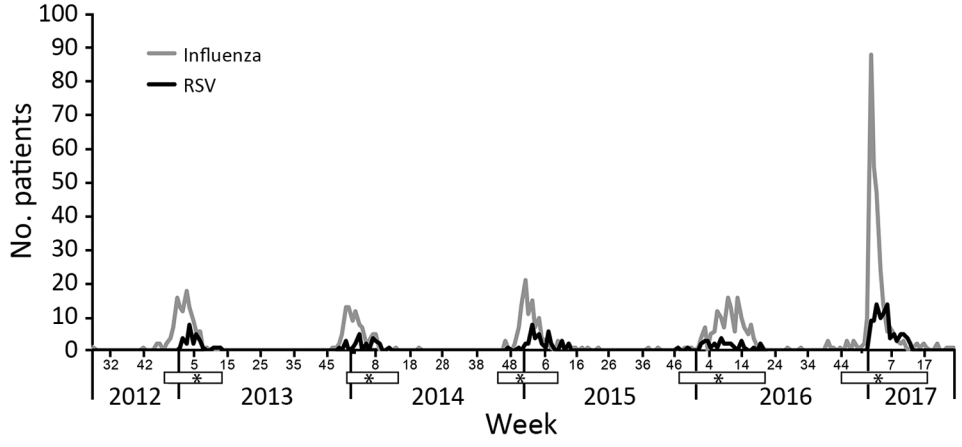


Figure 1. Detection of influenza and RSV in adults hospitalized at Harborview Medical Center, Seattle, WA, USA, July 2012–June 2017. White bars below the x-axis indicate RSV seasons; asterisks indicate weeks when cases of RSV infection peaked, on the basis of Centers for Disease Control and Prevention surveillance data in region 10 (Alaska, Idaho, Washington, and Oregon) (*7*,*8*) during 2012–2017. RSV, respiratory syncytial virus.

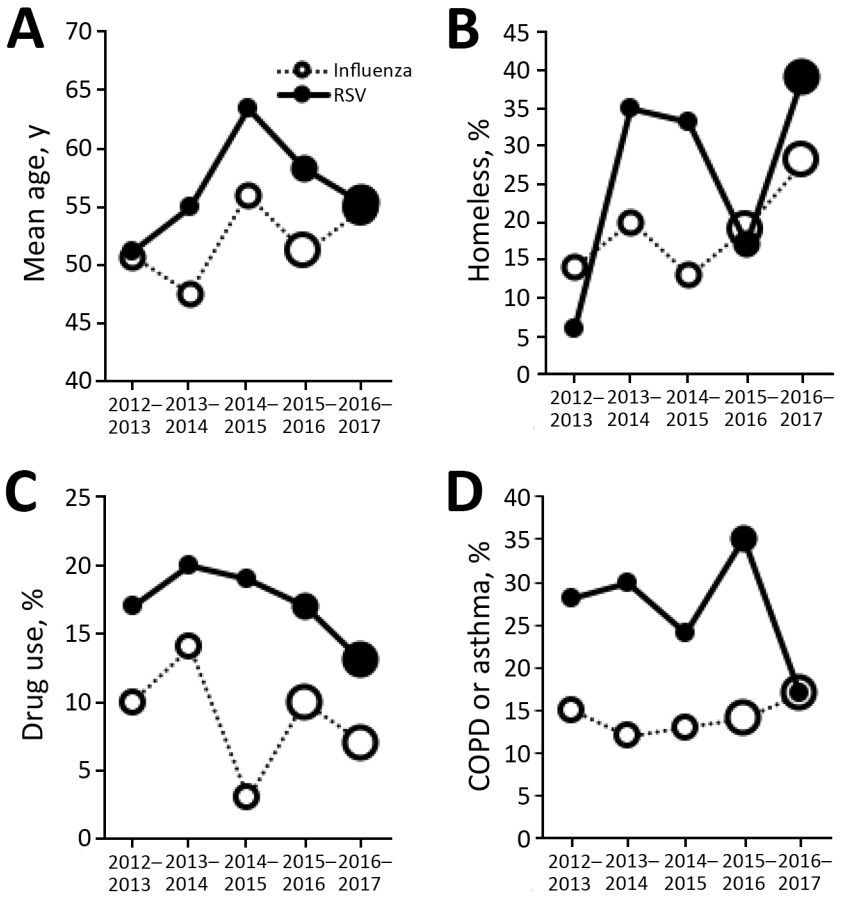


Figure 2. Sociodemographic characteristics of patients hospitalized with RSV infection or influenza across 5 seasons, 2012–2017, Washington, USA. A) Age; B) homelessness; C) drug use; and D) COPD or asthma. Size of each circle indicates number of patients for that data point: small circles indicate <50 patients, medium circles indicate 50–150 patients, and large circles indicate >150 patients. COPD, chronic obstructive pulmonary disease; RSV, respiratory syncytial virus.

Services and Hospital Quality Alliance (http://www.allhealthpolicy.org/glossary/hospital-quality-alliance). Other studies have found an association between lower education and unemployment with rehospitalization and that most rehospitalizations were related to concurrent conditions (*13*).

Limitations of this study include the retrospective study design, limiting analysis to only hospitalized patients with RSV infection or influenza. The overall number of hospitalizations annually for RSV infection and influenza increased during 2012–2017 and was likely caused by transition to on-site rapid testing, which might increase provider uptake (*14*). In addition, without hospitalized and community controls who do not have influenza or RSV infection and who are not homeless, we cannot definitively conclude that homelessness is associated with a greater risk for hospitalization for RSV infection compared with influenza. The association might have 3 possible interpretations: risk factors are associated with more severe disease caused by RSV than influenza; risk factors are associated with a higher risk for infection with RSV compared with influenza; or risk factors are associated with greater susceptibility to RSV infection and disease compared with influenza. In addition, this study was limited to a single site, although it is representative of public, safety-net hospitals. Additional limitations include clinician-initiated testing triggered by influenza-like symptoms rather than for detection of RSV

Table 2. Clinical characteristics of patients admitted with RSV infection or influenza, Washington, USA, 2012–2017*

Characteristic	RSV, n = 158	Influenza, n = 712	Univariable p value
Mean length of hospital stay, d	5.5	4.6	0.67
ICU admission	39 (25)	123 (17)	0.041
Mean length of ICU stay, d	3.5	3.6	0.86
Readmission within 30 d	40 (25)	79 (11)	<0.001
Patients fitting SIRS criteria at admission	78 (49)	309 (43)	0.18
Antimicrobial drugs used†	84 (53)	224 (31)	<0.001
Steroids used	22 (14)	61 (9)	0.05
Deaths	7 (4)	21 (3)	0.61

*Values are no. (%) unless otherwise noted. Four patients were admitted twice with influenza, and 1 patient was admitted twice with RSV infection. ICU, intensive care unit; RSV, respiratory syncytial virus; SIRS, systemic inflammatory response syndrome.
†Included vancomycin, ceftriaxone, meropenem, azithromycin, levofloxacin, ciprofloxacin, amoxicillin, piperacillin/tazobactam, or ampicillin.

infection, which is less likely to manifest with fever (*15*). Therefore, the true burden of RSV infection is likely higher than identified in this study.

In conclusion, homeless persons might represent a previously unrecognized population at increased risk for poor outcomes caused by infection with RSV. An effective vaccine or therapeutic in adults could benefit this medically underserved population. Further data on the impact of homelessness on respiratory virus infection severity and outcomes are needed to guide public health strategies and implementation.

Acknowledgments

We thank Ferric Fang and Tom Smith for providing assistance with obtaining residual nasal swabs specimens, and Larissa Lewis for reporting respiratory virus infections.

This study was supported by National Institutes of Health grants T32-AI007044 (to J.B.), T32-AI118690 (to J.B.), and K23-AI103105 (to H.Y.C.), and the University of Washington Chair of Medicine Scholars Award (to H.Y.C.).

H.Y.C. has received research support from GlaxoSmithKline, Novavax, Pfizer, and Sanofi.

About the Author

Dr. Boonyaratanakornkit is a senior fellow at the University of Washington and the Fred Hutchinson Cancer Research Center, Seattle, WA. His primary research interest is the epidemiology and immunology of respiratory viruses.

References

1. Falsey AR, Hennessey PA, Formica MA, Cox C, Walsh EE. Respiratory syncytial virus infection in elderly and high-risk adults. N Engl J Med. 2005;352:1749–59. http://dx.doi.org/10.1056/NEJMoa043951
2. Falloon J, Yu J, Esser MT, Villafana T, Yu L, Dubovsky F, et al. An adjuvanted, postfusion F protein-based vaccine did not prevent respiratory syncytial virus illness in older adults. J Infect Dis. 2017;216:1362–70. http://dx.doi.org/10.1093/infdis/jix503
3. Higgins D, Trujillo C, Keech C. Advances in RSV vaccine research and development: a global agenda. Vaccine. 2016;34:2870–5. http://dx.doi.org/10.1016/j.vaccine.2016.03.109
4. Coleman V. Annual homeless count reveals more people sleeping outside than ever before. The Seattle Times. May 31, 2018 [cited 2018 May 31]. https://www.seattletimes.com/seattle-news/homeless/new-homeless-count-in-king-county-shows-spike-in-number-of-people-sleeping-outside
5. Raoult D, Foucault C, Brouqui P. Infections in the homeless. Lancet Infect Dis. 2001;1:77–84. http://dx.doi.org/10.1016/S1473-3099(01)00062-7
6. Hadler SC, Castro KG, Dowdle W, Hicks L, Noble G, Ridzon R. Epidemic intelligence service investigations of respiratory illness, 1946–2005. Am J Epidemiol. 2011;174(Suppl):S36–46. http://dx.doi.org/10.1093/aje/kwr309
7. Rose EB, Wheatley A, Langley G, Gerber S, Haynes A. Respiratory syncytial virus seasonality—United States, 2014–2017. MMWR Morb Mortal Wkly Rep. 2018;67:71–6. http://dx.doi.org/10.15585/mmwr.mm6702a4
8. Haynes AK, Prill MM, Iwane MK, Gerber SI; Centers for Disease Control and Prevention. Respiratory syncytial virus—United States, July 2012–June 2014. MMWR Morb Mortal Wkly Rep. 2014;63:1133–6.
9. Donovan S, Shinseki EK. Homelessness is a public health issue. Am J Public Health. 2013;103(Suppl 2):S180.
10. Greenstone S. Seattle's homeless crisis the worst in the country? The Seattle Times. January 16, 2018 [cited 2019 Apr 3]. https://www.seattletimes.com/seattle-news/homeless/is-seattles-homeless-crisis-the-worst-in-the-country
11. Hall CB. The nosocomial spread of respiratory syncytial viral infections. Annu Rev Med. 1983;34:311–9. http://dx.doi.org/10.1146/annurev.me.34.020183.001523
12. Jasti H, Mortensen EM, Obrosky DS, Kapoor WN, Fine MJ. Causes and risk factors for rehospitalization of patients hospitalized with community-acquired pneumonia. Clin Infect Dis. 2008;46:550–6. http://dx.doi.org/10.1086/526526
13. Ackerson B, Tseng HF, Sy LS, Solano Z, Slezak J, Luo Y, et al. Severe morbidity and mortality associated with respiratory syncytial virus versus influenza infection in hospitalized older adults. Clin Infect Dis. 2018; [Epub ahead of print]. http://dx.doi.org/10.1093/cid/ciy991
14. Chu HY, Englund JA, Huang D, Scott E, Chan JD, Jain R, et al. Impact of rapid influenza PCR testing on hospitalization and antiviral use: a retrospective cohort study. J Med Virol. 2015;87:2021–6. http://dx.doi.org/10.1002/jmv.24279
15. Colosia AD, Yang J, Hillson E, Mauskopf J, Copley-Merriman C, Shinde V, et al. The epidemiology of medically attended respiratory syncytial virus in older adults in the United States: A systematic review. PLoS One. 2017;12:e0182321. http://dx.doi.org/10.1371/journal.pone.0182321

Address for correspondence: Jim Boonyaratanakornkit, Harborview Medical Center, MS 359779, Seattle, WA 98104, USA; email: jboonyar@fredhutch.org

Elizabethkingia bruuniana Infections in Humans, Taiwan, 2005–2017

Jiun-Nong Lin, Chung-Hsu Lai, Chih-Hui Yang, Yi-Han Huang, Hsi-Hsun Lin

Author affiliations: E-Da Hospital, Kaohsiung, Taiwan (J.-N. Lin, C.-H. Lai, Y.-H. Huang); I-Shou University, Kaohsiung (J.-N. Lin, C.-H. Lai, Y.-H. Huang); Meiho University, Pingtung, Taiwan (C.-H. Yang); Taipei Veterans General Hospital, Taipei, Taiwan (H.-H. Lin)

DOI: https://doi.org/10.3201/eid2507.180768

Using 16S rRNA and *rpoB* gene sequencing, we identified 6 patients infected with *Elizabethkingia bruuniana* treated at E-Da Hospital (Kaohsiung, Taiwan) during 2005–2017. We describe patient characteristics and the molecular characteristics of the *E. bruuniana* isolates, including their MICs. Larger-scale studies are needed for more robust characterization of this pathogen.

The *Elizabethkingia* genus comprises gram-negative, aerobic, nonmotile, nonspore-forming, nonfermenting rod-shaped bacteria (*1*). This genus previously comprised *E. meningoseptica*, *E. miricola*, and *E. anophelis*. In August 2017, Nicholson et al. proposed adding 3 new species, namely *E. bruuniana*, *E. ursingii*, and *E. occulta*, to this genus (*1*). However, little information exists about these species. In this study, we report the clinical characteristics and demographics of a group of patients with *E. bruuniana* infection in Taiwan and the molecular features of their *E. bruuniana* isolates.

We conducted this study at E-Da Hospital, a 1,000-bed university-affiliated medical center in Kaohsiung, Taiwan; this study was approved by the institutional review board of the hospital (no. EMRP-106-105). We searched the hospital database to identify microbial cultures performed during January 2005–December 2017 that yielded *Elizabethkingia*. The isolates were initially identified by staff in the clinical microbiology laboratory using API/ID32 phenotyping kits or VITEK MS (both from bioMérieux, https://www.biomerieux.com). We re-identified these species as *Elizabethkingia* using both 16S rRNA and *rpoB* gene sequencing. The primers and methods we used for amplification and sequencing of the 16S rRNA and *rpoB* genes were described previously (*1*,*2*). We compared the assembled 16S rRNA gene sequences with the nucleotide sequences of *Elizabethkingia*-type strains present in GenBank. We considered isolates with ≥99.5% similarity in the 16S rRNA gene sequence members of the same species, as recommended in a previous study (*3*). We constructed a phylogenetic tree using the *rpoB* genes of the isolates exhibiting ≥99.5% 16S rRNA gene sequence identity with the *E. bruuniana* type strain G0146^T. We calculated the average nucleotide identity using OrthoANI (*4*) and computed in silico DNA–DNA hybridization (DDH) using the Genome-to-Genome Distance Calculator (*5*), using the average nucleotide identity value of ≥95% and the DDH value of ≥70% separately as criteria for species delineation (*4*,*5*). We sequenced the quinolone resistance–determining regions of DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) to look for mutations associated with resistance (Appendix Table, https://wwwnc.cdc.gov/EID/article/25/7/18-0768-App1.pdf).

For the 13-year period, we found 103 nonduplicate *Elizabethkingia* isolates in the database of the clinical microbiology laboratory. Among these, 8 isolates shared ≥99.5% 16S rRNA gene sequence identity with *E. bruuniana* G0146^T, and an *rpoB* gene–based phylogenetic analysis revealed that 6 of the 8 isolates were more closely related to *E. bruuniana* G0146^T (Appendix Figure 1). We previously published the complete whole-genome sequence of 1 of these 6 isolates, EM798-26 (GenBank accession no. CP023746) (*6*). Using 16S rRNA gene sequence analysis, we initially identified this isolate as *E. miricola*. Average nucleotide identity analysis demonstrated that EM798-26 and *E. bruuniana* G0146^T share 97.7% whole-genome similarity (Appendix Figure 2). Using in silico DDH analysis, we predicted a DDH value of 81.7% for EM798-26 and *E. bruuniana* G0146^T (Appendix Figure 3). These results support that EM798-26 and the other 5 isolates (EM20-50, EM455-89, EM828-05, EM863-68, and EM891-63) are *E. bruuniana*.

These 6 isolates were collected from 6 (4 male and 2 female) patients (Table) with a mean age of 71.7 (SD ±11) years. The sources of isolation included bronchoalveolar lavage fluid (n = 2), blood (n = 2), urine (n = 1), and the tip of the central venous catheter (n = 1). All infections were healthcare associated. Two patients had septic shock, and all patients had ≥1 concurrent medical condition, such as hypertension, diabetes mellitus, or a malignancy. Antimicrobial therapy included piperacillin/tazobactam, trimethoprim/sulfamethoxazole, levofloxacin, or tigecycline, either singly or in combination. None of the patients died of *E. bruuniana* infection.

Most *E. bruuniana* isolates were resistant to β-lactams, β-lactam and lactamase inhibitors, carbapenems, aminoglycosides, and trimethoprim/sulfamethoxazole (Table). All isolates were susceptible to minocycline, 4 (67%) to tigecycline and levofloxacin, and 2 (33%) to ciprofloxacin. The antimicrobial susceptibility patterns we found are similar to those of other *Elizabethkingia* spp. identified

Table. Characteristics of patients infected with *Elizabethkingia bruuniana*, Taiwan, 2005–2017, and antimicrobial susceptibility of the *E. bruuniana* isolates*

Category	Patient no./isolate no.					
	No. 1/EM20-50	No. 2/EM455-89	No. 3/EM798-26	No. 4/EM828-05	No. 5/EM863-68	No. 6/EM891-63
Patient characteristics						
Year of illness	2005	2011	2015	2016	2016	2017
Age, y/sex	67/F	62/F	81/M	60/M	88/M	72/M
Site of microbe isolation	Urine	CVC tip	Blood	Blood	BAL fluid	BAL fluid
Clinical manifestations	Urinary tract infection	Septic shock	Primary bacteremia	Primary bacteremia	Pneumonia	Pneumonia, septic shock
Underlying conditions	Cervical cancer, hypertension	Maxillary osteosarcoma, hypothyroidism, hypertension	Lymphoma, chronic kidney disease	Brain meningioma, stroke, hypertension	Liver cirrhosis, hypertension, CHF	Esophageal cancer, diabetes mellitus, hypertension
Treatment	TMP/SMX	TZP	Levofloxacin	TZP, levofloxacin	Levofloxacin	Tigecycline, levofloxacin
Outcome	Survived	Survived	Survived	Survived	Survived	Survived
MIC, mg/L†						
Piperacillin	64	64	>64	64	>64	64
TZP	32/4	>128/4	32/4	32/4	64/4	128/4
Ceftazidime	>16	>16	>16	>16	>16	>16
Cefepime	>32	>32	>32	>32	>32	>32
Ceftriaxone	>32	>32	>32	>32	>32	>32
Aztreonam	>16	>16	>16	>16	>16	>16
Imipenem	>8	>8	>8	>8	>8	>8
Meropenem	>8	>8	>8	>8	>8	>8
Gentamicin	8	8	4	8	4	8
Tobramycin	>8	>8	>8	>8	>8	>8
Amikacin	<8	32	16	>32	16	32
Tetracycline	>8	>8	>8	>8	>8	>8
Minocycline	<1	<1	<1	4	<1	<1
Tigecycline	<1	8	2	4	<1	<1
Ciprofloxacin	1	2	2	>2	2	1
Levofloxacin	<1	8	<1	>8	<1	<1
TMP/SMX	>4/76	>4/76	>4/76	>4/76	>4/76	>4/76

*BAL, bronchoalveolar lavage; CHF, congestive heart failure; CVC, central venous catheter; TMP/SMX, trimethoprim/sulfamethoxazole; TZP, piperacillin/tazobactam.
†Light gray shading indicates intermediate susceptibility; dark gray, susceptible isolates; no shading, resistant isolates.

in previous studies (*7–10*). For example, reports from the United States, Hong Kong, and South Korea have revealed that *E. anophelis* and *E. meningoseptica* were frequently resistant to most β-lactams, including ceftazidime, ceftriaxone, and imipenem, but showed variable susceptibility to piperacillin/tazobactam, cefepime, ciprofloxacin, and levofloxacin (*7–10*).

To investigate the association between target gene mutations and fluoroquinolone resistance, we examined the mutations present in quinolone resistance–determining regions in these 6 isolates. We did not find nonsynonymous substitutions in the quinolone resistance–determining regions of *gyrA*, *gyrB*, *parC*, and *parE*, which suggests that mutations in these genes are not the cause of fluoroquinolone resistance.

In summary, our study demonstrates the clinical manifestations of *E. bruuniana* infection and the molecular characteristics of the pathogen. Because cases in our study were limited in number, further large-scale studies are necessary to investigate the antimicrobial susceptibility patterns of *E. bruuniana* and elucidate the clinical characteristics and treatment of *E. bruuniana* infection.

This work was supported by grants from E-Da Hospital (no. EDAHP106007) and the Ministry of Science and Technology, Taiwan (no. 106-2314-B-214-009-MY2).

About the Author

Dr. Jiun-Nong Lin is an associate professor at the School of Medicine, College of Medicine, I-Shou University, Kaohsiung, Taiwan. His research interests include infectious diseases and clinical microbiology.

