EMERGING INFECTIOUS DISEASES®

October 2012

Human–Primate Encounters

EMERGING
INFECTIOUS DISEASES®

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EMERGING INFECTIOUS DISEASES

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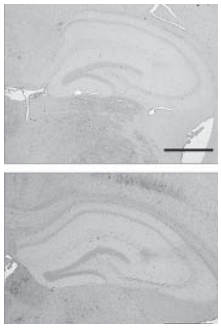
On the Cover

Mori Sosen (1747–1821)
Monkey Performing the Sanbasō Dance (Dated 1800, the first day of the Monkey Year)
Scroll painting, ink on paper (49.5 cm x 115.6 cm)
Pacific Asia Museum, Pasadena, California, USA, Gift of Mr. and Mrs. Bruce Ross
www.pacificasiamuseum.org

About the Cover p. 1711

Research



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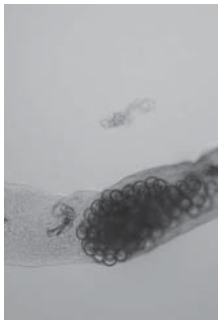

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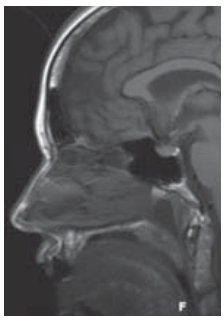

Another Dimension

Letters

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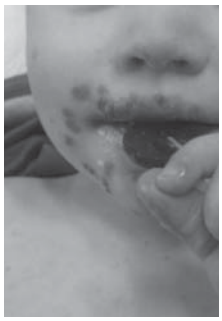
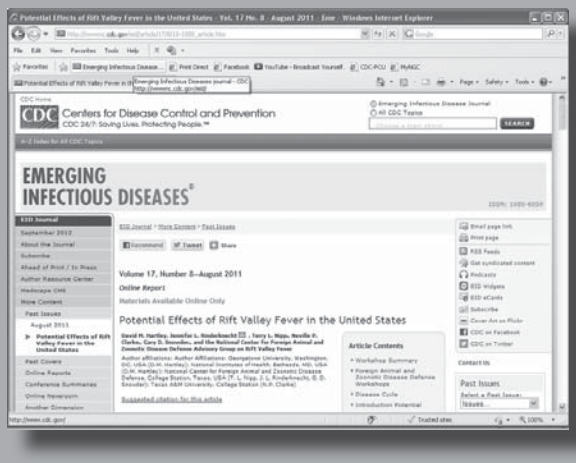


About the Cover

Etymologia



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JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

Infectious diseases whose incidence in humans has increased in the past 2 decades or threatens to increase in the near future have been defined as "emerging." These diseases, which respect no national boundaries, include

- New infections resulting from changes or evolution of existing organisms.
- Known infections spreading to new geographic areas or populations.
- Previously unrecognized infections appearing in areas undergoing ecologic transformation.
- Old infections reemerging as a result of antimicrobial resistance in known agents or breakdowns in public health measures.

Why an "Emerging" Infectious Diseases journal?

The Centers for Disease Control and Prevention (CDC), the agency of the U.S. Public Health Service charged with disease prevention and health promotion, leads efforts against emerging infections, from AIDS, hantavirus pulmonary syndrome, and avian flu, to tuberculosis and West Nile virus infection. CDC's efforts encompass improvements in disease surveillance, the public health infrastructure, and epidemiologic and laboratory training.

Emerging Infectious Diseases represents the scientific communications component of CDC's efforts against the threat of emerging infections. However, even as it addresses CDC's interest in the elusive, continuous, evolving, and global nature of these infections, the journal relies on a broad international authorship base and is rigorously peer-reviewed by independent reviewers from all over the world.

What are the goals of Emerging Infectious Diseases?

1) Recognition of new and reemerging infections and understanding of factors involved in disease emergence, prevention, and elimination. Toward this end, the journal

   - Investigates factors known to influence emergence: 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.
   - Reports laboratory and epidemiologic findings within a broader public health perspective.
   - Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

2) Fast and broad dissemination of reliable information on emerging infectious diseases. Toward this end, the journal

   - Publishes reports of interest to researchers in infectious diseases and related sciences, as well as to public health generalists learning the scientific basis for prevention programs.
   - Encourages insightful analysis and commentary, stimulating global interest in and discussion of emerging infectious disease issues.
   - Harnesses electronic technology to expedite and enhance global dissemination of emerging infectious disease information.