References

1. Nicholson AC, Gulvik CA, Whitney AM, Humrighouse BW, Graziano J, Emery B, et al. Revisiting the taxonomy of the genus *Elizabethkingia* using whole-genome sequencing, optical mapping, and MALDI-TOF, along with proposal of three novel *Elizabethkingia* species: *Elizabethkingia bruuniana* sp. nov., *Elizabethkingia ursingii* sp. nov., and *Elizabethkingia occulta* sp. nov. Antonie van Leeuwenhoek. 2018;111:55–72. http://dx.doi.org/10.1007/s10482-017-0926-3
2. Lin JN, Lai CH, Yang CH, Huang YH, Lin HF, Lin HH. Comparison of four automated microbiology systems with 16S rRNA gene sequencing for identification of *Chryseobacterium* and *Elizabethkingia* species. Sci Rep. 2017;7:13824.

http://dx.doi.org/10.1038/s41598-017-14244-9
3. Janda JM, Abbott SL. 16S rRNA gene sequencing for bacterial identification in the diagnostic laboratory: pluses, perils, and pitfalls. J Clin Microbiol. 2007;45:2761–4. http://dx.doi.org/10.1128/JCM.01228-07
4. Lee I, Ouk Kim Y, Park SC, Chun J. OrthoANI: an improved algorithm and software for calculating average nucleotide identity. Int J Syst Evol Microbiol. 2016;66:1100–3. http://dx.doi.org/10.1099/ijsem.0.000760
5. Meier-Kolthoff JP, Auch AF, Klenk H-P, Göker M. Genome sequence-based species delimitation with confidence intervals and improved distance functions. BMC Bioinformatics. 2013;14:60. http://dx.doi.org/10.1186/1471-2105-14-60
6. Lin JN, Lai CH, Yang CH, Huang YH, Lin HH. Complete genome sequence of *Elizabethkingia miricola* strain EM798-26 isolated from the blood of a cancer patient. Genome Announc. 2018;6:e01408-17. http://dx.doi.org/10.1128/genomeA.01408-17
7. Hsu MS, Liao CH, Huang YT, Liu CY, Yang CJ, Kao KL, et al. Clinical features, antimicrobial susceptibilities, and outcomes of *Elizabethkingia meningoseptica* (*Chryseobacterium meningosepticum*) bacteremia at a medical center in Taiwan, 1999–2006. Eur J Clin Microbiol Infect Dis. 2011;30:1271–8. http://dx.doi.org/10.1007/s10096-011-1223-0
8. Lau SK, Chow WN, Foo CH, Curreem SO, Lo GC, Teng JL, et al. *Elizabethkingia anophelis* bacteremia is associated with clinically significant infections and high mortality. Sci Rep. 2016;6:26045. http://dx.doi.org/10.1038/srep26045
9. Perrin A, Larsonneur E, Nicholson AC, Edwards DJ, Gundlach KM, Whitney AM, et al. Evolutionary dynamics and genomic features of the *Elizabethkingia anophelis* 2015 to 2016 Wisconsin outbreak strain. Nat Commun. 2017;8:15483. http://dx.doi.org/10.1038/ncomms15483
10. Han MS, Kim H, Lee Y, Kim M, Ku NS, Choi JY, et al. Relative prevalence and antimicrobial susceptibility of clinical isolates of *Elizabethkingia* species based on 16S rRNA gene sequencing. J Clin Microbiol. 2017;55:274–80. http://dx.doi.org/10.1128/JCM.01637-16

Address for correspondence: Jiun-Nong Lin, E-Da Hospital, Division of Infectious Diseases, Department of Internal Medicine, No. 1, Yida Road, Jiaosu Village, Yanchao District, Kaohsiung 824, Taiwan; email: jinoli@kmu.edu.tw

Human Enterovirus C105, China, 2017

Maozhong Li,[1] Tiegang Zhang,[1] Cheng Gong, Aihua Li, Ming Luo, Mei Dong, Fang Huang

Author affiliation: Beijing Center for Disease Prevention and Control, Beijing, China

[1]These authors contributed equally to this article.

DOI: https://doi.org/10.3201/eid2507.180874

We report a case of enterovirus C105 infection in an 11-year-old girl with lower respiratory tract symptoms that was identified through the Respiratory Virus Surveillance System, which covers 30 sentinel hospitals in all 16 districts of Beijing, China. The presence of this virus strain in China confirmed its geographically wide distribution.

Enteroviruses are small, nonenveloped RNA viruses that cause illnesses in humans ranging from mild to severe (*1*). Fifteen species of enterovirus are known, 7 of which are known to infect humans. These species include enterovirus A–D and rhinovirus A–C (*1*,*2*). The newly emerging genotype C105 (EV-C105) represents a novel monophyletic clade of enterovirus C; this strain was identified in 2010 in the Democratic Republic of the Congo (strain 34S) (*3,4*). EV-C105 cases from Italy (Pavia/8376, Pavia/9095), Romania (ROM31), the United States (USA/OK/2014-19362), New Zealand (strains not available), and Burundi (BU77, BU5) have been identified and characterized, suggesting that the spread of EV-C105 could be wider than previously hypothesized (*5*). Here, we report a detected case of EV-C105 in an 11-year-old girl with lower respiratory tract symptoms in Beijing, China.

The Beijing Center for Disease Prevention and Control established the Respiratory Virus Surveillance System (RVSS) in 2014. The RVSS tracks patients with respiratory tract infections (RTIs) and pneumonia in 30 sentinel hospitals throughout Beijing. The RVSS is an active system, designed to alert for future outbreaks of respiratory infections. To study enterovirus infections, we tested 24,093 clinical specimens (nasopharyngeal swab, sputum, and alveolar lavage fluid) from patients with RTIs that were reported through RVSS during June 2014–December 2017. RVSS classifies persons <14 years of age as children and those ≥14 years of age as adults. The ages of the reported patients ranged from 8 months to 93 years (median 33.5 years, mean 37.9 years).

We screened all samples using real-time PCR for influenza virus, parainfluenza virus types 1–4, respiratory syncytial virus, coronaviruses (229E, NL63, HKU1, and OC43), metapneumovirus, adenovirus, bocavirus, and enteroviruses (*6*). Overall, 445 (445/7,122; 6.2%) children and 276 (276/16,971; 1.6%) adults were positive for enterovirus or other respiratory viruses.

We further genotyped enterovirus-positive samples with primers sequentially targeting the viral protein (VP) 1 region (*7,8*). We obtained a 699-nt amplicon of EV-C105 from a nasopharyngeal swab sample collected at the time of a hospital visit (GenBank accession no. KX910099). The patient was an 11-year-old girl with no underlying disease who was brought to the outpatient clinic of the Beijing Children's

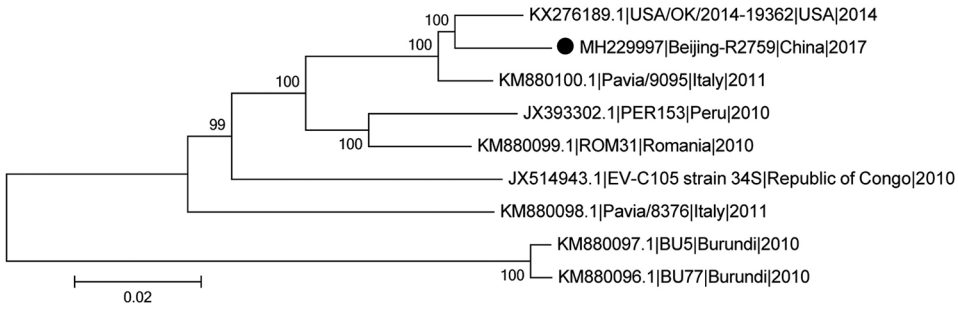


Figure. Phylogenetic tree of EV-C105 from a patient in Beijing, China (black dot) and reference isolates from different locations. We estimated the phylogenetic relationships of complete or near-complete EV-C105 genomes using the neighbor-joining method with 1,000 replicates bootstrapped by using MEGA version 6.06 software (http://www.megasoftware.net). Numbers along branches indicate bootstrap percentages. Isolates are identified by GenBank accession number, strain name, location, and year. Scale bar indicates nucleotide substitutions per site. EV-C105, enterovirus C105.

Hospital on May 23, 2016, with an 8-day history of fever (highest temperature 38.7°C), coughing, and difficulty breathing. Blood tests in the clinic showed total leukocyte count 1.41×10^{10} cells/L; neutrophils, 79.2%; lymphocytes, 12.1%; total platelet count, 5.2×10^{11}/L; and hemoglobin, 130 g/L. Chest radiographs showed thickness or turbulence in the texture in both lungs, which was diagnosed as pneumonia. She received supportive treatment and received antimicrobial drugs empirically before being sent home the same day. She was not hospitalized during her illness. According to a follow-up survey, she recovered 14 days later. We detected no other respiratory pathogens in this patient.

BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) of the amplicon (Beijing-R2759) showed that the sequences had 98% identity with the USA/OK/2014–19362 strain (GenBank accession no. KX276189.1) and 91.4% with the reference prototype genotype (EV-C105 strain 34S; accession no. JX514943). Phylogenetic analysis of the VP1 gene performed with MEGA version 6.06 software (http://www.megasoftware.net) showed that Beijing-R2759 belonged to genotype EV-C105.

To further characterize this virus strain, we amplified the genome sequence directly from the nasopharyngeal swab sample using reverse transcription PCR with overlapping primers; we sequenced each amplicon 4 times using the Sanger method. We assembled sequences using Lasergene version 5.01 (DNAStar Inc., https://www.dnastar.com). The genome of Beijing-R2759 (GenBank accession no. MH229997) was 7,316 nt, including 6,618 nt in open reading frame. The EV-C105 polyprotein sequence for this strain shares 96.6%–99.4% amino acid identity with 8 of the EV-C105 sequences in GenBank: 96.6% with accession no. KM880097 (Burundi); 96.8% with accession no. KM880096 (Burundi); 97.7% with accession no. JX393302 (Peru); 98.5% with accession no. JX514943 (Republic of the Congo); 98.6% with accession no. KM880098 (Italy); 98.8% with accession no. KM880099 (Romania); 99.3% with accession no. KX276189 (United States); and 99.4% with accession no. KM880100 (Italy).

The full length of the Beijing-R2759 VP1 gene was 888 nt. The deduced amino acid sequence in VP1 had 94.9%–100% identity with those from Italy, Peru, Republic of the Congo, and the United States. Alignment results analysis of VP1 aa sequences showed differences between the strains isolated in this study (Met^{25}, Asp^{138}, Ser^{207}) and EV-C105 strain 34S (Val^{25}, Glu^{138}, and Ala^{207}). In this study, we grouped Beijing-R2759 with the strain obtained from the United States in 2014 (Figure). We observed a similar relationship in the phylogenic tree of the VP1 gene. These findings indicate that Beijing-R2759 is closely related to the EV-C105 strain reported in the United States.

Our report confirms that the distribution of EV-C105 is geographically wider than previously believed. A greater awareness of EV-C105 may enable improved detection of this virus (*9*). In addition, our findings show the utility of the RVSS in assessing the patterns of circulation of enterovirus genotypes and detecting enterovirus outbreaks for the purpose of early warning.

This work was supported by the National Major Science and Technology Project for Control and Prevention of Major Infectious Diseases of China (2017ZX101304), the Beijing Municipal Science and Technology Commission (Z151100003915140), and the Capital Medical Development and Scientific Research Fund (2016-2-3011).

About the Author

Dr. Li is an assistant researcher in the Institute for Immunization and Prevention of the Beijing Center for Disease Prevention and Control. His research interests include early warning systems and respiratory pathogens.

References

1. Lukashev AN, Drexler JF, Kotova VO, Amjaga EN, Reznik VI, Gmyl AP, et al. Novel serotypes 105 and 116 are members of distinct subgroups of human enterovirus C. J Gen Virol. 2012;93:2357–62. http://dx.doi.org/10.1099/vir.0.043216-0
2. Van Leer-Buter CC, Poelman R, Borger R, Niesters HG. Newly identified enterovirus C genotypes, identified in the Netherlands

through routine sequencing of all enteroviruses detected in clinical materials from 2008 to 2015. J Clin Microbiol. 2016;54:2306–14. http://dx.doi.org/10.1128/JCM.00207-16
3. Grard G, Drexler JF, Lekana-Douki S, Caron M, Lukashev A, Nkoghe D, et al. Type 1 wild poliovirus and putative enterovirus 109 in an outbreak of acute flaccid paralysis in Congo, October–November 2010. Euro Surveill. 2010;15:pii=19723.
4. Richter J, Tryfonos C, Panagiotou C, Nikolaou E, Koliou M, Christodoulou C. Newly emerging C group enteroviruses may elude diagnosis due to a divergent 5′-UTR. Int J Infect Dis. 2013;17:e1245–8.
5. Todd A, Taylor S, Huang QS. Identification of enterovirus C105 for the first time in New Zealand. Western Pac Surveill Response J. 2015;6:60–2. http://dx.doi.org/10.5365/wpsar.2014.5.4.003
6. Zhang T, Ren L, Luo M, Li A, Gong C, Chen M, et al. Enterovirus D68–associated severe pneumonia, China, 2014. Emerg Infect Dis. 2015;21:916–8. http://dx.doi.org/10.3201/eid2105.150036
7. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. J Clin Microbiol. 2006;44:2698–704. http://dx.doi.org/10.1128/JCM.00542-06
8. Oberste MS, Penaranda S, Rogers SL, Henderson E, Nix WA. Comparative evaluation of Taqman real-time PCR and semi-nested VP1 PCR for detection of enteroviruses in clinical specimens. J Clin Virol. 2010;49:73–4.
9. Barnadas C, Midgley SE, Skov MN, Jensen L, Poulsen MW, Fischer TK. An enhanced enterovirus surveillance system allows identification and characterization of rare and emerging respiratory enteroviruses in Denmark, 2015–16. J Clin Virol. 2017;93:40–4.

Address for correspondence: Fang Huang, Beijing Center for Disease Prevention and Control, Institute for Communicable Disease Control and Prevention, No. 16, Hepingli Middle Av, Dongcheng District, Beijing 100013, China; email: hhffxdd@126.com

Recent Findings of Potentially Lethal Salamander Fungus *Batrachochytrium salamandrivorans*

David Lastra González, Vojtech Baláž, Milič Solský, Barbora Thumsová, Krzysztof Kolenda, Anna Najbar, Bartłomiej Najbar, Matej Kautman, Petr Chajma, Monika Balogová, Jiří Vojar

Author affiliations: Czech University of Life Sciences, Prague, Czech Republic (D. Lastra González, M. Solský, B. Thumsová, P. Chajma, J. Vojar); University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic (V. Baláž, M. Kautman); University of Wrocławski, Wroclaw, Poland (K. Kolenda, A. Najbar); University of Zielona Góra, Lubuskie, Poland (B. Najbar); Slovak Academy of Sciences, Košice, Slovakia (M. Kautman); Pavol Jozef Šafárik University in Košice, Košice (M. Balogová)

DOI: https://doi.org/10.3201/eid2507.181001

The distribution of the chytrid fungus *Batrachochytrium salamandrivorans* continues to expand in Europe. During 2014–2018, we collected 1,135 samples from salamanders and newts in 6 countries in Europe. We identified 5 cases of *B. salamandrivorans* in a wild population in Spain but none in central Europe or the Balkan Peninsula.

Chytridiomycosis, an amphibian disease caused by the chytrid fungi *Batrachochytrium dendrobatidis* and *B. salamandrivorans*, is responsible for declines of amphibian populations worldwide (*1*). The recently discovered *B. salamandrivorans* (*2*) is severely impacting salamanders and newts in Europe (*3,4*). This emerging fungal pathogen infects the skin of caudates and causes lethal lesions (*2*). It most likely was introduced to Europe by the pet salamander trade from Southeast Asia (*3*). In Europe, the Netherlands, Belgium, and Germany have confirmed *B. salamandrivorans* in wild caudates; the United Kingdom, Germany, and Spain have confirmed the fungus in captive animals (*5,6*). Several countries have established trade regulations (*5*) and a recent European Union decision, no. 2018/320, implements measures to protect against the spread of *B. salamandrivorans* via traded salamanders (*7*). The World Organisation for Animal Health listed infection with *B. salamandrivorans* as a notifiable disease in 2017. In addition to controlling the amphibian pet trade, surveillance of the pathogen is urgently needed to establish disease intervention strategies in affected areas and prevention in *B. salamandrivorans*–free regions.

During 2014–2018, we collected 1,135 samples directly for the detection of *B. salamandrivorans* or as a part of unrelated studies. Samples came from 10 amphibian species at 47 sites in 6 countries in Europe. Most samples came from the fire salamander, *Salamandra salamandra*, which is a known suitable host for *B. salamandrivorans* (*3*), and the palmate newt, *Lissotriton helveticus*, which is known to be resistant to *B. salamandrivorans* (Appendix Table 1, http://wwwnc.cdc.gov/EID/article/25/7/18-1001-App1.pdf).

Most samples were skin swabs collected by following the standard procedure for sampling of amphibian chytrid fungi (*8*). A smaller portion of samples was toe clippings (Appendix Table 2). We extracted genomic DNA following the protocol of Blooi et al. (*9*), and 2 laboratories with different equipment tested for *B. salamandrivorans*. Samples from Spain and the Czech Republic initially were analyzed at the Czech University of Life Sciences (Prague, Czech

Republic) by standard PCR with *B. salamandrivorans*–specific primers STerF and STerR, as described by Martel et al. (*2*), with subsequent electrophoresis on the amplified target. We reanalyzed samples that produced positive or equivocal results by using duplex quantitative PCR (qPCR) for *B. dendrobatidis* and *B. salamandrivorans* (*9*) at the University of Veterinary and Pharmaceutical Sciences (Brno, Czech Republic). Trenton Garner of the Institute of Zoology, Zoological Society of London (London, England), provided DNA for quantification standards of the *B. dendrobatidis* GPL lineage, strain IA042, and An Martel of Ghent University (Ghent, Belgium) provided quantification standards of *B. salamandrivorans*.

We directly analyzed samples from other countries by qPCR. We used negative and positive controls for standard PCR analyses and quantification standards for qPCR analyses. For *B. dendrobatidis*– or *B. salamandrivorans*–positive sites, we estimated prevalence and Bayesian 95% CIs using 3 parallel Markov chains with 2,000 iterations each, a burn-in of 1,000 iterations, and no thinning (Appendix Table 1). We performed all statistical analyses in R 3.3.1 using the R2WinBUGS package and WinBUGS 1.4.3 (*10*).

Samples from 5 *L. helveticus* newts tested positive for *B. salamandrivorans*, implying that this species is not resistant to this fungus as previously indicated by experimental exposures (*3*). The positive cases were found in populations from an isolated area encompassing 2 different regions in northern Spain, Cantabria and Asturias, with remote human populations. Four cases were found in livestock drinking troughs located 150–1,000 m above sea level, and 1 case was found in a pond in a private garden, 30 km from the nearest recorded case. We did not find *B. salamandrivorans*–positive cases in consecutive locations during our monitoring.

Although *B. salamandrivorans* cases have been reported in captive salamanders (*6*), our reported cases were >1,000 km from any area of known *B. salamandrivorans* occurrence (*7*). We also detected *B. dendrobatidis* by duplex qPCR in 11 samples from 3 newt species (*L. helveticus*, *L. vulgaris*, and *Triturus cristatus*) from Spain and Montenegro and 1 captive *Cynops ensicauda* newt from the Czech Republic. The *B. dendrobatidis*–positive cases did not involve co-infection with *B. salamandrivorans*.

We confirmed that the known distribution of *B. salamandrivorans* continues to expand in Europe, indicating that this fungus might be capable of dispersing over long distances (*4*), might be introduced by humans, or might even have been circulating in this geographic range with no detected deaths. Our results should alert the research and conservation community and motivate urgent action to identify regions with early emergence of the disease and implement mitigation measures to prevent further spread of this deadly pathogen.

Acknowledgments

We thank the Cantabria delegation of SEO/Birdlife; Fundación Zoo Santillana del Mar; workers from Marismas de Santoña, Victoria y Joyel Natural Park, with special thanks to Carlos Rubio; Pepo Nieto, Pedro Barreda, and his family; Elena Kulikova and Wiesław Babik; and also our friends Daniel Koleška, Kamila Šimůnková, Tomáš Holer, and Daniela Budská for fieldwork.

This work was performed with permission from the Nature Conservation Agency of the Czech Republic; Agency for Nature and Environment Protection of Montenegro permit no. 02 Broj UPI–321/4; Ministry of Environment of the Slovak Republic, permit no. 4924/2017–6.3; the Endangered Species Section of Environmental Service of Cantabria, Spain, permit no. EST–419/2017–SEP; the Environmental Service of Castilla y León, Spain, permit no. EP/LE/233/2017; Department of Nature Conservation of Poland, permit nos. DZP-WG.6401.02.7.2014. JRO, WPN.6401.211.2015.MR.2, 78/2014, and 68/2015; the Ministry of Protection of Environment of Croatia, permit no. UP/I–612–07/169–48/68; and agreements from other agencies, including Red Cambera, special thanks to Sergio Tejón and Tomás González; Fondo para la Protección de los Animales Salvajes; and Fundación Naturaleza y Hombre, Spain. The study was supported by the Czech University of Life Sciences, Prague, Czech Republic (grant nos. 20174218 and 20184247) and the Internal Grant Agency of the University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic (grant no. 224/2016/FVHE). K.K. was supported by MNiSW grant for Young Scientists no. 0420/1408/16; A.N. was supported by grant no. DS 1076/S/IBŚ/2014 and MNiSW grant for Young Scientists no. 0420/1409/16.

About the Author

Mr. Lastra González is a PhD candidate at Czech University of Life Sciences, Prague. His research focuses on amphibian conservation and emerging infectious diseases that affect them.