Methicillin-Resistant *Staphylococcus aureus* Sequence Type 239-III, Ohio, USA, 2007–2009[1]

Shu-Hua Wang, Yosef Khan, Lisa Hines, José R. Mediavilla, Liangfen Zhang, Liang Chen, Armando Hoet, Tammy Bannerman, Preeti Pancholi, D. Ashley Robinson, Barry N. Kreiswirth, and Kurt B. Stevenson, for the Prevention Epicenter Program of the Centers for Disease Control and Prevention

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Release date: September 20, 2012; Expiration date: September 20, 2013

Learning Objectives

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

- Distinguish the most common strains of methicillin-resistant *Staphylococcus aureus* (MRSA) in the United States
- Assess the clinical characteristics of infection with MRSA ST239-III
- Analyze the treatment and prognosis of MRSA ST239-III infection
- Evaluate molecular characteristics of MRSA ST239-III.

CME Editor

Thomas J. Gryczan, MS, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Thomas J. Gryczan, MS, has disclosed no relevant financial relationships*.

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Methicillin-resistant *Staphylococcus aureus* (MRSA) is a human pathogen that has diverse molecular heterogeneity. Most MRSA strains in the United States are pulsed-field gel electrophoresis USA100 sequence type (ST) 5 and USA300 ST8. Infections with MRSA ST239-III are common and found during health care–associated outbreaks. However, this strain has been rarely reported in the United States.

[1]Presented in part at the 48th Annual Meeting of the Infectious Diseases Society of America, Vancouver, British Columbia, Canada, October 21–24, 2010.

As part of a study supported by the Prevention Epicenter Program of the Centers for Disease Control and Prevention (Atlanta, GA, USA), which evaluated transmission of MRSA among hospitals in Ohio, molecular typing identified 78 (6%) of 1,286 patients with MRSA ST239-III infections. Ninety-five percent (74/78) of these infections were health care associated, and 65% (51/78) of patients had histories of invasive device use. The crude case-fatality rate was 22% (17/78). Identification of these strains, which belong to a virulent clonal group, emphasizes the need for molecular surveillance.

Staphylococcus aureus is a major human pathogen that possesses multiple toxins and virulence mechanisms (*1*). Antimicrobial drug resistance in *S. aureus* has added to the complexity of treating serious infections caused by this bacteria, and methicillin-resistant *S. aureus* (MRSA) appears to have greater virulence than methicillin-susceptible strains (*2*,*3*). Most MRSA strains in the United States are pulsed-field gel electrophoresis (PFGE) types USA100 and USA300, corresponding to multilocus sequence typing (MLST) ST5 and ST8, respectively (*4*). MRSA belonging to MLST ST239 and harboring staphylococcal cassette chromosome *mec* (SCC*mec*) type III (MRSA ST239-III) are associated with infections in health care settings, outbreaks, increased resistance to antimicrobial drugs, and capacity for invasive disease (*5*–*7*).

MRSA ST239-III has a history of successful dissemination in many regions, leading to a diverse array of regionally prevalent clones. These clones include the Brazilian; British Epidemic 1, 4, 7, 9, and 11; Canadian Epidemic 3/Punjab; Czech; Eastern Australian 2 and 3; Georgian; Hungarian; Lublin; Nanjing/Taipei (ST241); Portuguese; and Vienna clones (*8*,*9*). Although it is common worldwide, MRSA ST239-III has not played any predominant role in the United States; infections with MRSA ST239-III have been rarely reported in the United States since the 1990s (*9*–*13*). Recently, only 2 reports of this strain in the United States involving sporadic nasal colonization and bloodstream infections have been published (*13*,*14*).

In this study, we describe clinical epidemiologic characteristics and molecular analysis of clinical infections with MRSA ST239-III in the midwestern United States. Identification of a strain from such a virulent clonal group in the United States with wide dissemination in other parts of the world represents a potential public health concern.

Methods

Sampling Method

As part of Centers for Disease Control and Prevention (CDC) (Atlanta, GA, USA)–sponsored Prevention Epicenter Program study evaluating the transmission of MRSA between hospitals in Ohio, USA, molecular analysis was performed on a group of clinical MRSA isolates collected from The Ohio State Health Network (OSHN). The OSHN consists of The Ohio State University (OSU) Wexner Medical Center (WMC), which is a tertiary care medical center, and 7 smaller community hospitals located 30–120 miles from OSU. All MRSA specimens from community hospitals and selected OSU MRSA blood isolates and isolates from patients residing in the catchment areas of the outreach hospitals were prospectively collected during March 2009–February 2010 for genotyping by using a repetitive element PCR (rep-PCR). Among archived MRSA isolates from OSUWMC from January 2007 through February 2009, only a selection of isolates was chosen for genotyping (not a randomized sampling). The total number of isolates and the total number of ST239 from each time period was collected.

Data Collection

We performed medical record reviews for 1,286 patients. Patient demographic characteristics (presence of health care–related risk factors during the preceding 12 months, presence of an invasive device during the previous 7 days, and concurrent conditions) were collected. Patient-level data, including addresses for geocoding, were entered into a secure database within the OSUWMC Information Warehouse.

Classification of MRSA Infections

All MRSA cases were classified into 3 categories on the basis of accepted epidemiologic definitions (*11*). The first category was health care–associated, defined as a culture obtained >48 hours after admission. The second category was health care–associated community onset, defined as a culture obtained ≤48 hours after admission with identified health care–associated risk factors. The third category was community-associated, defined as a culture obtained ≤48 hours after admission without health care–associated risk factors. Health care–associated risk factors comprised presence of an invasive device, history of MRSA infection or colonization, surgery, hospitalization, dialysis, or residence in a long-term care facility in the 12 months preceding the culture.

Outcomes for MRSA infection were categorized as cure (complete resolution after antimicrobial drug treatment); failure (persistence of infection and change in antimicrobial drug regimen); relapse (resolution of infection after complete treatment with subsequent development of new symptoms); recurrent (redevelopment of MRSA at same or other site ≥2 weeks after completion of treatment for initial MRSA infection); indeterminate (unknown outcome); and death (death ≤30 days after diagnosis of MRSA infection because of any cause or during the same

hospitalization). Destination after hospital discharge, such as home or skilled nursing facility, was also noted.

Drug Susceptibility Testing

The respective OSHN Clinical Microbiology Laboratories initially identified all MRSA isolates by using standard microbiological methods. Antimicrobial drug susceptibility testing was performed at each institution, and results were interpreted according to Clinical and Laboratory Standards Institute break point guidelines (*15*). At OSUWMC, antimicrobial drug susceptibility testing was performed by using the automated Micro-Scan method (Siemens Diagnostics, Sacramento, CA, USA), and only constitutive clindamycin testing was performed. Linezolid MIC >4 mg/L were confirmed by using the Etest method (bioMérieux, Marcy l'Etoile, France).

Genotyping

The MRSA ST239 III isolates were genotyped initially by using rep-PCR, followed by PFGE, staphylococcal protein A sequencing (*spa* typing), SCC*mec* typing, and *mec*-associated direct repeat unit (*dru*) typing. Selected isolates were also characterized by MLST and single-nucleotide polymorphism (SNP) typing. Detection of genes encoding Panton–Valentine leukocidin (PVL), toxic shock syndrome toxin (TSST), arginine catabolic mobile element (ACME), and high-level mupirocin resistance (*mupA*) was also performed. Brief descriptions of each testing method are outlined below.

rep-PCR

The DiversiLab System (bioMérieux, Durham, NC, USA) was used for rep-PCR analysis according to described methods (*16*). Isolates belonging to designated rep-PCR clusters shared >95% similarity. In addition, comparison of matching patterns in the DiversiLab System library was initially used to infer the PFGE and SCC*mec* types, which were later validated by using appropriate testing methods. The numeric classification system used for rep-PCR analysis is unique to OSUWMC.

PFGE

The PulseNet protocol for molecular subtyping of *S. aureus* was followed. *Salmonella enterica* serotype Braenderup DNA was digested with *Xba*I (Roche, Indianapolis, IN, USA) and used as the normalization standard for gel analysis. *S. aureus* chromosomal DNA was digested with *Sma*I (Roche). Fragments were separated in a clamped homogenous electric field mapper unit (Bio-Rad Laboratories, Hercules, CA, USA). Fingerprint images were analyzed by using Bionumerics software version 4.61 (Applied Maths NV, Sint-Martens-Latem, Belgium). The traditional classification of PFGE subtypes was not used because we were not analyzing for an outbreak (*17*). Thus, interpretations of possibly or probably related PFGE subtypes between strains obtained by PFGE band patterns were not made. Each PFGE band difference was classified as a unique PFGE pattern.

spa Typing

spa typing (*18*) was performed on all MRSA isolates by using eGenomics software (www.egenomics.com) as described (*19*); Ridom *spa* types were subsequently assigned by using the SpaServer website (www.spaserver.ridom.de).