References

1. Berger L, Roberts AA, Voyles J, Longcore JE, Murray KA, Skerratt LF. History and recent progress on chytridiomycosis in amphibians. Fungal Ecol. 2016;19:89–99. http://dx.doi.org/10.1016/j.funeco.2015.09.007
2. Martel A, Spitzen-van der Sluijs A, Blooi M, Bert W, Ducatelle R, Fisher MC, et al. *Batrachochytrium salamandrivorans* sp. nov. causes lethal chytridiomycosis in amphibians. Proc Natl Acad Sci U S A. 2013;110:15325–9. http://dx.doi.org/10.1073/pnas.1307356110
3. Martel A, Blooi M, Adriaensen C, Van Rooij P, Beukema W, Fisher MC, et al. Recent introduction of a chytrid fungus endangers Western Palearctic salamanders. Science. 2014;346:630–1. http://dx.doi.org/10.1126/science.1258268
4. Stegen G, Pasmans F, Schmidt BR, Rouffaer LO, Van Praet S, Schaub M, et al. Drivers of salamander extirpation mediated by *Batrachochytrium salamandrivorans.* Nature. 2017;544:353–6. http://dx.doi.org/10.1038/nature22059

5. European Food Safety Authority Panel on Animal Health and Welfare, More S, Angel Miranda M, Bicout D, Bøtner A, Butterworth A, et al. Risk of survival, establishment and spread of *Batrachochytrium salamandrivorans* (*Bsal*) in the EU. EFSA Journal. 2018;16:5259. http://dx.doi.org/10.2903/j.efsa.2018.5259
6. Fitzpatrick LD, Pasmans F, Martel A, Cunningham AA. Epidemiological tracing of *Batrachochytrium salamandrivorans* identifies widespread infection and associated mortalities in private amphibian collections. Sci Rep. 2018;8:13845. http://dx.doi.org/10.1038/s41598-018-31800-z
7. Commission Implementing Decision (EU) 2018/320 of 28 February 2018 on certain animal health protection measures for intra-Union trade in salamanders and the introduction into the Union of such animals in relation to the fungus *Batrachochytrium salamandrivorans*. Official Journal of the European Union. 2018;L62:18–33 [cited 2018 Jun 1]. http://data.europa.eu/eli/dec_impl/2018/320/oj
8. Hyatt AD, Boyle DG, Olsen V, Boyle DB, Berger L, Obendorf D, et al. Diagnostic assays and sampling protocols for the detection of *Batrachochytrium dendrobatidis.* Dis Aquat Organ. 2007;73:175–92. http://dx.doi.org/10.3354/dao073175
9. Blooi M, Pasmans F, Longcore JE, Spitzen-van der Sluijs A, Vercammen F, Martel A. Duplex real-time PCR for rapid simultaneous detection of *Batrachochytrium dendrobatidis* and *Batrachochytrium salamandrivorans* in amphibian samples. J Clin Microbiol. 2013;51:4173–7. http://dx.doi.org/10.1128/JCM.02313-13
10. Lunn DJ, Thomas A, Best N, Spiegelhalter D. WinBUGS–a Bayesian modelling framework: concepts, structure, and extensibility. Stat Comput. 2000;10:325–37. http://dx.doi.org/10.1023/A:1008929526011

Address for correspondence: David Lastra González, Faculty of Environmental Sciences, Czech University of Life Sciences, Kamýcká 129, 165 21 Prague–Suchdol, Czech Republic; email: lastra_gonzalez@fzp.czu.cz

Crimean-Congo Hemorrhagic Fever Virus Genome in Tick from Migratory Bird, Italy

Elisa Mancuso, Luciano Toma, Andrea Polci, Silvio G. d'Alessio, Marco Di Luca, Massimiliano Orsini, Marco Di Domenico, Maurilia Marcacci, Giuseppe Mancini, Fernando Spina, Maria Goffredo, Federica Monaco

Author affiliations: Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale," Teramo, Italy (E. Mancuso, A. Polci, S.G. d'Alessio, M. Orsini, M. Di Domenico, M. Marcacci, G. Mancini, M. Goffredo, F. Monaco); Istituto Superiore di Sanità, Rome, Italy (L. Toma, M. Di Luca); Istituto Superiore per la Protezione e la Ricerca Ambientale, Bologna, Italy (F. Spina)

DOI: https://doi.org/10.3201/eid2507.181345

We detected Crimean-Congo hemorrhagic fever virus in a *Hyalomma rufipes* nymph collected from a whinchat (*Saxicola rubetra)* on the island of Ventotene in April 2017. Partial genome sequences suggest the virus originated in Africa. Detection of the genome of this virus in Italy confirms its potential dispersion through migratory birds.

Crimean-Congo hemorrhagic fever virus (CCHFV) is a vectorborne virus responsible for severe illness in humans, whereas other mammals usually act as asymptomatic reservoirs. The virus is transmitted through tick bites or by direct contact with blood or body fluids of infected vertebrate hosts. CCHFV, an *Orthonairovirus* within the Nairoviridae family, has a negative-sense tripartite RNA genome characterized by high genetic diversity. The sequences of the circulating strains cluster in 6 genotypes (I–VI) reflecting their geographic origin; worldwide distribution is the result of efficient dispersion through migratory birds, human travelers, and the trade and movement of livestock and wildlife (*1*,*2*). In Europe, CCHFV distribution was limited to the Balkan region until 2010, when the virus was identified in ticks collected from a red deer (*Cervus elaphus*) and, 6 years later, in 2 autochthonous human cases in the same region of Spain (*3*). Sequences from the Iberia strains clustered in the Africa genotype III (*4*), supporting the hypothesis of CCHFV dispersion through ticks hosted by migrating birds.

The role of birds in the potential spread of the virus was confirmed by CCHFV detection in ticks collected from migratory birds in Greece in 2009 (*5*) and Morocco in 2011 (*6*). Because Italy hosts an intense passage of birds migrating along major routes connecting winter quarters in Africa and breeding areas in Europe, the country is potentially exposed to the risk for virus introduction. We report the detection of CCHFV RNA in a tick collected in Italy from a migratory bird.

We conducted tick sampling during March–May 2017 on the island of Ventotene, where a ringing station has been operating since 1988 as part of the Small Islands Project, a large-scale and long-term effort to monitor spring migrations of birds across the central and western Mediterranean. We ringed 5,095 birds and checked ≈80% for ectoparasites. We collected 14 adults, 330 nymphs, and 276 larvae from 268 passerines belonging to 28 species; 18 species were trans-Saharan migrants. We stored ticks in 70% ethanol until morphologic identification and assignment to a genus or, whenever possible, a species (*7*). We then individually

homogenized the ticks and extracted nucleic acids by using the Maxwell16LEV simplyRNA Blood Kit with a Maxwell16 Instrument (Promega, https://twitter.com/promega). We assayed purified nucleic acids for the presence of CCHFV RNA and to refine the morphologic identification of the immature ticks. We used the RealStar CCHFV RT-PCR Kit 1.0 (Altona Diagnostics, https://www.altona-diagnostics.com) to reveal viral RNA and conducted molecular tick species identification by sequencing 340 bp of the 12S rDNA sequence (*8*). We detected CCHFV RNA in a half-fed nymph of *Hyalomma rufipes*, a member of the *H. marginatum* complex, collected from a whinchat (*Saxicola rubetra*), a trans-Saharan migratory bird distributed across Europe and western Asia that winters mainly in central Africa.

We further sequenced the sample by using the Illumina Next 500 platform (Illumina, https://www.illumina.com) with a slightly modified sequence-independent single-primer amplification protocol. We cleaned and de novo assembled raw reads by using SPADES 3.11 (http://cab.spbu.ru/software/spades). We identified 18 partial but reliably assembled contigs (97%–99% identity) that could be definitely assigned to CCHFV. We aligned partial sequences from small (485 bp) and medium (1,374 bp) segments with 13 virus sequences representative of the 6 circulating virus genotypes. We performed cladistics analysis by using MEGA7 (https://www.megasoftware.net). Whatever the genome segment involved, the cladograms revealed that the strain identified in Italy originated in Africa and had a close affinity with those strains segregated in genotype III (Figure) but excluded a direct introduction from eastern Europe.

The origin of the introduction of this virus is further supported by the geographic distribution of the infected tick, identified as *H. rufipes*. Adults of this species have been sporadically collected in Europe (*9*,*10*) but are mainly distributed in sub-Saharan Africa, whereas *H. marginatum* ticks are autochthonous in many Mediterranean countries. *H. marginatum* and *H. rufipes* ticks are competent vectors for CCHFV (*2*) and are characterized by a 2-host cycle, with birds usually acting as hosts of immature stages.

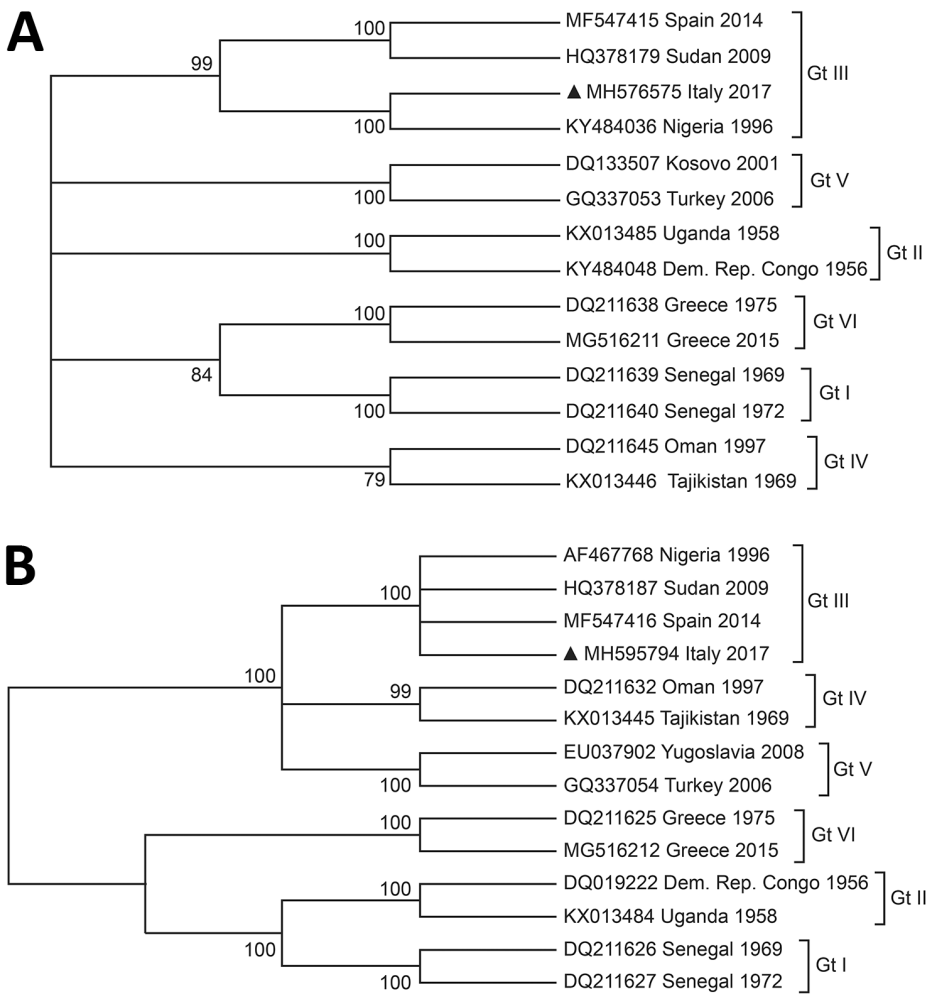


Figure. Cladistic relationship of Crimean-Congo hemorrhagic fever virus gene found in a *Hyalomma rufipes* nymph collected from a whinchat (*Saxicola rubetra)* in Italy (black triangles) with strains representative of the circulating genotypes. The analysis is based on partial sequences of the small segment (A) and medium segment (B) of the virus genomes. We used the neighbor-joining method based on the Tamura-Nei model. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The analysis involved 14 nucleotide sequences. Codon positions included were first, second, third, and noncoding. All positions containing gaps and missing data have been eliminated. A total of 485 positions (small segment) and 1,374 positions (medium segment) were included in the final dataset. GenBank accession numbers are provided. Gt, genotype.

Larvae feed on birds and molt to become nymphs, remaining on the same avian host up to 26 days, a period that usually lasts until the trans-Saharan migrating birds have reached Europe (*1*). Thus, the half-fed nymph probably was attached to the whinchat when migration started. Nymphs drop off the bird only after completion of the blood meal and molt on the ground before attaching to their second, final hosts, which are usually large mammals, including humans.

Although detection of virus genome does not necessarily imply the presence of live virus that is able to spread locally, our findings, consistent with the recent autochthonous cases in Spain, underscore the need to monitor any introduction and circulation of CCHFV in southwestern Europe. Such monitoring should focus on sites where migrants rest or nest and where a local population of competent ticks and their hosts interact. Raising awareness of possible outbreaks should also include specific surveillance and contingency plans focused on categories of persons and animals at elevated risk for CCHFV infection.

Acknowledgments

We gratefully acknowledge the support of Sara Riello and all volunteers operating the ringing station of Ventotene. We warmly thank the Natural Reserve of Ventotene and S. Stefano islands for logistic and financial support. All the ticks were collected during the ringing activities in the framework of the Small Island research project (ISPRA).

This work has been supported by Italy's Ministry of Health (research grant no. MSRCTE 03/14).

About the Author

Ms. Mancuso holds an MSc in biology and serves as a junior research assistant in the National Reference of Foreign Animals Diseases at the Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" in Teramo, Italy. Her research interests include bird migration ecology and the diagnosis of vectorborne pathogens.

References

1. Hoogstraal H. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. J Med Entomol. 1979;15:307–417. http://dx.doi.org/10.1093/jmedent/15.4.307
2. Hoogstraal H. Changing patterns of tickborne diseases in modern society. Annu Rev Entomol. 1981;26:75–99. http://dx.doi.org/10.1146/annurev.en.26.010181.000451
3. Negredo A, de la Calle-Prieto F, Palencia-Herrejón E, Mora-Rillo M, Astray-Mochales J, Sánchez-Seco MP, et al.; Crimean Congo Hemorrhagic Fever@Madrid Working Group. Autochthonous Crimean-Congo hemorrhagic fever in Spain. N Engl J Med. 2017;377:154–61. http://dx.doi.org/10.1056/NEJMoa1615162
4. Ramírez de Arellano E, Hernández L, Goyanes MJ, Arsuaga M, Cruz AF, Negredo A, et al. Phylogenetic characterization of Crimean-Congo hemorrhagic fever virus, Spain. Emerg Infect Dis. 2017;23:2078–80. http://dx.doi.org/10.3201/eid2312.171002
5. Lindeborg M, Barboutis C, Ehrenborg C, Fransson T, Jaenson TG, Lindgren PE, et al. Migratory birds, ticks, and Crimean-Congo hemorrhagic fever virus. Emerg Infect Dis. 2012;18:2095–7. http://dx.doi.org/10.3201/eid1812.120718
6. Palomar AM, Portillo A, Santibanez P, Mazuelas D, Crespo A, Gutierrez O, et al. CCHFV from migratory birds, Morocco. Emerg Infect Dis. 2013;19:260–3. http://dx.doi.org/10.3201/eid1902.121193
7. Manilla G. Acari Ixodida. In: Fauna d'Italia. Volume 36. Bologna (Italy): Calderini; 1998. p. 280.
8. Toma L, Mancini F, Di Luca M, Cecere JG, Bianchi R, Khoury C, et al. Detection of microbial agents in ticks collected from migratory birds in central Italy. Vector Borne Zoonotic Dis. 2014;14:199–205. http://dx.doi.org/10.1089/vbz.2013.1458
9. Chitimia-Dobler L, Nava S, Bestehorn M, Dobler G, Wölfel S. First detection of *Hyalomma rufipes* in Germany. Ticks Tick Borne Dis. 2016;7:1135–8. http://dx.doi.org/10.1016/j.ttbdis.2016.08.008
10. Hornok S, Horváth G. First report of adult *Hyalomma marginatum rufipes* (vector of Crimean-Congo haemorrhagic fever virus) on cattle under a continental climate in Hungary. Parasit Vectors. 2012;5:170. http://dx.doi.org/10.1186/1756-3305-5-170

Address for correspondence: Federica Monaco, Istituto Zooprofilattico Sperimentale dell'Abruzzo e del Molise "G. Caporale" Campo Boario, Teramo 64100, Italy; email: f.monaco@izs.it

Echinococcus canadensis G8 Tapeworm Infection in a Sheep, China, 2018

Ruiqi Hua,[1] Yue Xie,[1] Hongyu Song, Yuan Shi, Jiafei Zhan, Maodi Wu, Xiaobin Gu, Xuerong Peng, Guangyou Yang

Author affiliation: Sichuan Agricultural University, Chengdu, China

DOI: https://doi.org/10.3201/eid2507.181585

We report a sheep infected with *Echinococcus canadensis* G8 tapeworm in China in 2018. This pathogen was previously detected in moose, elk, muskox, and mule deer in Europe and North America; our findings suggest a wider host range and geographic distribution. Surveillance for the G8 tapeworm should be conducted in China.

[1]These authors contributed equally to this article.

Cystic echinococcosis (CE) is a zoonotic disease of worldwide distribution that causes disease, death, and economic loss in many domestic and wildlife ungulates and carnivore species, as well as in humans. Animals and humans can become infected through the accidental ingestion of *Echinococcus* tapeworm eggs (*1*,*2*). *Echinococcus granulosus* sensu stricto (G1, G3) tapeworms are considered the major cause of CE globally; however, cases attributable to *E. canadensis* genotypes within the *E. granulosus* tapeworm complex are increasingly being recognized (*3*). Overall, *E. canadensis* tapeworms comprise 4 genotypes (G6, G7, G8, G10), although the taxonomy is still being debated (*4*). *E. canadensis* G8 tapeworms were initially identified in 1994 in a moose (*Alces alces*) in Minnesota, USA (Appendix Table, https://wwwnc.cdc.gov/EID/article/25/7/18-1585-App1.pdf). Then, in 2002, two infections were reported in humans in Alaska. G8 tapeworms have also been found in elk (*Cervus canadensis*, 2006) and muskox (*Ovibos moschatus*, 2013) in Canada. Updated epidemiologic data show infections have also occurred in Estonia moose (2008), Russia moose (2013), and a US mule deer (*Odocoileus hemionus*, 2018). As of April 2019, at least 4 species (moose, elk, muskox, and mule deer) have been proven to serve as intermediate hosts of G8 tapeworms in Europe and North America. We report a potential new public health threat regarding sheep (*Ovis aries*) infected with *E. canadensis* G8 tapeworms in China and highlight the potential wider host range and geographic distribution of this species.

During 2017, we conducted a molecular epidemiologic survey of CE in northwestern China and collected 277 hydatid cysts from sheep (78 from Qinghai-Tibet Plateau, 60 from Xinjiang Autonomous Region) and yaks (*Bos mutus*; 139 from Qinghai-Tibet Plateau) at local slaughterhouses. During sampling, we handled all animals in strict accordance with the animal welfare laws of China. We genotyped the hydatid cysts using the partial mitochondrial *cox1* gene sequence, as described previously (*5*), and found that most cyst specimens were represented by *E. granulosus* G1 and G3 tapeworms (data not shown), and 1 sheep cyst was diagnosed as an *E. canadensis* G8–like tapeworm infection (herein designated sheep-XN) (Appendix Figure 1, panel A). To further investigate the genotype of tapeworm sheep-XN, we amplified the full-length *cox1* gene (1,608 bp) and the mitochondrial *nad1* gene (894 bp), a method proven effective for *Echinococcus* tapeworm genotyping (*4*). This analysis verified that sheep-XN clustered with *E. canadensis* G8 tapeworms (Appendix Figure 1, panel B). However, given that partial mitochondrial DNA (mtDNA) sequences are insufficient to identify genotype (because of limited loci information) (*6*), we amplified the complete mtDNA of sheep-XN and compared it with *Echinococcus* mtDNA sequences from GenBank. The resulting phylogenetic tree showed the same topologic structure as that acquired when using the *cox1* and *nad1* genes, suggesting that sheep-XN was an *E. canadensis* G8 tapeworm (Figure).

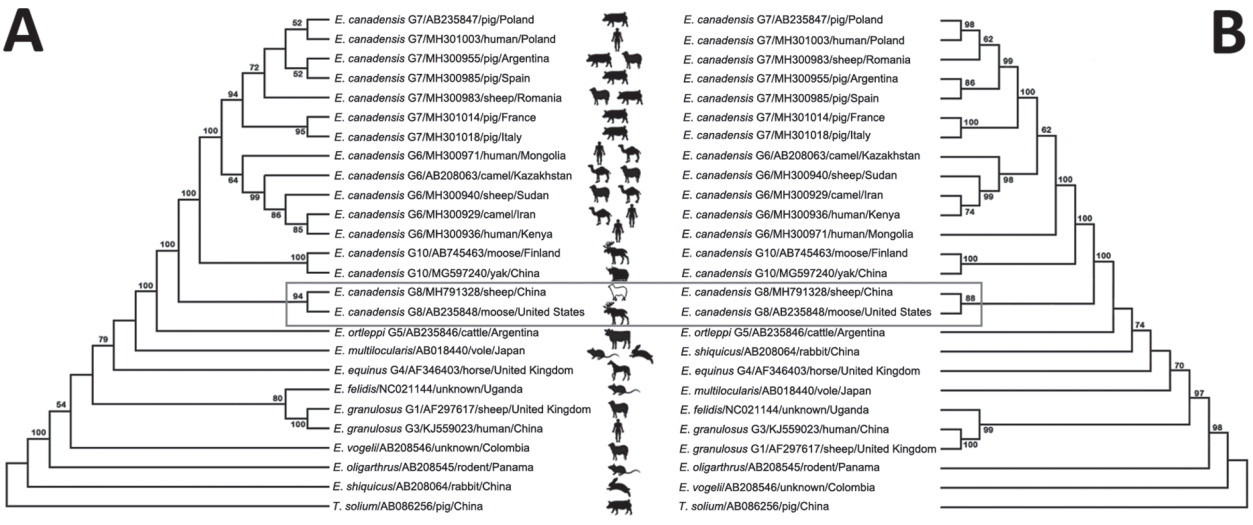


Figure. Phylogenetic analysis of *Echinococcus* species of different genotypes, strains, and host origins, including the *E. canadensis* G8 tapeworm identified in a sheep in China, 2018. Phylogenetic trees were inferred by maximum-likelihood analysis on the basis of concatenated amino acid data of 12 protein-coding genes by using the Jones-Taylor-Thornton model (A) and concatenated nucleotide data of 12 protein-coding genes by using the Tamura-Nei model (B) in MEGA7.0 (https://www.megasoftware.net). The reference species *Taenia solium* was used as the outgroup. We performed bootstrapping with 1,000 replicates to calculate the percentage reliability for each node in both data sets; only values of ≥50% are shown. Tree branch lengths are proportional to the evolutionary distance. The box contains the *E. canadensis* G8 tapeworm identified in this study (GenBank accession no. MH791328) and its closest relative from a moose in the United States (GenBank accession no. AB235848). Sheep shown in white represents a potential new intermediate host of *E. canadensis* G8.

We confirmed that the sheep-origin hydatid cyst was *E. canadensis* G8 tapeworm (Appendix Figure 1, panel C) and suggest that this pathogen potentially poses a new public health threat on the Qinghai-Tibet Plateau of China, where human echinococcosis is prevalent. Previous research has shown that sterile cysts usually result when *Echinococcus* spp. infect species not commonly infected (*7*). However, for the sheep-origin cyst, we found numerous protoscoleces in the hydatid fluid, indicating the cyst was fertile. Thus, sheep might serve as another intermediate host of the *E. canadensis* G8 tapeworm in nature and spread protoscoleces to definitive hosts, posing a threat to local herdsmen and livestock.

G6 and G7 tapeworms can circulate through the domestic cycle (in animals such as camels, pigs, and dogs) or the sylvatic cycle (in animals such as reindeer and wolves), and G8 and G10 tapeworms are generally believed to be restricted to the sylvatic cycle in circumpolar regions (Appendix Table) (*2*,*8*). Our finding of an *E. canadensis* G8 tapeworm in a sheep in China should not only alert the local population to be aware of this pathogen but also contributes to the discussion concerning *E. canadensis* tapeworm taxonomy. Further research is required to determine the transmission dynamics of this pathogen and determine whether the domestic life cycle of *E. canadensis* G8 tapeworm (circulation through sheep and dogs) has been or is present.

Since 2017, a mandatory vaccination campaign of sheep and goats with the CE vaccine EG95 has been sponsored in high-prevalence areas of China because of China's policy, the National Medium- and Long-Term Plan for Animal Disease Control (2012–2020) (*9*). However, EG95 was developed against the *E. granulosus* G1 tapeworm (*10*) and might not provide effective protection against the *E. canadensis* G8 tapeworm. Our findings indicate the G8 tapeworm might be prevalent in sheep in China, suggesting a wider host range and geographic distribution (Appendix Table; Appendix Figure 2). Thus, we propose the need for increased surveillance of the *E. canadensis* G8 tapeworm in China and that integration of this pathogen into ongoing echinococcosis programs is essential for tapeworm prevention and control.

This work was supported by grants from the Key Technology Research and Development Program of Sichuan Province in China (grant no. 2015NZ0041) and the National Natural Science Foundation of China (grant no. 31672547).

About the Author

Mr. Hua is a graduate student studying at the Department of Parasitology, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, Sichuan, China. His primary research interest is parasite genomics.

References

1. Cardona GA, Carmena D. A review of the global prevalence, molecular epidemiology and economics of cystic echinococcosis in production animals. Vet Parasitol. 2013;192:10–32. http://dx.doi.org/10.1016/j.vetpar.2012.09.027
2. Cerda JR, Buttke DE, Ballweber LR. *Echinococcus* spp. tapeworms in North America. Emerg Infect Dis. 2018;24:230–5. http://dx.doi.org/10.3201/eid2402.161126
3. Alvarez Rojas CA, Romig T, Lightowlers MW. *Echinococcus granulosus* sensu lato genotypes infecting humans—review of current knowledge. Int J Parasitol. 2014;44:9–18. http://dx.doi.org/10.1016/j.ijpara.2013.08.008
4. Davidson RK, Lavikainen A, Konyaev S, Schurer J, Miller AL, Oksanen A, et al. *Echinococcus* across the north: current knowledge, future challenges. Food Waterborn Parasitol. 2016;4:39–53. http://dx.doi.org/10.1016/j.fawpar.2016.08.001
5. Bowles J, Blair D, McManus DP. Genetic variants within the genus *Echinococcus* identified by mitochondrial DNA sequencing. Mol Biochem Parasitol. 1992;54:165–73. http://dx.doi.org/10.1016/0166-6851(92)90109-W
6. Laurimäe T, Kinkar L, Romig T, Omer RA, Casulli A, Umhang G, et al. The benefits of analysing complete mitochondrial genomes: deep insights into the phylogeny and population structure of *Echinococcus granulosus* sensu lato genotypes G6 and G7. Infect Genet Evol. 2018;64:85–94. http://dx.doi.org/10.1016/j.meegid.2018.06.016
7. Bružinskaitė R, Šarkūnas M, Torgerson PR, Mathis A, Deplazes P. Echinococcosis in pigs and intestinal infection with *Echinococcus* spp. in dogs in southwestern Lithuania. Vet Parasitol. 2009;160:237–41. http://dx.doi.org/10.1016/j.vetpar.2008.11.011
8. Oksanen A, Lavikainen A. *Echinococcus canadensis* transmission in the North. Vet Parasitol. 2015;213:182–6. http://dx.doi.org/10.1016/j.vetpar.2015.07.033
9. Zhong Z. The national medium- and long-term plan for animal disease control (2012–2020). 2014 Nov 4 [cited 2018 Nov 11]. http://ap.fftc.agnet.org/ap_db.php?id=308
10. Alvarez Rojas CA, Gauci CG, Lightowlers MW. Antigenic differences between the EG95-related proteins from *Echinococcus granulosus* G1 and G6 genotypes: implications for vaccination. Parasite Immunol. 2013;35:99–102. http://dx.doi.org/10.1111/pim.12009

Address for correspondence: Guangyou Yang, Department of Parasitology, College of Veterinary Medicine, Sichuan Agricultural University, 211 Huimin Rd, Chengdu, Sichuan, 611130, China; email: guangyouyang@hotmail.com

Zoonotic Bacteria in Fleas Parasitizing Common Voles, Northwestern Spain

Ruth Rodríguez-Pastor,[1] François Mougeot, Mª Dolors Vidal, Isabel Jado, Rosa M. González-Martín-Niño, Raquel Escudero,[2] Juan José Luque-Larena[2]

Author affiliations: Universidad de Valladolid and Instituto Universitario de Investigación en Gestión Forestal Sostenible, Palencia, Spain (R. Rodríguez-Pastor, J.J. Luque-Larena); Instituto de Investigación en Recursos Cinegéticos, Ciudad Real, Spain (F. Mougeot); Universidad de Castilla-La Mancha, Ciudad Real (M.D. Vidal); Instituto de Salud Carlos III, Madrid, Spain (I. Jado, R.M. González-Martín-Niño, R. Escudero)

DOI: https://doi.org/10.3201/eid2507.181646

We detected *Francisella tularensis* and *Bartonella* spp. in fleas parasitizing common voles (*Microtus arvalis*) from northwestern Spain; mean prevalence was 6.1% for *F. tularensis* and 51% for *Bartonella* spp. Contrasted vector–host associations in the prevalence of these bacteria suggest that fleas have distinct roles in the transmission cycle of each pathogen in nature.

A dynamic prevalence of *Francisella tularensis* and *Bartonella* spp. was reported in irruptive common vole (*Microtus arvalis*) populations during 2013–2015 from agricultural landscapes of northwestern Spain (*1*,*2*). In that area, notifiable tularemia has been endemic since 1997, and human cases periodically occur during outbreaks in voles (*3*,*4*). Prevalence of *F. tularensis* and *Bartonella* spp. in voles increases with vole density (*1,2*), highlighting the key role of fluctuating rodents in shaping zoonoses dynamics (*1*–*4*). Rodent ectoparasites often play a major role in transmitting zoonotic pathogens. In the population studied, ticks rarely infest voles (2% prevalence), whereas fleas are much more prevalent (68%) (*2*). Nevertheless, any potential role for vole fleas in the circulation of *F. tularensis* or *Bartonella* spp. in natural environments remains unknown. To elucidate realistic transmission route scenarios in host-dynamic environments (*5*–*8*), we investigated whether zoonotic bacteria occur concomitantly in voles and fleas.

Our main goal was to study the prevalence of *F. tularensis* in fleas collected from voles previously tested for tularemia (*1*). We screened flea DNA in search of 6 main zoonotic bacteria simultaneously (*Anaplasma phagocytophilum*, *Bartonella* spp., *Borrelia* spp., *Coxiella burnetii*, *F. tularensis*, and *Rickettsia* spp.), following the same molecular procedure (multiplex PCR) (*9*) previously used to screen vole pathogens (*1,2*). Voles and fleas were live-trapped in northwestern Spain during March 2013–March 2015 (Appendix, https://wwwnc.cdc.gov/EID/article/25/7/18-1646-App1.pdf). We collected fleas from each individual vole and identified and grouped them in pools (pool = total fleas/vole). Three flea species parasitize common voles in the area: *Ctenophthalmus apertus*, *Nosopsyllus fasciatus*, and *Leptopsylla taschenbergi* (*2*). We screened monospecific pools (all fleas in a pool belonged to the same species and came from the same vole host), for a sample size of 90 vole hosts (pools) and 191 fleas. We screened 78 *C. apertus* fleas (39 pools) and 113 *N. fasciatus* fleas (51 pools). Among the 90 voles providing fleas, 27 were *F. tularensis* PCR–positive; the remaining 63 were negative (*1*). Of these same 90 voles, 45 were *Bartonella* PCR–positive and 45 were negative. Seventeen were positive for both *F. tularensis* and *Bartonella* spp. (*2*).

Flea pools had an average of 2.12 fleas (range 1–9); however, most (>70%) contained 1 (51%) or 2 (22%) fleas (Table). We did not detect DNA from pathogens other than *F. tularensis* and *Bartonella* spp. in fleas. Three (3%) flea pools harbored *F. tularensis* DNA; we estimated the overall prevalence at 6%. *F. tularensis* prevalence in both flea species was low (1 positive pool of 51 in *N. fasciatus* and 2 of 39 in *C. apertus*). All *F. tularensis* PCR–positive flea pools came from *F. tularensis* PCR–positive voles, and prevalence of *F. tularensis* in fleas was significantly associated with its prevalence in voles (analysis of variance [ANOVA], $R^2 = 0.072$, $F_{0.05, 1, 88} = 6.81$; $p = 0.011$). Of note, all fleas containing *F. tularensis* DNA were collected during July 2014, when vole populations reached top densities and tularemia prevalence peaked among them (33%) (*1*). The low prevalence of *F. tularensis* detected in fleas carried by infected hosts (3 of 27 pools) and the detection of infected flea pools only when abundance of the bacterium in the environment was highest (during vole peaks) (*1*,*4*) suggest that the quantitative role of fleas in the circulation of *F. tularensis* might be modest.

Conversely, the role of fleas in the circulation of *Bartonella* spp. seems much more relevant. We detected *Bartonella* spp. in 28 (37%) flea pools and in both flea species (37% of *N. fasciatus* and 23% of *C. apertus*) (Table). We detected *Bartonella* spp. in fleas collected from *Bartonella* PCR–positive and *Bartonella* PCR–negative voles in nearly equal proportions (51% vs. 44%) (Table). The average prevalence of *Bartonella* spp. in fleas was not associated with its prevalence in voles (ANOVA, $R^2 = 0.006$, $F_{0.05, 1, 88} = 0.53$; $p = 0.467$). We found a higher *Bartonella* spp. prevalence in *N. fasciatus* (65%) than in *C. apertus* (33%).

[1]Current affiliation: Ben-Gurion University of the Negev, Midreshet Ben-Gurion, Israel.

[2]These authors contributed equally to this article.

Table. Detection of *Francisella tularensis* and *Bartonella* spp. in 2 species of fleas from live common voles (*Microtus arvalis*), northwestern Spain, 2013–2015*

		Flea pools			Fleas		
Voles	Flea species	No.	*F. tularensis*–positive, %	*Bartonella* spp.–positive, %	No.	*F. tularensis* prevalence, % (range)	*Bartonella* spp. prevalence, % (range)
All	All	90	3.3	31.1	191	6.1 (3.3–8.8)	51.1([31.1–71.1)
	Nosopsyllus fasciatus	51	2.6	37.3	113	6.9 (3.9–9.8)	64.7 (37.3–92.2)
	Ctenophthalmus apertus	39	3.9	23.1	78	5.1 (2.6–7.7)	33.3 (23.1–43.6)
F. tularensis–negative	All	63			127	0	
	N. fasciatus	32			71	0	
	C. apertus	31			56	0	
F. tularensis–positive	All	27			64	20.4 (11.1–29.6)	
	N. fasciatus	19			42	18.4 (10.5–26.3)	
	C. apertus	8			22	25.0 (12.5–37.5)	
Bartonella spp.–negative	All	45			93		44.4 (26.7–62.2)
	N. fasciatus	21			53		71.4 (38.1–100)
	C. apertus	24			40		20.8 (16.7–25.0)
Bartonella spp.–positive	All	45			98		51.1 (31.1–71.1)
	N. fasciatus	30			60		60 (36.7–83.3)
	C. apertus	15			38		53.3 (33.3–73.3)

*Blank cells indicate that nothing can be calculated for that option.

We identified 3 *Bartonella* species among fleas (*B. taylorii* [17%], *B. grahamii* [14%], and *B. rochalimae* [3%]), as well as mixed infections (Appendix). These findings are in accordance with other research showing fleas as a main vector of *Bartonella* spp. (*5*). Although *F. tularensis* and *Bartonella* spp. have been simultaneously detected in ≈13% of voles during population density peaks (*2*), we identified no co-infection among flea pools (ANOVA, $R^2 = 0.011$, $F_{0.05, 1, 88} = 0.97$; $p = 0.328$).

Our data show that *F. tularensis* and *Bartonella* spp. occur in the fleas infesting wild common voles in northwestern Spain, with notable differences in prevalence (6% and 51%, respectively) and associations with prevalence in vole hosts. Future studies are needed to determine the role of fleas in the circulation of these pathogens in nature and in particular to ascertain any effective vectoring of *F. tularensis*.

Acknowledgments

We thank Fabio Flechoso for helping with ectoparasite counts and flea identification.

This work was supported by ECOVOLE (Factores ecologicos que influyen en la reproducción y dinámica poblacional del topillo campesino (*Microtus arvalis*) en medios agrarios; CGL2012-35348), ECOTULA (Ecología de la Tularemia: dinámica espacio-temporal, ciclos ecológicos de transmisión y mapas de riesgo en ecosistemas agrarios del NO de España; CGL2015-66962-C2-1-R), and RESERTULA (Microbiología de la Tularemia: circulación de *Francisella tularensis* en los ecosistemas agrarios del NO de España. Estudio d relaciones epidemiológicas y filogenéticas; CLG2015-66962-C2-2-R) projects funded by the Government of Spain (lMINECO/FEDER). R.R.-P. was supported by a PhD studentship from the University of Valladolid (co-funded by Banco Santander, RR 30/04/2014).

About the Author

Dr. Rodríguez-Pastor is a postdoctoral student at the Mitrani Department of Desert Ecology, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben-Gurion, Israel. Her research interests include the ecology of zoonoses and disease dynamics in animal populations, specifically parasite and infectious pathogen dynamics in wild rodents.

References

1. Rodríguez-Pastor R, Escudero R, Vidal D, Mougeot F, Arroyo B, Lambin X, et al. Density-dependent prevalence of *Francisella tularensis* in fluctuating vole populations, northwestern Spain. Emerg Infect Dis. 2017;23:1377–9. http://dx.doi.org/10.3201/eid2308.161194
2. Rodríguez-Pastor R, Escudero R, Lambin X, Vidal MD, Gil H, Jado I, et al. Zoonotic pathogens in fluctuating common vole (*Microtus arvalis*) populations: occurrence and dynamics. Parasitology. 2019;146:389–98.
3. Luque-Larena JJ, Mougeot F, Roig DV, Lambin X, Rodríguez-Pastor R, Rodríguez-Valín E, et al. Tularemia outbreaks and common vole (*Microtus arvalis*) irruptive population dynamics in northwestern Spain, 1997–2014. Vector Borne Zoonotic Dis. 2015;15:568–70. http://dx.doi.org/10.1089/vbz.2015.1770
4. Luque-Larena JJ, Mougeot F, Arroyo B, Vidal MD, Rodríguez-Pastor R, Escudero R, et al. Irruptive mammal host populations shape tularemia epidemiology. PLoS Pathog. 2017;13:e1006622. http://dx.doi.org/10.1371/journal.ppat.1006622
5. Gutiérrez R, Krasnov B, Morick D, Gottlieb Y, Khokhlova IS, Harrus S. *Bartonella* infection in rodents and their flea ectoparasites: an overview. Vector Borne Zoonotic Dis. 2015;15:27–39. http://dx.doi.org/10.1089/vbz.2014.1606
6. Silaghi C, Pfeffer M, Kiefer D, Kiefer M, Obiegala A. *Bartonella*, rodents, fleas and ticks: a molecular field study on host–vector-pathogen associations in Saxony, eastern Germany. Microb Ecol. 2016;72:965–74. http://dx.doi.org/10.1007/s00248-016-0787-8
7. Hornok S, Földvári G, Rigó K, Meli ML, Gönczi E, Répási A, et al. Synanthropic rodents and their ectoparasites as carriers

of a novel haemoplasma and vector-borne, zoonotic pathogens indoors. Parasit Vectors. 2015;8:27. http://dx.doi.org/10.1186/s13071-014-0630-3
8. Stevenson HL, Bai Y, Kosoy MY, Montenieri JA, Lowell JL, Chu MC, et al. Detection of novel *Bartonella* strains and *Yersinia pestis* in prairie dogs and their fleas (Siphonaptera: Ceratophyllidae and Pulicidae) using multiplex polymerase chain reaction. J Med Entomol. 2003;40:329–37. http://dx.doi.org/10.1603/0022-2585-40.3.329
9. Anda P, Escudero R, Rodriguez-Moreno I, Jado I, Jimenez-Alonso MI. Method and kit for the detection of bacterial species by means of DNA analysis. European patent EP1895015B1. 2012 July 25.

Address for correspondence: Ruth Rodríguez-Pastor, Mitrani Department of Desert Ecology, Jacob Blaustein Institutes for Desert Research, Ben-Gurion University of the Negev, Midreshet Ben-Gurion, 84990, Israel; email: ruth.r.pastor@gmail.com

Mycobacterium bovis Infection in African Wild Dogs, Kruger National Park, South Africa

Roxanne L. Higgitt, O. Louis van Schalkwyk, Lin-Mari de Klerk-Lorist, Peter E. Buss, Peter Caldwell, Leana Rossouw, Tebogo Manamela, Guy A. Hausler, Jennie Hewlett,[1] Emily P. Mitchell,[1] Paul D. van Helden, Sven D.C. Parsons,[2] Michele A. Miller[2]

Author affiliations: Stellenbosch University, Cape Town, South Africa (R.L. Higgitt, G.A. Hausler, P.D. van Helden, S.D.C. Parsons, M.A. Miller); Department of Agriculture, Forestry and Fisheries, Skukuza, South Africa (O.L. van Schalkwyk, L.-M. de Klerk-Lorist); South African National Parks, Skukuza (P.E. Buss, L. Rossouw, T. Manamela, J. Hewlett); Old Chapel Veterinary Clinic, Pretoria, South Africa (P. Caldwell); National Zoological Gardens of South Africa, Pretoria (E.P. Mitchell)

DOI: https://doi.org/10.3201/eid2507.181653

We screened African wild dogs (*Lycaon pictus*) in Kruger National Park, South Africa, for *Mycobacterium bovis* infection using an interferon-gamma release assay. We detected *M. bovis* sensitization in 20 of 21 packs; overall apparent infection prevalence was 83%. These animals experience high infection pressure, which may affect long-term survival and conservation strategies.

The African wild dog (*Lycaon pictus*) is an endangered carnivore occurring in fragmented, small populations (in South Africa, <500 animals). These factors make them susceptible to adverse factors, such as infectious diseases, that may threaten their long-term survival (*1,2*). Of particular concern are diseases caused by multihost pathogens that are capable of persisting in reservoir host species, such as *Mycobacterium bovis*, the causative agent of bovine tuberculosis (bTB). This pathogen may pose a major threat to the conservation of endangered host populations (*3*).

Since 2012, sporadic cases of wild dogs with macroscopic and histological lesions consistent with tuberculosis (TB) have been recorded in South Africa, specifically in Kruger National Park (KNP; n = 8), uMkuze Game Reserve (n = 1), and Hluhluwe-iMfolozi Park (HiP; n = 2). *M. bovis* infection is endemic in these parks and occurs in multiple species that are preyed upon by wild dogs, such as warthogs, which have an estimated *M. bovis* seroprevalence up to 38% in KNP (*4,5*). In 2 cases from KNP, acid-fast bacilli were associated with granulomatous lymphadenitis, and spoligotype analysis of *M. bovis* isolates from lesions in affected wild dogs from KNP (strain type SB0121) and HiP (strain type SB0130) were the same as those found in local prey (*6*).

M. bovis is a novel pathogen of wild dogs; understanding the impact of bTB disease in wild dogs is imperative to making informed management decisions regarding these animals' conservation. Estimation of prevalence would provide a starting point for this investigation but requires diagnostic tools for accurate detection of *M. bovis* infection. To estimate prevalence in the KNP wild dog population, we assessed sensitization to TB antigens ESAT-6 and CFP-10.

During July 2016–January 2018, we tested blood samples from 77 wild dogs from KNP using an interferon-gamma release assay (IGRA) developed by our group (*7*). We tested animals from 21 wild dog packs; 20 of these included ≥1 IGRA-positive animal, indicating widespread exposure to *M. bovis* throughout KNP (Figure). We observed no significant difference in IGRA results based on sex (p = 0.79 by 2-tailed Mann-Whitney test). Overall, the apparent prevalence of *M. bovis* infection was 82% (63/77; 95% CI 72%–89% by modified Wald test).

Few reports of active bTB disease and related deaths have been documented in wild dogs, so the high apparent

[1]Current affiliation: University of Pretoria, Onderstepoort, South Africa.

[2]These authors were co–principal investigators for this article.

prevalence of *M. bovis* infection in the KNP population is surprising. However, similarly high prevalence estimates have been reported for African lions (*Panthera leo leo*) in KNP using the tuberculin skin test (55%; 95% CI 45%–65%) (*8*) and a cytokine gene expression assay (44%; 95% CI 32%–57%) (*9*). These results highlight the high infection pressure for carnivores within KNP.

The prevalence estimate in wild dogs was based on detection of immune sensitization to *M. bovis*–specific antigens. Although the infection status of a small number of animals was confirmed by antemortem mycobacterial culture of oropharyngeal swabs (4 [7.0%] of 57 wild dogs tested), we could not confirm the infection status of all of the animals included in this study. Furthermore, during the time of the study, most animals included in this analysis appeared to be healthy. Therefore, further investigations will be required to clarify the progression of *M. bovis* infection and risk of bTB disease in this species.