SCC*mec* Typing

SCC*mec* typing was performed on all MRSA isolates by using a described multiplex real-time PCR (*20*). This PCR is specific for 2 essential gene complexes (*ccr* and *mec*) found in all SCC*mec* elements.

dru Typing

MRSA isolates were also characterized by sequencing the hypervariable *dru* repeat region within the SCC*mec* element (*21*) and using DruID software (*9*). New *dru* types were submitted to www.dru-typing.org.

MLST and PVL, TSST, ACME, and Mupirocin Resistance

MLST was performed on representative isolates as described (*22*) by using the MLST database (http://saureus.mlst.net). PCR-based detection of PVL (*23*), TSST (*24*), ACME (*25*), and *mupA* (*26*) was performed on all isolates as described.

SNP Typing

A panel of 43 SNPs for describing the global population structure of MRSA ST239-III (*9*) was used to identify the haplotypes of 22 isolates. These SNPs were typed by using Golden-Gate Genotyping Assay (Illumina, San Diego, CA, USA) and conventional Sanger sequencing.

Statistical Analysis

All patient demographic, clinical, and molecular typing data were aggregated in tabular format, and descriptive statistics were generated by using SAS version 9.2 (SAS Institute Inc., Cary NC, USA), for demographic and risk factor history. A significant difference between ST239 and other strains (US300, US100, and all other strains) was examined by using χ^2 tests for categorical variables and *t*-tests for continuous variables. An α level of 0.5 was used.

Human Subjects Protection

We obtained approval for this study from the OSU Office of Responsible Research Practices' Biomedical

Institutional Review Board. The OSU Information Warehouse has established honest broker status with the OSU Institutional Review Board, enabling storage of fully identifiable data and presentation of patient data to investigators in a coded format that maintains patient confidentiality.

Results

Patient Characteristics

Of 1,286 clinical MRSA isolates, 78 (6%) were identified as MRSA ST239-III; 71 (91%) were obtained from OSU and 7 (9%) from community hospitals. Seven (2%) of 397 isolates were obtained from outreach community hospitals, 37 (6%) of 613 from OSUWMC, and 34 (12%) of 276 from OSUWMC archives.

These strains were first recognized by rep-PCR as possible SCC*mecA* type III isolates from the DiversiLab System library with imputed Brazilian PFGE types. Additional molecular typing identified MRSA ST239-III in a clade different from that containing the Brazilian strains.

Demographics and clinical characteristics of 78 patients infected with MRSA ST239-III are shown in the Table. Among patients with MRSA ST239-III infections, 46 (59%) were male, 65 (83%) were white, 31 (40%) disabled, and 29 (37%) were retired. Seventy-four (95%) had health care–associated isolates and 51 (65%) had histories of use of invasive devices within the previous 7 days. Distribution of specimen types include 37 (47%) bloodstream infections (BSIs), 19 (24%) lower respiratory tract infections (LRTIs), 11 (14%) skin and soft tissue infections, and 11 (14%) other infections. Seventeen (22%) patients died, and 18 (23%) patients had treatment failures, recurring infections, or relapses. Only 32% of the patients were discharged home.

Comparison of clinical characteristics of MRSA ST239-III with those of PFGE types USA100 and USA300 and other non–ST239 infections are shown in the Table. Most ST239 isolates were health care–associated MRSA and had characteristics and concurrent conditions similar to those associated with USA100. MRSA ST239-III did not have virulent determinants often associated with health care–associated or community-associated MRSA strains, such as PVL, TSST, ACME, or *mupA*.

Characteristics of MRSA ST239-III Isolates

Resistance was observed to clindamycin (76/76, 100%), moxifloxacin (47/47, 100%), gentamicin (74/77, 96%), tetracycline (70/74, 95%), and trimethoprim–sulfamethoxazole (62/77, 81%). All MRSA ST239-III isolates were susceptible to vancomycin. For daptomycin, 64 (98%) of 65 isolates were susceptible, and 1 blood isolate with an MIC of 2 mg/L was classified as uninterpretable. For linezolid, 69 (97%) of 71 isolates were susceptible and 2 blood isolates with MICs >4 mg/L were classified as uninterpretable.