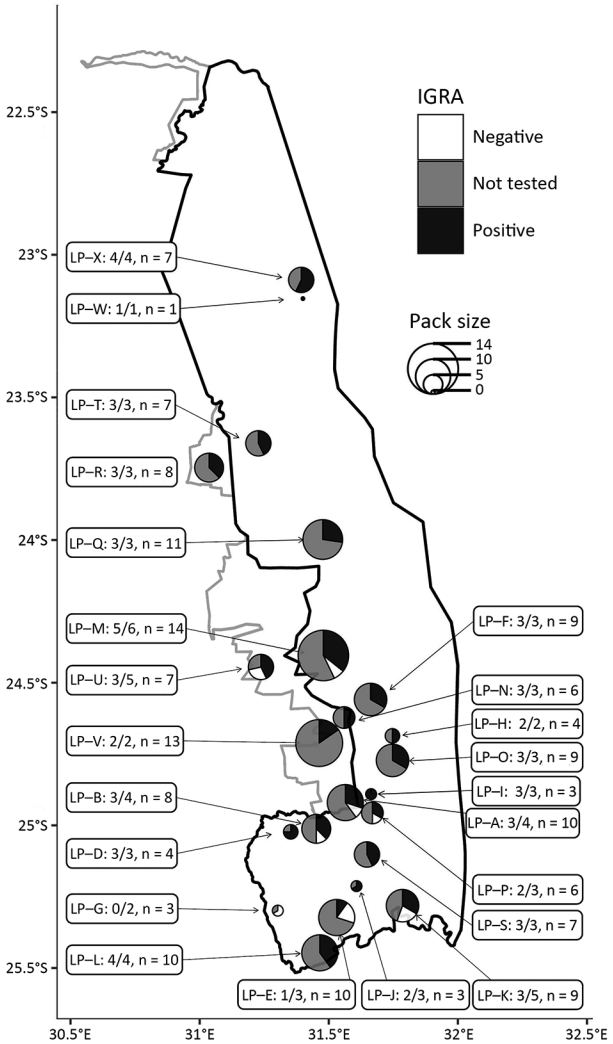


Figure. *Mycobacterium bovis* infection in African wild dog packs, Kruger National Park, South Africa. Each pie chart indicates the position of a pack at time of sampling; the size of the pack (n value); and the proportion of test-positive, test-negative, and untested animals. The pack name (e.g., LP-A) and the proportion of tested animals that were test-positive (e.g., 2/2) are shown. A single wild dog that was not part of a pack was included. IGRA, interferon-gamma release assay.

These results have implications for managing the wild dog metapopulation in South Africa, which involves translocation of animals across the country to maintain genetic diversity and to achieve conservation goals (*2*). The risk of introducing *M. bovis* into previously uninfected areas by an infected wild dog is unknown, and studies on transmission will be crucial in assessing this risk.

Survival of a species is affected by a complex array of factors, of which disease is only one. Currently, the KNP wild dog population appears to be stable (*2*), despite the apparent high prevalence of *M. bovis* infection. Favorable conditions, such as abundance of prey, may support high reproductive rates. However, with environmental changes, such as prolonged drought, the vulnerability of host populations to infectious disease may be more pronounced (*10*).

In conclusion, this study shows widespread exposure of wild dog packs to *M. bovis* in KNP, with high infection pressure to individual dogs. Although the impact of disease on population numbers is unknown, wild dogs infected with *M. bovis*, even young animals, have been observed to have generalized disease leading to death. Further investigations into the progression of *M. bovis* infection, the risk for transmission, and the probability of developing progressive disease are needed to assess the threat of this emerging disease in African wild dog populations.

Acknowledgments

We acknowledge the contributions made by the veterinary technicians of the Kruger National Park State Veterinary Office in Skukuza, South Africa, as well as David Cooper, Birgit Eggers, and Ezemvelo KZN Wildlife for assistance with wild dog sampling and data collection.

Research protocols were approved by the University of Stellenbosch Animal Care and Use Committee (SU-ACUD16-00076) and the Department of Agriculture, Forestry and Fisheries, South Africa (12/11/1/7/2). This research was funded (partially or fully) by the South African government through the South African Medical Research Council and the National Research Foundation of South Africa (grant no. 86949).

About the Author

Ms. Higgitt is a research assistant at the Department of Biomedical Sciences, Stellenbosch University. Her current research interests are understanding immune responses of South Africa carnivores to *M. bovis* infection and the development of diagnostic assays for these species.

References

1. Woodroffe R, Ginsberg J, Macdonald D. Status survey and conservation action plan: the African wild dog. Gland (Switzerland): International Union for Conservation of Nature/ Species Survival Commission Canid Specialist Group; 1997.
2. Davies-Mostert H, Page-Nicholson S, Marneweck D, Marnewick K, Cilliers D, Whittington-Jones B, et al. A conservation assessment of *Lycaon pictus*. In: Child M, Roxburgh L, Do Lihn San E, Raimondo D, Davies-Mostert H, editors. The red list of mammals of South Africa, Swaziland and Lesotho. Pretoria (South Africa): South African National Biodiversity Institute and Endangered Wildlife Trust; 2016 [cited 2018 Aug 20]. https://pdfs.semanticscholar.org/7259/e724f3111c2e68609d41cb630f91692f1892.pdf
3. Woodroffe R. Managing disease threats to wild mammals. Anim Conserv. 1999;2:185–93. http://dx.doi.org/10.1111/ j.1469-1795.1999.tb00064.x
4. Renwick AR, White PCL, Bengis RG. Bovine tuberculosis in southern African wildlife: a multi-species host–pathogen system. Epidemiol Infect. 2007;135:529–40. http://dx.doi.org/10.1017/S0950268806007205
5. Roos EO, Olea-Popelka F, Buss P, de Klerk-Lorist L-M, Cooper D, van Helden PD, et al. Seroprevalence of *Mycobacterium bovis* infection in warthogs (*Phacochoerus africanus*) in bovine tuberculosis-endemic regions of South Africa. Transbound Emerg Dis. 2018;65:1182–9. http://dx.doi.org/10.1111/tbed.12856
6. Michel AL, Coetzee ML, Keet DF, Maré L, Warren R, Cooper D, et al. Molecular epidemiology of *Mycobacterium bovis* isolates from free-ranging wildlife in South African game reserves. Vet Microbiol. 2009;133:335–43. http://dx.doi.org/10.1016/j.vetmic.2008.07.023
7. Higgitt RL, van Schalkwyk OL, deKlerk-Lorist LM, Buss PE, Caldwell P, Rossouw L, et al. An interferon gamma release assay for the detection of immune sensitization to *Mycobacterium bovis* in African wild dogs (*Lycaon pictus*). J Wildl Dis. 2018:55. http://dx.doi.org/10.7589/2018-03089
8. Keet D, Davies-Mostert H, Bengis R, Funston P, Buss P, Hofmeyr M, et al. Disease risk assessment workshop report: African lion (*Panthera leo*) bovine tuberculosis. Conservation Breeding Specialist Group/CBSG Southern Africa, Endangered Wildlife Trust. 2009 [cited 2018 Aug 20]. http://www.wcs-ahead.org/gltfca_grants/pdfs/lion_tb_risk_report_final.pdf
9. Sylvester TT, Martin LER, Buss P, Loxton AG, Hausler GA, Rossouw L, et al. Prevalence and risk factors for *Mycobacterium bovis* infection in African lions (*Panthera leo*) in the Kruger National Park. J Wildl Dis. 2017;53:372–6. http://dx.doi.org/10.7589/2016-07-159
10. de La Rocque S, Rioux JA, Slingenbergh J. Climate change: effects on animal disease systems and implications for surveillance and control. Rev Sci Tech. 2008;27:339–54.

Address for correspondence: Michele A. Miller, Stellenbosch University DST-NRF Centre of Excellence for Biomedical TB Research/MRC Centre for Tuberculosis Research/Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, PO Box 241, Cape Town 8000, South Africa; email: miller@sun.ac.za

Identification of Internationally Disseminated Ceftriaxone-Resistant *Neisseria gonorrhoeae* Strain FC428, China

Shao-Chun Chen, Yan Han, Liu-Feng Yuan, Xiao-Yu Zhu, Yue-Ping Yin

Author affiliations: Chinese Academy of Medical Sciences and Peking Union Medical College, Nanjing, China (S.-C. Chen, Y. Han, X.-Y. Zhu, Y.-P. Yin); Chinese Center for Disease Control and Prevention, Nanjing (S.-C. Chen, Y. Han, X-.Y. Zhu, Y.-P. Yin); Beijing Ditan Hospital Capital Medical University, Beijing, China (L.-F. Yuan)

DOI: https://doi.org/10.3201/eid2507.190172

In 2016, we identified a ceftriaxone-resistant *Neisseria gonorrhoeae* isolate in China. The strain genotype was identical to the resistant clone FC428 that originated in Japan. Enhanced international collaborative surveillance programs are crucial to track the transmission of the ceftriaxone-resistant clones.

Ceftriaxone has been used to treat gonorrhea in China and most other countries for >1 decade, but the level of decreased susceptibility or clinical resistance to ceftriaxone has increased (*1*). Moreover, the international spread of ceftriaxone-resistant clones has been recognized as a threat to effective control of gonorrhea (*2*). We describe an imported ceftriaxone-resistant *N. gonorrhoeae* strain isolated in China in 2016.

The patient was a heterosexual man in his late twenties. He reported unprotected 1-night heterosexual sex in Beijing in July 2016. Urethral discharge with dysuria occurred 3 days after the sexual activity. He was prescribed oral cephalosporin when he visited a private clinic in July. Because the urethral discharge did not resolve, he visited the sexually transmitted diseases clinic in Beijing Ditan Hospital (Beijing, China) in August.

Laboratory analysis of a urethral swab sample found gram-negative diplococci within leukocytes. Culture and nucleic acid amplification test were positive for *N. gonorrhoeae*. Screening for other sexually transmitted infections by nucleic acid amplification test was negative for *Chlamydia trachomatis*, *Ureaplasma urealyticum*, and *Mycoplasma hominis*.

The patient was treated with a 1-g intravenous dose of ceftriaxone once per day for 3 days. His symptoms improved after 3 days, and a test-of-cure by culture showed

Table. Antimicrobial susceptibility and molecular characteristics of ceftriaxone-resistant *Neisseria gonorrhoeae*, China*

Strains	Year	Country (reference)	Sexual contact history	MIC, mg/L						MLST	*porB*	*tbpB*	NG-MAST	*penA*	NG-STAR
				TET	SPT	CRO	CIP	AZI	CEF						
Japan-FC428	2015	Japan (*6*)	NA	0.5	8	0.5	>32	0.25	1	1903	1053	21	3435	60	233
Japan-FC460	2015	Japan (*6*)	NA	0.5	8	0.5	>32	0.25	1	1903	1053	21	3435	60	233
China-BJ16148	2016 Aug	China (this study)	China	4	16	0.5	>32	0.25	1	1903	1053	21	3435	60	233
Denmark-GK124	2017 Jan	Denmark (*7*)	Denmark, China, Australia	NA	8	0.5	>32	0.5	1	1903	1053	33	1614	60	233
Canada-47707	2017 Jan	Canada (*8*)	China, Thailand	4	16	1	>32	0.5	2	1903	1053	33	1614	60	233
Australia-A7846	2017 Apr	Australia (*9*)	Cambodia, Philippines	2	8	0.5	>32	0.25	NA	1903	1053	33	1614	60	233
Australia-A7536	2017 Aug	Australia (*9*)	China	4	8	0.5	>32	0.25	NA	1903	9300	21	15925	60	233

*AZI, azithromycin; CEF, cefixime; CIP, ciprofloxacin; CRO, ceftriaxone; MLST, multilocus sequence typing; NA, not available; NG-MAST, *N. gonorrhoeae* multiantigen sequence typing; NG-STAR, *N. gonorrhoeae* sequence typing for antimicrobial resistance; SPT, spectinomycin; TET, tetracycline.

the treatment was successful. A telephone follow-up after 1 month indicated a lack of urethral discharge, and the patient provided information that his female sexual partner worked in a nightclub and had sexual contact with men from foreign countries.

The bacterial isolate was transferred to the reference laboratory at the National Center for Sexually Transmitted Disease Control, Chinese Center for Disease Control and Prevention (Nanjing, China). Gram staining and a carbohydrate utilization test confirmed *N. gonorrhoeae*. We confirmed antimicrobial susceptibilities to ceftriaxone, cefixime, spectinomycin, azithromycin, ciprofloxacin, and tetracycline for this isolate by using the agar dilution method. The strain was resistant to ceftriaxone (MIC 0.5 mg/L), cefixime (MIC 1 mg/L), tetracycline (4 mg/L), and ciprofloxacin (>32 mg/L) and susceptible to azithromycin (MIC 0.25 mg/L) and spectinomycin (MIC 16 mg/L) in accordance with the European Committee on Antimicrobial Susceptibility Testing protocol (http://www.eucast.org/clinical_breakpoints).

We performed *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) (*3*) and multilocus sequence typing (MLST) (*4*) to identify the sequence types (STs). The MLST type was ST1903, and the NG-MAST type was ST3435. We used *N. gonorrhoeae* sequence typing for antimicrobial resistance (NG-STAR) (*5*) to identify the characteristics of resistance determinants. The NG-STAR type was ST233, which contains a type 60 mosaic *penA* allele (*penA* 60.001), -35A Del in the *mtrR* promoter (*mtrR*1), G120K-A121D in PorB (PorB8), L421P in PonA (PonA1), S91F-D95A in GyrA (GyrA7), S87R in ParC (ParC3), and wild-type 23srRNA (23 srRNA0).

The genotype (MLST1903/NG-MAST3435/NG-STAR233) of this isolate was identical to the 2 ceftriaxone-resistant *N. gonorrhoeae* (FC428 and FC460) isolated in 2015 in Japan (*6*) and similar to other resistant strains isolated in 2017 in Denmark (*7*), Canada (*8*), and Australia (*9*) (Table). Type 60 mosaic PenA (*penA* 60.001), which contained A311V and T483S alterations, was the key ceftriaxone resistance mutation and typical of this internationally disseminated resistant clone.

The timeline and epidemiologic data of all previous reports of the infections suggest this clone originated in Japan in 2015 and was disseminated to China, Denmark, Canada, and Australia afterward. Moreover, this resistant clone may have a fitness advantage over previously reported "superbug" H041 and has successfully spread worldwide (*9*). Accordingly, enhancing international collaborative surveillance on the ceftriaxone-resistant clone is crucial.

In conclusion, we identified a ceftriaxone-resistant *N. gonorrhoeae* strain that has sustainably transmitted in several countries for ≈3 years. These findings indicate an imported risk and a further transmission of resistant clones in China and demonstrate the need for enhanced local and global gonococcal antimicrobial surveillance to track the emergence and dissemination of resistant strains for timely control of spread (*10*).

Acknowledgments

We are grateful to Beijing Ditan Hospital, a member of China's Gonococcal Resistance Surveillance Program, for providing the isolate and making the study possible. We thank William Shafer for his valuable comments.

This study was supported by a grant from the Chinese Academy of Medical Sciences Initiative for Innovative Medicine (2016-I2M-3-021) and the Jiangsu Natural Science Foundation (BK20171133).

About the Author

Dr. Chen is an associate professor at the National Center for STD Control, Chinese Center for Disease Control and Prevention. His primary research interests include molecular epidemiology and the antimicrobial resistance mechanism of *N. gonorrhoeae*.

References

1. Yin YP, Han Y, Dai XQ, Zheng HP, Chen SC, Zhu BY, et al. Susceptibility of *Neisseria gonorrhoeae* to azithromycin and ceftriaxone in China: a retrospective study of national surveillance data from 2013 to 2016. PLoS Med. 2018;15:e1002499. http://dx.doi.org/10.1371/journal.pmed.1002499
2. Chen SC, Yin YP, Chen XS. Cephalosporin-resistant *Neisseria gonorrhoeae* clone, China. Emerg Infect Dis. 2018;24:804–6. http://dx.doi.org/10.3201/eid2404.171817
3. Martin IM, Ison CA, Aanensen DM, Fenton KA, Spratt BG. Rapid sequence-based identification of gonococcal transmission clusters in a large metropolitan area. J Infect Dis. 2004;189: 1497–505. http://dx.doi.org/10.1086/383047
4. Jolley KA, Maiden MC. BIGSdb: Scalable analysis of bacterial genome variation at the population level. BMC Bioinformatics. 2010;11:595. http://dx.doi.org/10.1186/1471-2105-11-595
5. Demczuk W, Sidhu S, Unemo M, Whiley DM, Allen VG, Dillon JR, et al. *Neisseria gonorrhoeae* sequence typing for antimicrobial resistance, a novel antimicrobial resistance multilocus typing scheme for tracking global dissemination of *N. gonorrhoeae* strains. J Clin Microbiol. 2017;55:1454–68. http://dx.doi.org/10.1128/JCM.00100-17
6. Nakayama S, Shimuta K, Furubayashi K, Kawahata T, Unemo M, Ohnishi M. New ceftriaxone- and multidrug-resistant *Neisseria gonorrhoeae* strain with a novel mosaic *penA* gene isolated in Japan. Antimicrob Agents Chemother. 2016;60:4339–41. http://dx.doi.org/10.1128/AAC.00504-16
7. Terkelsen D, Tolstrup J, Johnsen CH, Lund O, Larsen HK, Worning P, et al. Multidrug-resistant *Neisseria gonorrhoeae* infection with ceftriaxone resistance and intermediate resistance to azithromycin, Denmark, 2017. Euro Surveill. 2017;22. http://dx.doi.org/10.2807/1560-7917.ES.2017.22.42.17-00659
8. Lefebvre B, Martin I, Demczuk W, Deshaies L, Michaud S, Labbé AC, et al. Ceftriaxone-resistant *Neisseria gonorrhoeae*, Canada, 2017. Emerg Infect Dis. 2018;24:381–3. http://dx.doi.org/10.3201/eid2402.171756
9. Lahra MM, Martin I, Demczuk W, Jennison AV, Lee KI, Nakayama SI, et al. Cooperative recognition of internationally disseminated ceftriaxone-resistant *Neisseria gonorrhoeae* strain. Emerg Infect Dis. 2018;24:735–40. http://dx.doi.org/10.3201/eid2404.171873
10. Wi T, Lahra MM, Ndowa F, Bala M, Dillon JR, Ramon-Pardo P, et al. Antimicrobial resistance in *Neisseria gonorrhoeae*: Global surveillance and a call for international collaborative action. PLoS Med. 2017;14:e1002344. http://dx.doi.org/10.1371/journal.pmed.1002344

Address for correspondence: Yue-Ping Yin, National Center for STD Control, China CDC—Reference Laboratory, 12 Jiangwangmiao St, Nanjing, Jiangsu 210042 China; email: yinyp@ncstdlc.org

Disseminated Metacestode *Versteria* Species Infection in Woman, Pennsylvania, USA[1]

Bethany Lehman, Sixto M. Leal, Jr., Gary W. Procop, Elise O'Connell, Jahangheer Shaik, Theodore E. Nash, Thomas B. Nutman, Stephen Jones, Stephanie Braunthal, Shetal N. Shah, Michael W. Cruise, Sanjay Mukhopadhyay, Jona Banzon

Author affiliations: Cleveland Clinic Foundation, Cleveland, Ohio, USA (B. Lehman, G.W. Procop, S. Jones, S. Braunthal, S.N. Shah, M.W. Cruise, S. Mukhopadhyay, J. Banzon); University of Alabama Medical Center, Tuscaloosa, Alabama, USA (S.M. Leal, Jr.); National Institutes of Health, Bethesda, Maryland, USA (E. O'Connell, J. Shaik, T.E. Nash, T.B. Nutman)

DOI: https://doi.org/10.3201/eid2507.190223

A patient in Pennsylvania, USA, with common variable immunodeficiency sought care for fever, cough, and abdominal pain. Imaging revealed lesions involving multiple organs. Liver resection demonstrated necrotizing granulomas, recognizable tegument, and calcareous corpuscles indicative of an invasive cestode infection. Sequencing revealed 98% identity to a *Versteria* species of cestode found in mink.

In July 2017, a 68-year-old woman in Pennsylvania, USA, sought care for fever, fatigue, cough, and abdominal pain. Her medical history was significant for common variable immunodeficiency and splenic B cell lymphoma that had been treated with R-CHOP (rituximab, cyclophosphamide, hydroxydaunorubicin, vincristine, and prednisone); treatment was completed in December 2016.

Imaging showed extensive nodular disease of the lungs and liver and a hepatic abscess. Examination of a fine-needle aspirate of the hepatic lesion detected hepatocytes with focal atypia on a background of marked acute inflammation and necrosis, suggestive of an active infectious process. Subsequent percutaneous needle biopsy samples of the liver, bronchoalveolar lavage and transbronchial biopsy samples, and surgical biopsy samples of the left lower lobe showed necrotizing granulomas and reactive/reparative tissue changes. All histochemically stained slides (Gomori-methenamine silver, Gram, periodic acid Schiff, Warthin-Starry, Ziehl-Neelsen, Fite) yielded negative results for microorganisms. Results of broad-range PCR for bacteria

[1]This manuscript was originally presented at the Infectious Disease Society of America IDWeek 2018; October 3–7, 2018; San Francisco, CA, USA.

(16S rDNA), fungi (28S rDNA), and mycobacteria (16S rDNA, *rpo*B, and *hsp*65) were also negative.

Four months later, after the patient had been receiving broad-spectrum antibacterial and antifungal medications, she sought a second opinion at the Cleveland Clinic (Cleveland, OH, USA), where repeat cross-sectional imaging showed progressive nodular disease within the lungs, liver, and kidneys and cyst-like lesions in the eyes and brain (Appendix Figure 1, https://wwwnc.cdc.gov/EID/article/25/7/19-0223-App1.pdf). Gross examination of a liver sample from a right partial hepatectomy performed for diagnosis revealed multifocal tan-white nodules and necrotic or cystic spaces. Microscopic analysis identified extensive necrotizing granulomatous inflammation and multifocal cystic spaces, which enclosed material characteristic of the tegument of a cestode. In a separate location within otherwise nondescript necrotic tissue was a focal collection of round basophilic concretions with concentric layers of deposited material characteristic of calcareous corpuscles, pathognomonic for a cestode infection (Appendix Figure 2). Additional histochemical studies for microorganisms detected no microorganisms.

These findings were consistent with a disseminated proliferating invasive cestode infection; the metacestode most closely resembled the cysticercus larva that lacks a scolex (i.e., the racemose form of cysticercosis). The presence of racemose-like disseminated involvement of multiple visceral organs was concerning because this feature is not common in patients with cysticercosis. Results of an enzyme-linked immunotransfer blot for *Taenia solium* cestodes were negative. Cysticercus-specific IgG was not elevated, and antibodies against echinococci were not found, although these tests are unreliable in a patient who has common variable immunodeficiency and is receiving intravenous immunoglobulin. Therefore, we considered the possibility of another cestode species.

The patient received praziquantel and albendazole for 1 month. Initially, dexamethasone (10 mg) was concurrently administered for the neurologic and ocular involvement. Treatment resolved the abdominal pain, fatigue, and fever. Follow-up imaging showed vast improvement in the brain, lung, kidney, and liver lesions. Imaging findings continued to improve after corticosteroids were tapered off after 3 months, and symptoms continued to improve 6 months after treatment. However, serial eye examinations revealed a new cystic lesion in the eye. The cyst was extracted; histopathologic examination did not detect a scolex but did detect an identical tegument, again appearing as an aberrant form (Appendix Figure 2). As of April 2019, the patient was continuing to receive albendazole and praziquantel and monthly intravenous immunoglobulin.

Because of the unusual histopathologic findings and clinical course, we performed molecular analysis. We extracted DNA from formalin-fixed, paraffin-embedded liver tissue and then performed partial mitochondrial

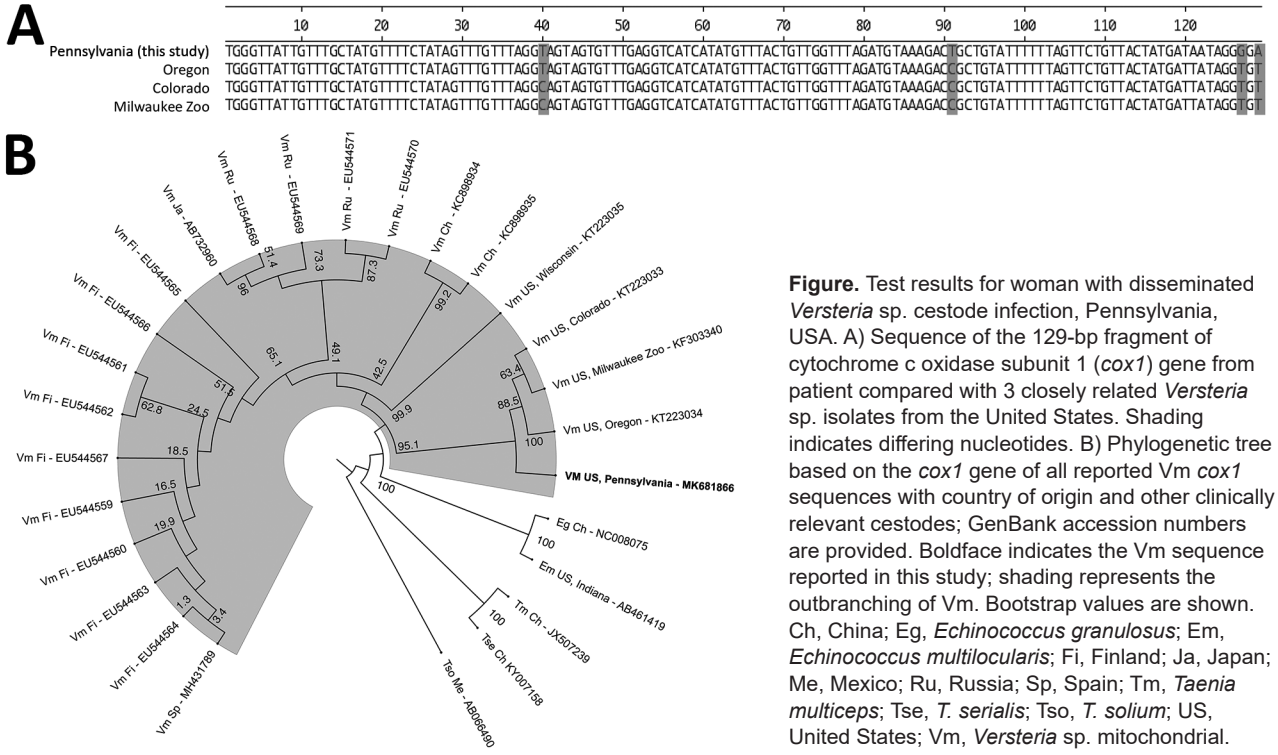


Figure. Test results for woman with disseminated *Versteria* sp. cestode infection, Pennsylvania, USA. A) Sequence of the 129-bp fragment of cytochrome c oxidase subunit 1 (*cox1*) gene from patient compared with 3 closely related *Versteria* sp. isolates from the United States. Shading indicates differing nucleotides. B) Phylogenetic tree based on the *cox1* gene of all reported Vm *cox1* sequences with country of origin and other clinically relevant cestodes; GenBank accession numbers are provided. Boldface indicates the Vm sequence reported in this study; shading represents the outbranching of Vm. Bootstrap values are shown. Ch, China; Eg, *Echinococcus granulosus*; Em, *Echinococcus multilocularis*; Fi, Finland; Ja, Japan; Me, Mexico; Ru, Russia; Sp, Spain; Tm, *Taenia multiceps*; Tse, *T. serialis*; Tso, *T. solium*; US, United States; Vm, *Versteria* sp. mitochondrial.

cytochrome (*cox1*) gene amplification. (*1*). PCR products were inserted into pCR 2.1 TOPO (https://www.thermofisher.com), cloned, and sequenced (at Macrogen USA, Rockville, MD, USA; https://www.macrogenusa.com). Our search for a 128-bp consensus sequence by using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) found a 98% match to the *Versteria* species *cox1* gene (GenBank accession no. KT223034). After disease recurrence and soon after extraction of the ocular cyst, we subsequently subjected DNA from the preserved ocular cyst to Nanopore sequencing (Oxford Nanopore Technologies, https://nanoporetech.com) and assembled the complete mitochondrial genome, which we deposited at GenBank (accession no. MK681866) (Figure).

The definitive hosts of the new *Versteria* (*Taenia mustelae*) cestodes are usually mustelids (*2*), a family of carnivorous mammals including weasels, ermine, mink, and others, which are found throughout the northern United States (*3*). This patient reported exposure to fishers in her residence in western Pennsylvania, where a resurgence in the population of these members of the family Mustelidae has been observed. Her husband was screened for signs of a parasitic infection and results were negative. The only other reported human infection with *Versteria* sp. involved a kidney transplant patient, who also had lung and liver lesions. Histopathologic examination of that patient's liver lesions revealed focal necrotizing granulomas with hooklets and a protoscolex (*4*).

The diagnosis of a cestode infection is usually suggested by the presence of specific cestode structures (e.g., a protoscolex, tegument, or calcareous corpuscles). However, unlike the previous report of human infection, histopathologic examination of the liver lesion and ocular cyst from this patient did not detect hooklets or protoscoleces, mimicking the histopathologic appearance of racemose disease sometimes seen in patients with subarachnoid neurocysticercosis. Because histopathologic examination is insufficient for species-level identification (specific cestode structures), molecular testing is necessary for definitive diagnosis of *Versteria* sp. cestode infection.

Acknowledgments

We thank Kevin El-Hayek, who performed the liver biopsy, and Sunil Srivastava for the fundus photograph.

This study was partially funded through the Division of Intramural Research, National Institute of Allergy and Infectious Disease, National Institutes of Health.

About the Author

Dr. Lehman is an infectious disease physician at the Cleveland Clinic. Her primary research interest is osteoarticular infections.

References

1. Poon RWS, Tam EWT, Lau SKP, Cheng VCC, Yuen KY, Schuster RK, et al. Molecular identification of cestodes and nematodes by *cox1* gene real-time PCR and sequencing. Diagn Microbiol Infect Dis. 2017;89:185–90. http://dx.doi.org/10.1016/j.diagmicrobio.2017.07.012
2. Lee LM, Wallace RS, Clyde VL, Gendron-Fitzpatrick A, Sibley SD, Stuchin M, et al. Definitive hosts of *Versteria* tapeworms (Cestoda: Taeniidae) causing fatal infection in North America. Emerg Infect Dis. 2016;22:707–10. http://dx.doi.org/10.3201/eid2204.151446
3. Bininda-Emonds OR, Gittleman JL, Purvis A. Building large trees by combining phylogenetic information: a complete phylogeny of the extant Carnivora (Mammalia). Biol Rev Camb Philos Soc. 1999;74:143–75. http://dx.doi.org/10.1017/S0006323199005307
4. Barkati S, Gottstein B, Mü Ller N, Sheitoyan-Pesant C, Metrakos P, Chen T, et al. First human case of metacestode infection caused by *Versteria* sp. in a kidney transplant recipient. Clin Infect Dis. 2019;68:680–3. http://dx.doi.org/10.1093/cid/ciy602

Address for correspondence: Bethany Lehman, Cleveland Clinic, Department of Infectious Disease, 9500 Euclid Ave/G21, Cleveland, OH 44195, USA; email: lehmanb@ccf.org

Increased Threat of Urban Malaria from *Anopheles stephensi* Mosquitoes, Africa

Willlem Takken, Steve Lindsay

Author affiliations: Wageningen University & Research, Wageningen, the Netherlands (W. Takken); Durham University, Durham, UK (S. Lindsay)

DOI: https://doi.org/10.3201/eid2507.190301

Malaria continues to be a major health threat in Africa, mainly in rural areas. Recently, the urban malaria vector *Anopheles stephensi* invaded Djibouti and Ethiopia, potentially spreading to other areas of Africa. Urgent action is needed to prevent urban malaria epidemics from emerging and causing a public health disaster.

The pernicious life-threatening disease malaria continues to place a heavy burden on communities in Africa, where >92% of malaria cases occur today (*1*). Mosquitoes of the genus *Anopheles* transmit malaria parasites to humans. Africa has ≥128 indigenous *Anopheles* species (*2*), several of which, *An. gambiae sensu stricto*, *An. coluzzii*,

and *An. funestus sensu stricto*, are among the world's most efficient malaria vectors. These species are found predominantly in rural areas, where they thrive in a variety of natural and manmade aquatic sites. Because mosquito densities fluctuate with rainfall, malaria is prevalent in rural areas in Africa with strong seasonal variations (*3*).

Malaria also occurs in urban centers in Africa, but at much lower levels, mostly in the peripheries, where small-scale commercial gardens collect surface water (*4*). Malaria is not the only mosquito-borne disease threat in urban Africa. The *Aedes aegypti* mosquito is a vector for dengue, yellow fever, chikungunya, and Zika viruses in urban settings.

Many countries in Africa are experiencing rapid urban development because people from the countryside, attracted by opportunities for work and education, are moving into urban centers. According to the United Nations, cities like Nairobi, Kenya; Dar es Salaam, Tanzania; Kinshasa, Democratic Republic of the Congo; Lagos, Nigeria; Abidjan, Côte d'Ivoire; and Dakar, Senegal, have doubled in population during the last decade and are predicted to expand further (https://population.un.org/wup).

The global malaria eradication campaign, launched in 2005, has led to major reductions in malaria prevalence (*5*), but recent data on malaria in Africa suggest that further reductions are less clear. In many parts of sub-Saharan Africa, progress in malaria control has stalled, and malaria is still widespread (*1*). In addition, the campaign does not focus on urban areas, where malaria prevalence is low or absent.

In 2016, *An. stephensi* mosquitoes were found for the first time in Ethiopia, where this species has since become established (*6*). This discovery followed earlier reports of the species in neighboring Djibouti (*7*). *An. stephensi* mosquitoes are native to southern and western Asia, where the species serves as an efficient malaria vector (*8*). Unlike other malaria vectors in Africa, *An. stephensi* mosquitoes are found not only in rural areas but also in cities, where they breed in manmade water containers, such as household water storage containers and garden reservoirs. The *An. stephensi* mosquito is considered to be the main malaria vector in urban centers in India and Pakistan (*8*). Recently, the species was recorded for the first time in Sri Lanka, demonstrating its ability to disperse across large bodies of water and establish successfully in new geographic regions (*9*).

Because Africa currently does not have a malaria vector adapted to urban centers, establishment of *An. stephensi* mosquitoes on the continent poses considerable health risks. If the species disperses beyond its current distribution in eastern Ethiopia and successfully invades large cities, such as Khartoum, Sudan; Mombasa, Kenya; and Dar es Salaam, the region could face malaria outbreaks of unprecedented size. Because of relatively high levels of malaria prevalence in persons of all ages in rural areas, high mobility between rural and urban areas, and inadequate healthcare, countries in Africa are unprepared to deal with rapid spread of malaria in their cities and towns by a vector species well adapted to urban infrastructures.

To halt the potential risk and prevent further spread of this vector requires urgent action. Historic examples demonstrate that a well-coordinated eradication of a species is possible, such as elimination of invasive *An. gambiae* mosquitoes from Brazil, as well as their eradication from Egypt. However, once a species disperses and covers larger geographic areas, eradication becomes nearly impossible. For example, the *Ae. albopictus* mosquito, a vector of chikungunya and dengue, has spread globally from its original location in Southeast Asia and has become a threat in many countries.

The World Health Organization's Global Vector Control Response 2017–2030 (GVCR; https://www.who.int/vector-control/publications/global-control-response/en) calls for multisectoral approaches to vector control. Urban mosquito control programs in Africa can use GVCR strategies to closely examine mosquito vectors thriving in cities and develop programs to reduce the threat to public health. In our view, surveillance for mosquito vectors in urban centers is essential for preventing outbreaks of infectious vectorborne diseases by eliminating newly established foci of vectors while they are still small (*10*). The invasion of *An. stephensi* mosquitoes on the African continent is a threat to health in tropical Africa but also provides an opportunity to build out vector control strategies as outlined in the GVCR.

S.L. is supported by the Grand Challenges Research Fund (https://grandchallenges.org).

About the Authors

Dr. Takken is a professor in Medical and Veterinary Entomology at Wageningen University & Research, Wageningen, the Netherlands. He is actively involved in studies on alternative strategies for malaria control, and his research interests are in the biology and control of vectorborne diseases, with an emphasis on malaria.

Dr. Lindsay is a professor in public health entomology at Durham University in the United Kingdom where he researches the behavior, ecology, and control of mosquito vectors, particularly those that transmit malaria. His research interests focus on interventions that could be used outside the health sector and on improvements to the built environment to help control vectorborne diseases.

References

1. World Health Organization. World Malaria Report 2018. Geneva: The Organization; 2018.
2. Kyalo D, Amratia P, Mundia CW, Mbogo CM, Coetzee M, Snow RW. A geo-coded inventory of anophelines in the

Afrotropical Region south of the Sahara: 1898–2016. Wellcome Open Res. 2017;2:57. https://doi.org/10.12688/wellcomeopenres.12187.1
3. Fillinger U, Lindsay SW. Larval source management for malaria control in Africa: myths and reality. Malar J. 2011;10:353–53. http://dx.doi.org/10.1186/1475-2875-10-353
4. Hay SI, Guerra CA, Tatem AJ, Atkinson PM, Snow RW. Urbanization, malaria transmission and disease burden in Africa. Nat Rev Microbiol. 2005;3:81–90. http://dx.doi.org/10.1038/nrmicro1069
5. Bhatt S, Weiss DJ, Cameron E, Bisanzio D, Mappin B, Dalrymple U, et al. The effect of malaria control on *Plasmodium falciparum* in Africa between 2000 and 2015. Nature. 2015;526:207–11. http://dx.doi.org/10.1038/nature15535
6. Carter TE, Yared S, Gebresilassie A, Bonnell V, Damodaran L, Lopez K, et al. First detection of *Anopheles stephensi* Liston, 1901 (Diptera: Culicidae) in Ethiopia using molecular and morphological approaches. Acta Trop. 2018;188:180–6. http://dx.doi.org/10.1016/j.actatropica.2018.09.001
7. Seyfarth M, Khaireh BA, Abdi AA, Bouh SM, Faulde MK. Five years following first detection of *Anopheles stephensi* (Diptera: Culicidae) in Djibouti, Horn of Africa: populations established—malaria emerging. Parasitol Res 2019;118:725–32. https://doi.org/10.1007/s00436-019-06213-0
8. Kiszewski A, Mellinger A, Spielman A, Malaney P, Sachs SE, Sachs J. A global index representing the stability of malaria transmission. Am J Trop Med Hyg. 2004;70:486–98. http://dx.doi.org/10.4269/ajtmh.2004.70.486
9. Gayan Dharmasiri AG, Perera AY, Harishchandra J, Herath H, Aravindan K, Jayasooriya HTR, et al. First record of *Anopheles stephensi* in Sri Lanka: a potential challenge for prevention of malaria reintroduction. Malar J. 2017;16:326. http://dx.doi.org/10.1186/s12936-017-1977-7
10. Flores HA, O'Neill SL. Controlling vector-borne diseases by releasing modified mosquitoes. Nat Rev Microbiol. 2018;16:508–18. http://dx.doi.org/10.1038/s41579-018-0025-0

Address for correspondence: Willem Takken, Wageningen University & Research, Laboratory of Entomology, PO Box 16, Wageningen 6700 AA, the Netherlands; email: willem.takken@wur.nl

Outbreak of African Swine Fever, Vietnam, 2019

Van Phan Le,[1] Dae Gwin Jeong,[1] Sun-Woo Yoon, Hye-Min Kwon, Thi Bich Ngoc Trinh, Thi Lan Nguyen, Thi To Nga Bui, Jinsik Oh, Joon Bae Kim, Kwang Myun Cheong, Nguyen Van Tuyen, Eunhye Bae, Thi Thu Hang Vu, Minjoo Yeom, Woonsung Na, Daesub Song

Author affiliations: Vietnam National University of Agriculture, Hanoi, Vietnam (V.P. Le, T.B.N. Trinh, T.L. Nguyen, T.T.N. Bui); Korea Research Institute of Bioscience and Biotechnology, Daejeon, South Korea (D.G. Jeong, S.-W. Yoon, H.-M. Kwon); Median Diagnostics, Chuncheon-si, South Korea (J. Oh, J.B. Kim, K.M. Cheong); Gold Coin, Hai Duong, Vietnam (N.V. Tuyen); Korea University, Sejong, South Korea (E. Bae, T.T.H. Vu, M. Yeom, D. Song); Chonnam National University, Gwangju, South Korea (W. Na)

DOI: https://doi.org/10.3201/eid2507.190303

African swine fever is one of the most dangerous diseases of swine. We confirmed the 2019 outbreak in Vietnam by real-time reverse transcription PCR. The causative strain belonged to p72 genotype II and was 100% identical with viruses isolated in China (2018) and Georgia (2007). International prevention and control collaboration is needed.

Since its first identification in East Africa in the early 1900s, African swine fever (ASF) spread to Kenya in the 1920s; transcontinental outbreaks in Europe and South America in the 1960s and in Georgia (Caucasus) in 2007 led to subsequent transmission to neighboring countries east of Georgia. Along with the outbreaks in the eastern territory of the Russian Federation, acute ASF outbreaks were reported in China in 2018 (*1*).

During January 15–31, 2019, a disease outbreak at a family-owned backyard pig farm in Hung Yen Province, Vietnam, was reported. The farm, ≈50 km from Hanoi and 250 km from the China border, housed 20 sows. In the early stage of the outbreak, 1 piglet and 1 sow exhibited marked redness all over the body, conjunctivitis, and hemorrhagic diarrhea. Breeding gilts demonstrated anorexia, cyanosis, and fever (>40.5°C).

On February 1, 2019, after confirming that the mortality rate at this farm had surpassed 50%, we collected organ samples (e.g., spleen, liver, kidney, tonsil, and lymph nodes) from dying pigs and submitted them to the diagnostic laboratory at the Vietnam National University of Agriculture for ASF diagnosis. All specimens underwent homogenization, followed by extraction of viral DNA (*2*). ASF virus DNA was identified by routine PCR, as recommended by the Office International des Epizooties (Paris, France), and by commercialized real-time PCR (Median Diagnostics Inc., http://www.mediandiagnostics.com). We named the detected ASF virus VNUA/HY-ASF1 and deposited the following complete genome sequences into GenBank: p10 (accession no. MK795932), p11.5 (MK795933), p12 (MK795934), p14.5 (MK795935), p17 (MK795936), p22 (MK795937), pE248R (MK795938), p30 (MK757460), p54

[1]These authors contributed equally to this article.

(MK554697), p72 (MK554698), and Cd2v (MK757459). We aligned the nucleotide sequences by using BioEdit version 7.2 (Ibis Biosciences, http://www.mbio.ncsu.edu/bioedit/bioedit.html) with ClustalW (http://clustal.org) and calculated sequence identity. Using MEGA7 (https://www.megasoftware.net) and the neighbor-joining method, we based phylogenetic analysis on the genetic information and calculated bootstrap values with 1,000 replicates. The genotype was determined by p72 gene characterization as reported previously (*3*,*4*). Phylogenetic trees revealed that the VNUA/HY-ASF1 strain belonged to p72 genotype II (Figure) and was 100% identical to China strains SY18/China/2018 (GenBank accession no. MH713612) and AnhuiXCGQ/China/2018 (MK128995) and other genotype II strains of Europe: Georgia/2007/1 (GenBank accession no. FR682468.1), Russia/2012 (KJ195685), Estonia/2014 (LS478113), and Poland/2015 (MH681419).

The clinical signs and necropsy findings of the pigs involved in the 2019 outbreak in Vietnam were similar to those caused by the virus strains in China and Georgia (e.g., high mortality rates over a short period and multifocal hemorrhagic lesions in many organs). However, the clinical forms and pathophysiology of ASF varied according to virus virulence, exposure dose, and transmission route.

Considering the epidemiologic features of the site where ASF has recently occurred, the virus is highly likely to have reached Vietnam via infected wild boar, by movement of pigs and pork products, or by infected fomites (*5*). The most probable source and major cause

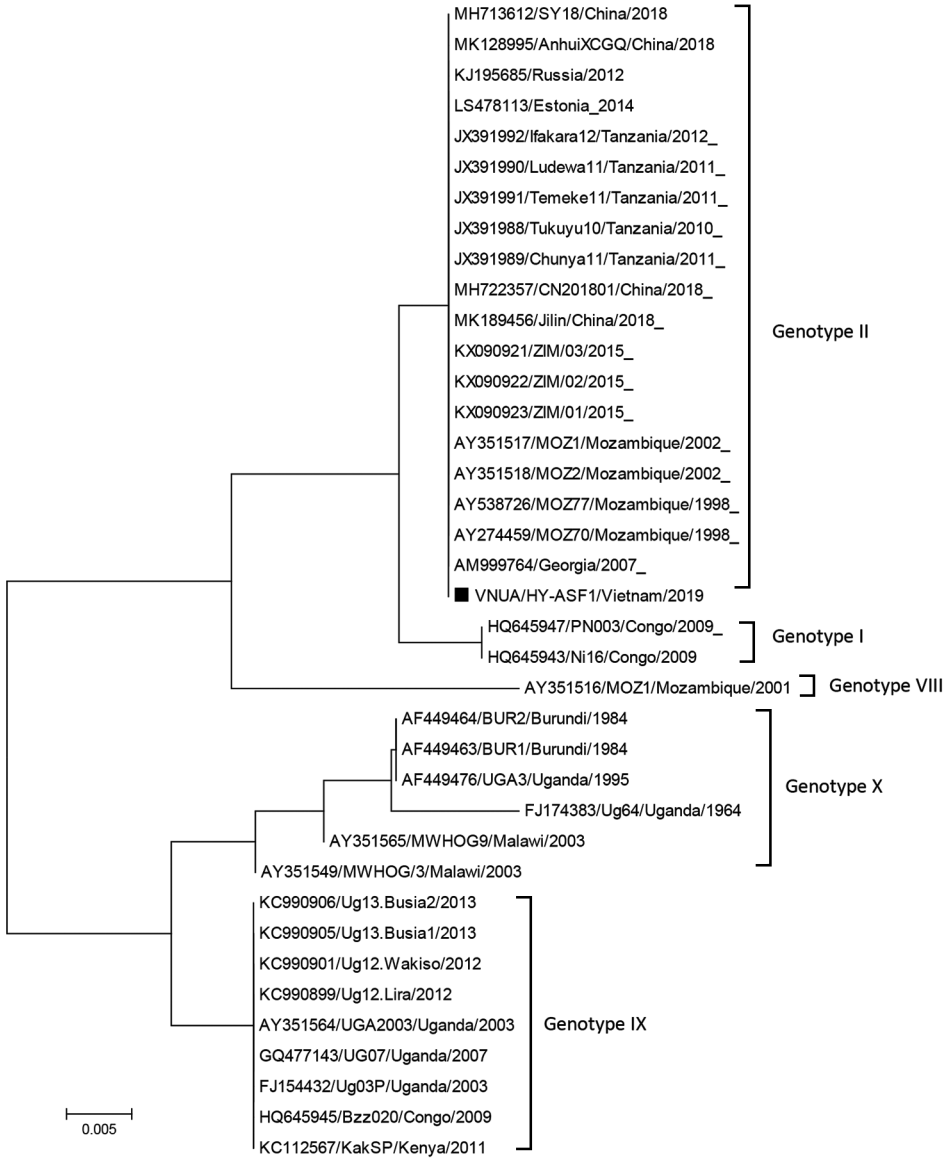


Figure. Phylogenetic analysis of major capsid protein gene (p72) of African swine fever virus isolated during outbreak in Vietnam in 2019 (VNUA HY-ASF1; black square) and reference isolates. The phylogenetic tree was constructed by using the neighbor-joining method in MEGA7 (http://www.megasoftware.net). Bootstrap values were calculated with 1,000 replicates. GenBank accession numbers, strain name, country, and year of collection are indicated. Scale bars indicate nucleotide substitutions per site.

of transmission across the countries is thought to be ASF virus–contaminated pork products (*2*). The outbreak in Vietnam was confirmed in the northern part of the country, near China, where many instances of illegal movement of animals and meat products across the China–Vietnam border have been reported (http://www.fao.org/3/i8805en/I8805EN.pdf). Therefore, it is likely that the virus originated in China.

Although the p30, p54, and p72 sequences were 100% identical to those from China and Georgia, whole genomes must be monitored for possible changes and further spread of the ASF virus. Since the 2018 outbreak in China, the subsequent ASF outbreak in Vietnam (February 1, 2019) increases the possibility of virus spread to nearby swine-raising Southeast Asia countries, including Laos, Thailand, Cambodia, and Myanmar. Although ASF has occurred in many countries, including Russia and Europe, its outbreak in Asia is far more critical because 60% of the world's pig population is concentrated in that area and the socioeconomic effects of swine disease would be greater than that in other regions. Therefore, to avoid great economic losses worldwide, we highly recommend that preventive and control measures be developed and implemented through international collaboration.

This research was supported by the Vietnam National University of Agriculture, Vietnam, and by BioNano Health Guard Research Center, funded by the Ministry of Science and Information and Communications Technology of South Korea, as a Global Frontier Project (2013M3A6B2078954).

About the Author

Dr. Le is a veterinarian and associate professor at Vietnam National University of Agriculture, with a main research interest in virology, including viruses of swine and birds.

References

1. Ge S, Li J, Fan X, Liu F, Li L, Wang Q, et al. Molecular characterization of African swine fever virus, China, 2018. Emerg Infect Dis. 2018;24:2131–3. http://dx.doi.org/10.3201/eid2411.181274
2. Kolbasov D, Titov I, Tsybanov S, Gogin A, Malogolovkin A. African swine fever virus, Siberia, Russia, 2017. Emerg Infect Dis. 2018;24:796–8. http://dx.doi.org/10.3201/eid2404.171238
3. Bastos ADS, Penrith M-L, Crucière C, Edrich JL, Hutchings G, Roger F, et al. Genotyping field strains of African swine fever virus by partial p72 gene characterisation. Arch Virol. 2003148:693–706.
4. Malogolovkin A, Burmakina G, Titov I, Sereda A, Gogin A, Baryshnikova E, et al. Comparative analysis of African swine fever virus genotypes and serogroups. Emerg Infect Dis. 2015;21:312–5. http://dx.doi.org/10.3201/eid2102.140649
5. Guinat C, Gogin A, Blome S, Keil G, Pollin R, Pfeiffer DU, et al. Transmission routes of African swine fever virus to domestic pigs: current knowledge and future research directions. Vet Rec. 2016;178:262–7. http://dx.doi.org/10.1136/vr.103593

Address for correspondence: Van Phan Le, Vietnam National University of Agriculture, Hanoi, Vietnam; email: letranphan@gmail.com; and Daesub Song, Korea University, College of Pharmacy, 2511, Sejong-ro Sejong 30019, South Korea; email: sds1@korea.ac.kr

Low-Grade Endemicity of Opisthorchiasis, Yangon, Myanmar

Woon-Mok Sohn, Bong-Kwang Jung, Sung-Jong Hong, Keon-Hoon Lee, Jong-Bok Park, Hyun-Seung Kim, Seon Cho, Thi Thi Htoon, Htay Htay Tin, Jong-Yil Chai

Author affiliations: Gyeongsang National University College of Medicine, Jinju, South Korea (W.-M. Sohn); Korea Association of Health Promotion, Seoul, South Korea (B.-K. Jung, K.-H. Lee, J.-B. Park, H.-S. Kim, S. Cho, J.-Y. Chai); Chung-Ang University, Seoul (S.-J. Hong); National Health Laboratory, Yangon, Myanmar (T.T. Htoon, H.H. Tin)

DOI: https://doi.org/10.3201/eid2507.190495

We performed an epidemiologic survey of opisthorchiasis in Yangon, Myanmar. The fecal egg-positive rate of residents was 0.7%, and we recovered an adult fluke after chemotherapy and purging of an egg-positive resident. We detected *Opisthorchis viverrini* metacercariae in freshwater fish. We found the Yangon area to have low-grade endemicity of opisthorchiasis.

The liver fluke *Opisthorchis viverrini*, a well-known cause of cholangiocarcinoma, is distributed predominantly in Southeast Asia countries (*1,2*). In Myanmar, health officials thought that opisthorchiasis might not occur because the population traditionally does not consume raw or undercooked fish. However, 2 recent reports have documented the presence of *O. viverrini* eggs or flukes in Myanmar (*3,4*). In 2017, a molecular study detected a mitochondrial cytochrome *c* oxidase subunit I (*cox1*) gene of *O. viverrini* from the fecal samples of persons in a rural area near Yangon (*3*); however, adult flukes were not recovered from the egg-positive persons. Another study in 2018 detected *O. viverrini* metacercariae from freshwater fish (*Puntius brevis*) caught in central

Table. Rates of helminth egg infection, by species, among 2,057 persons in 3 districts of Yangon, Myanmar

District	No. persons examined	No. (%) positive					
		Ascaris lumbricoides	*Trichuris trichiura*	*Enterobius vermicularis*	*Opisthorchis viverrini*	Other*	Total
Hlaing-Thayar	682	17 (2.5)	90 (13.2)	2 (0.3)	2 (0.3)	2 (0.3)	113 (16.6)
South Dagon	672	83 (13.2)	90 (14.4)	11 (1.8)	8 (1.3)	4 (0.6)	196 (31.3)
North Dagon	748	66 (8.8)	94 (12.6)	6 (0.8)	4 (0.5)	5 (0.7)	175 (23.4)
Total	2,057	166 (8.1)	274 (13.3)	19 (0.9)	14 (0.7)	11 (0.5)	484 (23.5)

*Includes 2 cases of hookworm infection and 1 case each of *Taenia* sp. and *Trichostrongylus* sp. infection.

Myanmar and obtained adult flukes from experimentally infected hamsters (*4*).

We recently observed a low-grade endemicity of *O. viverrini* infection among residents in the Yangon area. We also recovered an adult fluke (Appendix Figure, panel A, https://wwwnc.cdc.gov/EID/article/25/7/19-0495-App1.pdf) from an egg-positive resident and detected metacercariae in freshwater fish caught in Yangon.

In December 2015, we performed fecal examinations on 2,057 residents in 3 districts of Yangon (North Dagon, South Dagon, and Hlaing-Thayar) by using the Kato–Katz technique. The total number of helminth egg–positive cases was 484 (23.5%); we recovered eggs of *Trichuris trichiura* whipworms (13.3%), *Ascaris lumbricoides* roundworms (8.1%), *Enterobius vermicularis* pinworms (0.9%), *O. viverrini* flukes (0.7%), and other helminth species (0.5%) (Table).

Among the 14 residents positive for *O. viverrini* eggs (some possibly having mixed infections with minute intestinal fluke species such as *Haplorchis* spp.) (Table; Appendix Figure, panel B), 2 agreed to undergo worm recovery after treatment with praziquantel (40 mg/kg in a single dose) and purging with 25–30 g of $MgSO_4$. Fecal examination and anthelmintic treatment of the residents were officially approved by Myanmar's Ministry of Health and Sport, under the agreement of the South Korea–Myanmar International Collaboration on Intestinal Parasite Control for Schoolchildren in Myanmar (Ethics Review Committee approval no. 005117). Informed consent was received from each person.

The procedure of the worm recovery was as described previously (*5*). One adult fluke that looked like a liver fluke was recovered from 1 of these 2 residents. Minute intestinal fluke species, including *Haplorchis* spp., were not recovered. The adult fluke (Appendix Figure, panel A) was slender (11.1 × 1.5 mm) and had a small oral sucker (0.20 × 0.29 mm), large ventral sucker (0.51 × 0.59 mm), lobed ovary, 2 lobed testes, and a well-developed uterus with numerous eggs (25 × 14 μm). We confirmed the fluke to be an adult specimen of *O. viverrini*.

We also examined 10 species of freshwater fish (n = 160) purchased in a local market of North Dagon to detect *O. viverrini* metacercariae. The fish were transported on ice to Gyeongsang National University College of Medicine (Jinju, South Korea), and examined by using the artificial digestion method (*6*). We detected *O. viverrini* metacercariae in 4 species of fish (forest snakehead [*Channa lucius*], 5/5, 100%; striped snakehead [*C. striata*] 1/29, 3.5%; climbing perch [*Anabas testudineus*] 1/14, 7.1%; and unspecified *Puntioplites* sp., 1/15, 6.7%) (Appendix Figure, panels C, D). In forest snakehead fish, the average metacercarial density per fish was 24.4 (range 1–52). The metacercariae were round to elliptical and were 150–188 μm (average 165 μm) × 98–140 μm (average 122 μm) in size.

The metacercariae were fed orally to 2 golden hamsters (*Mesocricetus auratus*) to recover adult flukes. At day 50 postinfection, 20 adult flukes were recovered from the biliary tracts of the hamsters. The animal experiment was performed in accordance with the guidelines of Gyeongsang National University College of Medicine. The adult flukes were slender (average size 5.1 × 1.2 mm) and had the characteristic features of *O. viverrini* (Appendix Figure, panel F).

Opisthorchiasis is one of the most prevalent foodborne helminthiases in Thailand, Laos, Cambodia, and Vietnam (*2,5–9*). For example, in Laos, opisthorchiasis is prevalent in the central and southern lowlands along the Mekong River, including Vientiane Municipality and Savannakhet Province, where the rates of *O. viverrini* egg recovery (mixed with some minute intestinal flukes) among residents along rivers were 53.3% (Vientiane) and 67.1% (Savannakhet) (*5,7*). In Cambodia, eastern localities (e.g., Kratie Province, 4.6% egg-positive rate) and southern localities (Kampong Cham Province, 24.0% egg-positive rate, and Takeo Province, 23.8%–47.5% egg-positive rates) along the Mekong River were found to be endemic foci (*8,9*). From 2 egg-positive residents in Takeo Province, 34 adult *O. viverrini* flukeswere recovered (*10*).

In our study, the *O. viverrini* egg-positive rate of residents in surveyed areas of Myanmar was 0.7%, much lower than the 4.6%–67.1% rates in Laos and Cambodia (*5–9*). Also, only 1 adult fluke was recovered in 1 egg-positive case, whereas 34 adult specimens were recovered in 2 residents in Cambodia (*10*). Thus, we concluded that the Yangon area of Myanmar has low-grade endemicity of *O. viverrini*.

Acknowledgments

We appreciate the kind help of the staff of the National Health Laboratory of the Ministry of Health and Sport, Yangon, Myanmar, in the collection of fecal samples of the residents. We also appreciate the staff of the Korea

Association of Health Promotion, Seoul, South Korea, who participated in this survey. Special thanks to the staff of Department of Parasitology and Tropical Medicine, Gyeongsang National University College of Medicine, for their help in experimental studies with hamsters.

About the Author

Dr. Sohn is a professor of parasitology and tropical medicine at Gyeongsang National University College of Medicine, Jinju, South Korea. His primary research interests are fishborne parasites and parasite fauna.

References

1. Fürst T, Keiser J, Utzinger J. Global burden of human foodborne trematodiasis: a systematic review and meta-analysis. Lancet Infect Dis. 2012;12:210–21. http://dx.doi.org/10.1016/S1473-3099(11)70294-8
2. Sripa B, Echaubard P. Prospects and challenges towards sustainable liver fluke control. Trends Parasitol. 2017;33:799–812. http://dx.doi.org/10.1016/j.pt.2017.06.002
3. Aung WPP, Htoon TT, Tin HH, Thinn KK, Sanpool O, Jongthawin J, et al. First report and molecular identification of *Opisthorchis viverrini* infection in human communities from lower Myanmar. PLoS One. 2017;12:e0177130 http://dx.doi.org/10.1371/journal.pone.0177130
4. Sanpool O, Aung WPP, Rodpai R, Maleewong W, Intapan PM. Human liver fluke *Opisthorchis viverrini* (Trematoda, Opisthorchiidae) in central Myanmar: new records of adults and metacercariae identified by morphology and molecular analysis. Acta Trop. 2018;185:149–55. http://dx.doi.org/10.1016/j.actatropica.2018.05.009
5. Chai JY, Park JH, Han ET, Guk SM, Shin EH, Lin A, et al. Mixed infections with *Opisthorchis viverrini* and intestinal flukes in residents of Vientiane Municipality and Saravane Province in Laos. J Helminthol. 2005;79:283–9. http://dx.doi.org/10.1079/JOH2005302
6. Sohn WM, Yong TS, Eom KS, Pyo KH, Lee MY, Lim H, et al. Prevalence of *Opisthorchis viverrini* infection in humans and fish in Kratie Province, Cambodia. Acta Trop. 2012;124:215–20 http://dx.doi.org/10.1016/j.actatropica.2012.08.011
7. Chai JY, Han ET, Guk SM, Shin EH, Sohn WM, Yong TS et al. High prevalence of liver and intestinal fluke infections among residents of Savannakhet Province in Laos. Korean J Parasitol. 2007;45:213–8.
8. Yong TS, Shin EH, Chai JY, Sohn WM, Eom KS, Lee DM, et al. High prevalence of *Opisthorchis viverrini* infection in a riparian population in Takeo Province, Cambodia. Korean J Parasitol. 2012;50:173–6. http://dx.doi.org/10.3347/kjp.2012.50.2.173
9. Yong TS, Chai JY, Sohn WM, Eom KS, Jeoung HG, Hoang EH, et al. Prevalence of intestinal helminths among inhabitants of Cambodia (2006–2011). Korean J Parasitol. 2014;52:661–6. http://dx.doi.org/10.3347/kjp.2014.52.6.661
10. Sohn WM, Shin EH, Yong TS, Eom KS, Jeong HG, Sinuon M, et al. Adult *Opisthorchis viverrini* flukes in humans, Takeo, Cambodia. Emerg Infect Dis. 2011;17:1302–4. http://dx.doi.org/10.3201/eid1707.102071

Address for correspondence: Jong-Yil Chai, Korea Association of Health Promotion, Institute of Parasitic Diseases, 333 Hwagok-ro, Seoul 07649, South Korea; email: cjy@snu.ac.kr

LETTERS

Nontoxigenic *Corynebacterium diphtheriae* Infections, Europe

Aleksandra A. Zasada, Magdalena Rzeczkowska

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

DOI: https://doi.org/10.3201/eid2507.180995

To the Editor: We read with interest the article by Dangel et al. analyzing nontoxigenic *Corynebacterium diphtheriae* infections in northern Germany during 2016–2017 (*1*). Among the cases, 2 patients originated from Poland; each experienced an invasive disease, 1 endocarditis and 1 sepsis. Poland and Germany are neighboring countries. In Poland, we also observed an accumulation of nontoxigenic *C. diphtheriae* infections during 2016–2017. In both countries, most infections were caused by isolates belonging to sequence type (ST) 8 biotype gravis, which we previously suspected of having increased pathogenic properties (*2*).

ST8 has been causing infection in Poland since 2004 and was isolated in Russia before that (*2*,*3*). However, the first ST8 isolate was not obtained in northern Germany until 2015, suggesting spread of pathogenic ST8 from eastern to western Europe. Comparing epidemiologic data from Poland during 2012–2017, we confirmed 48 cases of nontoxigenic *C. diphtheriae*, increasing from 3 cases in 2012 to 20 in 2017. As seen in northern Germany, most affected patients in Poland were male (>80%), and ≈30% of patients were homeless, alcohol addicted, or both. We did not identify HIV as a risk factor. We saw a sharp increase in cases during the time of the Dangel et al. report as well, from 10 cases in 2016 to 20 in 2017. Nevertheless, in Poland, 40% of isolates (19/48) during 2012–2017 were obtained from invasive infections, whereas in Germany only 9 isolates (≈12%) were obtained from cases with severe invasive complications. None of the cases in Poland were related epidemiologically.

We hypothesize that pathogenic ST8 could spread to other countries in Europe in a few years and that persistence of ST8 isolates in the population might be related to increases in the number of invasive infections. The scale of the problem of nontoxigenic *C. diphtheriae* infections in Europe remains unknown because only toxigenic infections are registered. Lack of registration leads to lack of prevention and, thus, to outbreak development and spread.

References

1. Dangel A, Berger A, Konrad R, Bischoff H, Sing A. Geographically diverse clusters of nontoxigenic *Corynebacterium diphtheriae* infection, Germany, 2016–2017. Emerg Infect Dis. 2018;24:1239–45. http://dx.doi.org/10.3201/eid2407.172026
2. Zasada AA. Nontoxigenic highly pathogenic clone of *Corynebacterium diphtheriae*, Poland, 2004–2012. Emerg Infect Dis. 2013;19:1870–2. http://dx.doi.org/10.3201/eid1911.130297
3. Czajka U, Wiatrzyk A, Mosiej E, Formińska K, Zasada AA. Changes in MLST profiles and biotypes of *Corynebacterium diphtheriae* isolates from the diphtheria outbreak period to the period of invasive infections caused by nontoxigenic strains in Poland (1950–2016). BMC Infect Dis. 2018;18:121. http://dx.doi.org/10.1186/s12879-018-3020-1

Address for correspondence: Aleksandra A. Zasada, National Institute of Public Health–National Institute of Hygiene, Department of Sera and Vaccines Evaluation, Chocimska 24, 00-791 Warsaw, Poland; email: azasada@pzh.gov.pl

Racial/Ethnic Disparities in Antimicrobial Drug Use, United States, 2014–2015

Mark Thomas, Naomi Whyler, Andrew Tomlin, Murray Tilyard

Author affiliations: University of Auckland, Auckland, New Zealand (M. Thomas); Auckland City Hospital, Auckland (M. Thomas, N. Whyler); Best Practice Advocacy Centre, Dunedin, New Zealand (A. Tomlin, M. Tilyard)

DOI: https://doi.org/10.3201/eid2507.181775

To the Editor: We read with interest the article by Olesen and Grad (*1*), which reported that, in the United States during 2014–2015, the rate of antimicrobial drug use by white persons was twice that of persons of other races. The authors did not relate this finding to previous reports of ≈2 times lower incidence of sepsis (*2*) and ≈1.5 times lower incidence of death from infectious diseases (*3*) in white persons in the United States.

A national study of community antibacterial dispensing in relation to ethnicity in New Zealand (*4*) found that the dispensing rates were highest in Pacific people and Maori, consistent with their higher incidence of infectious diseases. However, the ethnic disparities in dispensing rates were substantially less than the ethnic disparities in the incidence of some infections. For example, even though the incidence of hospitalization for rheumatic fever was 63 times higher for Pacific people and 27 times higher for Maori than for persons of all other ethnicities combined, the annual dispensing rates of penicillins for Pacific people and Maori were <1.5 times higher than in other ethnicities.

Olesen and Grad suggest that ethnic disparities in antimicrobial drug use will lead to disparities in the prevalence of colonization (and disease) by antimicrobial-resistant bacteria. The New Zealand study found that dispensing rates of topical antimicrobial agents (predominantly fusidic acid) for Pacific and Maori children were approximately twice those for children of other ethnicities and suggested that these high dispensing rates might have contributed to the higher proportion of staphylococcal infections caused by methicillin-resistant (and fusidic acid–resistant) *Staphylococcus aureus* in Pacific people and Maori (*5*). We suggest that improved understanding of ethnic disparities in the incidence of infectious diseases and in the level of consumption of antimicrobial agents might usefully inform antimicrobial stewardship targets and strategies.

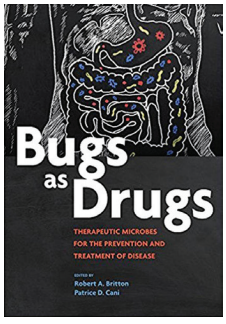
References

1. Olesen SW, Grad YH. Racial/ethnic disparities in antimicrobial drug use, United States, 2014–2015. Emerg Infect Dis. 2018;24:2126–8. http://dx.doi.org/10.3201/eid2411.180762
2. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med. 2003;348:1546–54. http://dx.doi.org/10.1056/NEJMoa022139
3. Richardus JH, Kunst AE. Black-white differences in infectious disease mortality in the United States. Am J Public Health. 2001;91:1251–3. http://dx.doi.org/10.2105/AJPH.91.8.1251
4. Whyler N, Tomlin A, Tilyard M, Thomas M. Ethnic disparities in community antibacterial dispensing in New Zealand, 2015. N Z Med J. 2018;131:50–60.
5. Williamson DA, Monecke S, Heffernan H, Ritchie SR, Roberts SA, Upton A, et al. High usage of topical fusidic acid and rapid clonal expansion of fusidic acid-resistant *Staphylococcus aureus*: a cautionary tale. Clin Infect Dis. 2014;59:1451–4. http://dx.doi.org/10.1093/cid/ciu658

Address for correspondence: Mark Thomas, Department of Molecular Medicine and Pathology, Faculty of Medical and Health Sciences, University of Auckland, 85 Park Rd, Grafton, Auckland 1023, New Zealand; email: mg.thomas@auckland.ac.nz

Bugs as Drugs: Therapeutic Microbes for the Prevention and Treatment of Disease

Robert A. Britton, Patrice D. Cani, editors; ASM Press, Washington, DC, USA, 2018; ISBN-10: 1555819699; ISBN-13: 978-1555819699; e-ISBN: 978-1555819705; Pages: 514; Price: $120.00



The role of microbes and the microbiome in various aspects of human health and disease is a subject of ongoing intense study. The complexity of microbial communities and their interactions with the host present exceptional challenges for conducting these investigations that will require multipronged strategies to resolve. As the field undergoes the much-needed transition beyond association studies to a mechanistic understanding of how microbes influence their hosts, manipulating commensal microbes and using them for diagnostics and therapeutic interventions is the logical next step. Because microbiome research studies are being published at an unprecedented rate, a reliable book that reviews the seminal work and knowledge gaps in this field is pressingly needed. In *Bugs as Drugs: Therapeutic Microbes for the Prevention and Treatment of Disease*, the authors address major themes in microbiome research, particularly focusing on therapeutic applications.

This ≈500-page volume, edited by Robert A. Britton and Patrice D. Cani, comprises 5 main sections of varying scope and length. The introductory section reviews pathways used by commensals, particularly *Lactobacilli* and *Bifidobacteria* spp., to benefit the host. The first chapter is particularly impressive, providing a thorough and well-cited overview of the various microbial metabolites and how they influence the host.

The theme of section 2, the longest and most wide-ranging part, is the role of the microbiome in chronic diseases. Given the ambitious scope of this section, the authors selectively covered a range of diseases (from nutritional disorders to colorectal cancer and osteoporosis) for which the host–microbe interactions have been described. Although this section might seem fragmented, it succeeds in providing the reader with a sense of the breadth of diseases in which therapeutic microbes might play a role. When links between microbes or the microbiome and human ailments are tentative or controversial, the authors do a sound job describing the conflicting reports within their respective fields.

Section 3 covers the vital function of the gut microbiome, namely the control of infectious diseases, with a focus on *Clostridioides difficile* and *Enterococcus* spp. infections. In this section, the authors tackle the seminal studies of fecal microbiota transplantation to control *C. difficile* infections and questions that need to be answered to evaluate the long-term consequences of this therapy, extending beyond *C. difficile* control.

Section 4 provides insight on the tools and techniques used to study and manipulate the microbiome, including strategies to genetically modify microbiota to design diagnostics and therapeutic applications, innovative genetic tools to engineer probiotics, and CRISPR (clustered regularly interspaced short palindromic repeats) technologies to modify microbes. Moreover, a chapter in this section is devoted to reviewing US regulatory considerations for developing live biotherapeutics-based drugs; however, regulatory constraints outside the United States are not covered in the book. The final section covers indirect strategies to functionally target the gut microbiome, including the use of bacteriophages as antibacterial agents and prebiotics to modulate the microbiome.

Overall, this book is a commendable and timely volume of well-sourced reviews written by experts in the field well organized into broad research themes. This work will serve as a helpful resource for both scientists and clinicians interested in using microbes for therapeutic applications.

A.S.B. is supported by the Damon Runyon Cancer Research Foundation, Emerson Collective, and a Baxter Foundation fellowship.

Soumaya Zlitni, Ami S. Bhatt

Author affiliation: Stanford University, Stanford, California, USA

DOI: https://doi.org/10.3201/eid2507.190582

Address for correspondence: Ami S. Bhatt, 269 Campus Dr, Department of Medicine (Hematology, Blood, and Marrow Transplantation) and Department of Genetics, Stanford University, Stanford, CA 94305, USA; email: asbhatt@stanford.edu

Amie Esslinger (b. 1984), *Hydro Vents and Other Difficult Places* (2016) (detail)**.** Acrylic, ink, paper, gel medium, thread, 30 in x 22 in/76 cm x 56 cm. Digital image courtesy of the artist.

Difficult Places, Unexpected Discoveries

Byron Breedlove, J. Todd Weber

Microbes, including myriad pathogens, have demonstrated their tenacity and malleability to endure, even flourish, under extreme conditions thought to be inhospitable to life. These microbes evolve at a pace that proves hard to fathom: they can undergo as many as 500,000 generations during a single human generation.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: https://doi.org/10.3201/eid2507.AC2507

The proliferation and abundance of modern antibiotics have accelerated the pace of pathogens' evolutionary adaptation through mutation and acquisition of genetic material conferring resistance from other species. The World Health Organization notes that new resistance mechanisms are emerging and spreading around the world and that without effective antimicrobials, treating infectious diseases is becoming increasingly challenging.

Researchers Julian and Dorothy Davies offer this perspective: "What happened during the evolution of

bacteria and other microbes and organisms over several billions of years cannot be compared to the phenomenon of antibiotic resistance development and transfer over the last century. Contemporary selection pressure of antibiotic use and disposal is much more intense; selection is largely for survival in hostile environments rather than for traits providing fitness in slowly evolving populations."

The concept of life thriving and adapting under extreme conditions resonates with Atlanta-based artist Amie Esslinger. Her painting "Hydro Vents and Other Difficult Places" appears as this month's cover art. Esslinger explains that her approach "interjects activity not visible to the human eye into the real space of the gallery." She works carefully and meticulously, creating complex, multilayered works based on "organic cell structures, aerial landscapes, and other hidden spaces." Esslinger labels her work as "promiscuous pattern realism" and cites among her influences 19th century Victorian illustrations, in particular *Kunstformen der Natur* (*Artforms of Nature*) by Ernst Haeckel (A. Esslinger, pers. comm. email, 2019 May 16).

Esslinger explains what inspired her to create this painting. "Hydrothermal vents exist on the deepest sea floors. Under the cover of complete darkness, they emit magma, minerals, and chemicals while geothermally heating the water to temperatures often above the boiling point. At first blush, these vents would seem to create toxic, uninhabitable environments, yet, surprisingly, the areas around them are biologically more productive than elsewhere in the deep sea. Despite the extreme temperatures, high acidity, and lack of sunlight, microorganisms adapt and thrive there. This natural phenomenon surprises and excites me. I wanted to capture the hyperactivity involved in the survival and adaptation of the organisms in these seemingly contradictory environments" (A. Esslinger, pers. comm. email, 2019 May 16).

Her painting evokes the teeming life forms that surround deep ocean oases. Lines, shapes, and textures converge and overlap. Awash in colors and shapes, the overall visual effect suggests pulsation and displacement. A filigree of irregular yellow-rimmed, reddish-brown masses frames the top and right sides of the image, and a dense cacophony of speckled crimson rises from the bottom. Reddish ribbons of scaly cellular forms cluster together, and flowerlike forms float at the end of undulating tendrils. Wreathed by interlaced white and black lines, a collection of organisms resembling sliced geodes dominates the left center, fighting with the strange starburst just above it for the viewer's attention. Cerulean-edged indigo seawater appears through this dense curtain of exotic shapes.

The artist notes that although she was not striving to portray actual life-forms realistically, elements in the painting are based on various bacteria and tubeworms. In her words, "I want to represent the vitality and potentiality of simple cell organisms. The piece is an expressive attempt to capture the activity and chance mutations that allow life to make these strange places home."

This issue of the journal lays out the diversity of those mutations and spread of antimicrobial resistance through environmental pressure and horizontal gene transfer with articles on *Aspergillus fumigatus*, *Campylobacter jejuni*, *Candida auris*, *Enterobacteriaceae*, ceftriaxone-resistant *Neisseria gonorrhoeae*, carbapenem-resistant *Pseudomonas aeruginosa*, tuberculosis, and the gut microbiome. This selected list overlaps with pathogens that can travel via water to cause infection (e.g., *Aspergillus fumigatus*, *Campylobacter*, *Escherichia coli*, and *Pseudomonas aeruginosa*), a list that further includes pathogens such as *Legionella pneumophila*, nontuberculous mycobacteria, and *Vibrio* spp., among others.

On the flip side, extreme conditions may also harbor solutions. Researchers have postulated that deep-sea organisms, perhaps similar to those depicted in "Hydro Vents and Other Difficult Places," could be sources of novel bioactive compounds that could aid in the development of new antimicrobial drugs. Curiosity about the interactions between pathogens and cellular life forms in difficult places creates an interesting bridge between art and science and may lead to discoveries that could forge unexpected solutions to the global crisis of antimicrobial resistance.

Bibliography

1. Centers for Disease Control and Prevention. Antibiotic/antimicrobial resistance (AR/AMR) [cited 2019 May 15]. https://www.cdc.gov/drugresistance/about.html
2. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74:417–33. https://doi.org/10.1128/MMBR.00016-10
3. Esslinger A. About the work [cited 2019 May 15]. http://www.amieesslinger.com/about
4. National Ocean Service. What is a hydrothermal vent? [cited 2019 May 15]. https://oceanservice.noaa.gov/facts/vents.html
5. Pedersen A. Art the science. Creators—Amie Esslinger [2019 May 15]. https://artthescience.com/blog/2017/01/04/creators-amie-esslinger
6. Sommerstein R, Rüegg C, Kohler P, Bloemberg G, Kuster SP, Sax H. Transmission of *Mycobacterium chimaera* from heater–cooler units during cardiac surgery despite an ultraclean air ventilation system. Emerg Infect Dis. 2016;22:1008–13. https://doi.org/10.3201/eid2206.160045
7. Tortorella E, Tedesco P, Palma Esposito F, January GG, Fani R, Jaspars M, et al. Antibiotics from deep-sea microorganisms: current discoveries and perspectives. Mar Drugs. 2018;16:pii; E355. https://doi.org/10.3390/md16100355
8. World Health Organization. Antimicrobial resistance [cited 2019 May 17]. https://www.who.int/en/news-room/fact-sheets/detail/antimicrobial-resistance

Address for correspondence: Byron Breedlove, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop H16-2, Atlanta, GA 30329-4027, USA; email: wbb1@cdc.gov

EMERGING INFECTIOUS DISEASES®

Upcoming Issue

- Zika Virus Infection in Pregnant Women, Yucatan, Mexico
- Multistate Outbreak of Listeriosis Associated with Packaged Leafy Green Salads, United States and Canada, 2015–2016
- *Pseudomonas poae*–Associated Fatal Septic Transfusion Reaction, Peoria, Illinois, USA, 2017
- Cross-Protection of Dengue Virus Infection against Congenital Zika Syndrome, Northeastern Brazil
- Direct Medical Costs of Reported Travel-Related Infections in Ontario, Canada, 2012–2014
- Natural Vertical Transmission of Zika Virus in Larval *Aedes aegypti* Populations, Morelos, Mexico
- *Artyfechinostomum sufrartyfex* Trematode Infection in Children, Bihar, India
- Wild-Type Yellow Fever Virus in Cerebrospinal Fluid of Child
- Emergent Invasive Group A *Streptococcus dysgalactiae* Subspecies *equisimilis* Recovered, United States, 2015–2018
- Case Series Study of Melioidosis, Colombia
- Lethal Encephalitis in Seals with Japanese Encephalitis Virus Infection, China
- Marburgvirus in Egyptian Fruit Bats, Zambia
- Kaposi Sarcoma in Mantled Guereza
- Evaluating Temperature Sensitivity of Vesicular Stomatitis Virus–Based Vaccines
- Poliomyelitis-Like Presentation of Powassan Virus with Anterior Horn Cell Involvement, Canada
- Intact *Mycobacterium leprae* Isolated from Placenta in a Pregnant Woman, China
- *Erwinia billingiae* as an Unusual Cause of Septic Arthritis, France, 2017
- Bejel, a Nonvenereal Treponematosis, among Men Who Have Sex with Men, Japan
- Chikungunya Fever Outbreak, Zhejiang Province, China, 2017
- No Evidence for Role of Cutavirus in Malignant Melanoma

Complete list of articles in the August issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

July 14–17, 2019
STI and HIV 2019 World Congress
Vancouver, Canada
http://stihiv2019vancouver.com

July 20–24, 2019
American Society for Virology
Minneapolis, MN, USA
http:// www.asv.org

July 21–24, 2019
International AIDS Society 2019
Mexico City, Mexico
http:// www.ias2019.org

August 12–23, 2019
16th International Course on Dengue, Zika and other Emergent Arboviruses
Havana, Cuba
http://instituciones.sld.cu/ipk/16th-international-course-on-dengue-zika-and-other-emergent-arboviruses/

August 28–September 1, 2019
OPTIONS X for the Control of Influenza
Suntec, Singapore
https://www.isirv.org/site/

October 2–6, 2019
ID Week
Washington, DC, USA
https://idweek.org/

November 20–24, 2019
ASTMH
American Society of Tropical Medicine and Hygiene
68th Annual Meeting
National Harbor, MD, USA
https://www.astmh.org/

February 20–23, 2020
International Society for Infectious Diseases
Kuala Lumpur, Malaysia
https://www.isid.org/

Announcements

Email announcements to EIDEditor (eideditor@cdc.gov). Include the event's date, location, sponsoring organization, and a website. Some events may appear only on EID's website, depending on their dates.

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.

You must be a registered user on http://www.medscape.org. If you are not registered on http://www.medscape.org, please click on the "Register" link on the right hand side of the website.

Only one answer is correct for each question. Once you successfully answer all post-test questions, you will be able to view and/or print your certificate. For questions regarding this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please go to https://www.ama-assn.org. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the AMA PRA CME credit certificate, and present it to your national medical association for review

Article Title

Prescribing Patterns for Treatment of *Mycobacterium avium* Complex and *M. xenopi* Pulmonary Disease in Ontario, Canada, 2001–2013

CME Questions

1. You are advising a public health department regarding the need for physician education on the proper treatment of *Mycobacterium avium* complex pulmonary disease (MAC-PD) and *M. xenopi* pulmonary disease (Mx-PD). According to the retrospective cohort study by Brode and colleagues, which of the following statements about older adults (≥66 years) with MAC-PD and Mx-PD in Ontario, Canada, who received antimicrobial therapy during 2001–2013 and factors associated with receipt of treatment is correct?

A. Of 2,834 patients with MAC-PD and 928 patients with Mx-PD who met microbiologic criteria during 2001–2013, 24.2% and 15.3% received treatment
B. Treated vs untreated patients with MAC-PD were younger and more likely to be female and to live in higher-income neighborhoods and rural settings
C. Treated vs untreated patients with MAC-PD were more likely to have diabetes
D. Among treated patients with MAC-PD, the most commonly used drug in the first treatment episode was a rifamycin

2. According to the retrospective cohort study by Brode and colleagues, which of the following statements about older adults with MAC-PD and Mx-PD in Ontario, Canada, who received guideline-recommended treatment and comparison with that in other countries is correct?

A. Recommended standard triple therapy was the most commonly used regimen (47% with MAC-PD and 36% with Mx-PD)
B. 23% of treated patients with MAC-PD received standard triple therapy for initial treatment
C. Use of standard triple therapy for MAC-PD in this study was less common than reported in the United States or the European Union
D. Standard triple therapy for MAC-PD was given for a mean of 165 days before a regimen switch or discontinuation

3. According to the retrospective cohort study by Brode and colleagues, which of the following statements about older adults with MAC-PD and Mx-PD in Ontario, Canada, who received treatment that could lead to macrolide resistance and factors associated with receipt of such treatment is correct?

A. Less than one-fifth of patients treated with MAC-PD received regimens associated with emergent macrolide resistance
B. Among treated patients with Mx-PD, 18.2% received nonmacrolide monotherapy and 13.9% received macrolide monotherapy
C. In adjusted analyses, diabetes and COPD were significantly associated with a starting regimen of macrolide monotherapy
D. Increased focus is needed to disseminate knowledge contained in treatment guidelines to physicians who treat MAC-PD and Mx-PD

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.

You must be a registered user on http://www.medscape.org. If you are not registered on http://www.medscape.org, please click on the "Register" link on the right hand side of the website.

Only one answer is correct for each question. Once you successfully answer all post-test questions, you will be able to view and/or print your certificate. For questions regarding this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please go to https://www.ama-assn.org. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the AMA PRA CME credit certificate, and present it to your national medical association for review

Article Title

Hospital-Associated Multicenter Outbreak of Emerging Fungus *Candida auris*, Colombia, 2016

CME Questions

1. You are advising a large South American hospital regarding prevention, detection, and control of a *Candida auris* outbreak. On the basis of the clinical case series by Armstrong and colleagues, which one of the following statements about clinical characteristics and outcomes of a hospital-associated *C. auris* outbreak in Colombia in 2015 to 2016 is correct?

A. Median age was 63 years; 50% were male
B. 88% had a preexisting comorbidity such as hemodialysis and immunocompromise
C. Age or having a comorbid condition predicted mortality
D. In-hospital mortality was 18%; 30-day mortality was 13%

2. According to the clinical case series by Armstrong and colleagues, which one of the following statements about likely mode of transmission and associated findings in a hospital-associated *C. auris* outbreak in Colombia in 2015 to 2016 is correct?

A. There was evidence of temporal, but not spatial, clustering of cases
B. Whole-genome sequencing (WGS) showed a low degree of relatedness of case isolates within hospitals
C. All cases had an invasive procedure (for example, central venous catheter placement and intubation)
D. Fecal-oral transmission was most likely

3. On the basis of the clinical case series by Armstrong and colleagues, which one of the following statements about treatment and other clinical and public health implications of a hospital-associated *C. auris* outbreak in Colombia in 2015 to 2016 is correct?

A. The most common treatments for *C. auris* candidemia were fluconazole and caspofungin
B. The findings suggest good adherence to infection control recommendations
C. Choice of antimicrobial agents was appropriate in nearly all cases
D. Outbreaks are likely to occur only in pediatric hospitals or units

Emerging Infectious Diseases is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Summary of Authors' Instructions

Authors' Instructions. For a complete list of EID's manuscript guidelines, see the author resource page: http://wwwnc.cdc.gov/eid/page/author-resource-center.

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words in the main body of the text or include more than 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (not to exceed 150 words), a 1-line summary of the conclusions, and a brief biographical sketch of first author or of both authors if only 2 authors. This section comprises case series papers and concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome. If detailed methods are included, a separate section on experimental procedures should immediately follow the body of the text.

Research. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 50 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Research Letters Reporting Cases, Outbreaks, or Original Research. EID publishes letters that report cases, outbreaks, or original research as Research Letters. Authors should provide a short abstract (50-word maximum), references (not to exceed 10), and a short biographical sketch. These letters should not exceed 800 words in the main body of the text and may include either 1 figure or 1 table. Do not divide Research Letters into sections.

Letters Commenting on Articles. Letters commenting on articles should contain a maximum of 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) 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.

In This Issue

Synopses

Research

Historical Review

Dispatches

ISSN 1080-6040

DEPARTMENT OF HEALTH & HUMAN SERVICES
Public Health Service
Centers for Disease Control and Prevention (CDC)
Mailstop D61, Atlanta, GA 30329-4027

Official Business
Penalty for Private Use $300
Return Service Requested

MEDIA MAIL
POSTAGE & FEES PAID
PHS/CDC
Permit No. G 284

Vol 25, No 7, July 2019

EMERGING INFECTIOUS DISEASES

Pages 1261–1444