Molecular Typing of MRSA ST239-III Isolates

Eight rep-PCR patterns (6, 19, 22, 42, 53, 78, 79, and 97) were detected among MRSA ST239-III isolates, and 39 (50%) isolates had rep-PCR pattern 6. Similarly, isolates were distributed among 9 PFGE patterns (A, B, C, D, E, F, G, H, and I), and 58 (74%) had PFGE pattern A. The rep-PCR and PFGE patterns are shown in Figure 1. PFGE pattern F is <80% similar with other PFGE subtypes in the dendrogram and was identified as an unknown PFGE type by the CDC database. However, molecular testing confirmed it to be MRSA ST239-III: MLST 239, SCC*mec* type III, *spa* type 3(t037), *dru* type dt15b, and SNP haplotype H9.

Four *spa t*ypes, including eGenomics *spa* types 3 (Ridom t037), 314 (t363), 121 (t421), and 1256 (t631) were identified, and 74 (95%) isolates were classified as *spa* type 3 (t037). In contrast, isolates were grouped into 13 *dru* types (dt2c, dt6n, dt9g, dt12i, dt13k, dt14 g, dt15b, dt15h, dt15i, dt15j, dt15k, dt16a, and dt23a), and 60 (77%) isolates were classified as dt15b. MLST typing of selected representative isolates confirmed 13 to be MRSA ST239-III (2-3-1-1-4-4-3), 3 as ST1801 (2-3-1-1-4-19-3), and 1 as ST2017 (2-3-1-1-4-19-227). ST1801 is a single-locus variant of MRSA ST239-III, and ST2017 is a novel single-locus variant of ST1801 and a double-locus variant of MRSA ST239. All were SCC*mec* type III, and the *ccrC* locus was not detected. The genes that encode PVL, TSST, ACME, or high-level mupirocin resistance were not found in any isolates.

A total of 33 unique genotypic combinations from 78 patients infected with MRSA ST239-III were represented among isolates with complete SCC*mec*, rep-PCR, PFGE, *spa*, and *dru* data. This information and year of isolation are shown in Figure 2. The genotype cluster sizes ranged from 2 to 18 isolates; 23 of the isolates were unique. In contrast, SNP typing of 22/78 isolates with a panel of 43 SNPs showed them to be indistinguishable from each other. All isolates tested belonged to haplotype 9 (H9) within MRSA ST239-III clade II (Figure 3). This clade was composed of isolates from many continents, including many from sources in Asia (*9*).

Discussion

MRSA ST239-III has demonstrated epidemic potential worldwide, and identification of this strain in the United States might represent a major public health concern. Although the precise time frame of emergence of this strain in Ohio is unknown, it appears to have been present before the study because it was identified in archived

specimens dating back to 2007. The origin of the MRSA ST239-III clonal group has been dated to the mid-20th century in 2 studies (*9*,*27*). Thus, the Ohio strain might have been introduced into the region anytime during the past 40 years. Because of incomplete sampling during the study, the transmission dynamics of Ohio MRSA ST239 III are uncertain. Whether the prevalence would have been >6% if complete sampling was conducted is unknown. Furthermore, we found no epidemiologic evidence of clustering of cases consistent with an outbreak of infection with this strain. The large number of isolates at OSUWMC merely reflects its role as a large referral center.

Clinically, MRSA ST239-III is primarily health care associated, causes major outbreaks, shows increased

Table. Characteristics of 1,286 patients with MRSA ST239-III and non–MRSA ST239-III infections, Ohio, USA, 2007–2009*

Characteristic	ST239, n = 78	USA100, n = 481	p value	USA300, n = 574	p value	All other, n = 153	p value
Outreach hospital isolates	7 (9)	81 (17)	NA	262 (46)	NA	47 (31)	NA
Patient demographics							
Mean age, y (range)	58 (19–90)	61 (18–99)	0.06	43 (18–92)	<0.0001	49 (18–94)	0.001
Male sex	46 (59)	259 (54)	0.39	311 (54)	0.42	80 (52)	0.33
White race	65 (83)	393 (82)	0.85	441 (77)	0.61	126 (82)	0.77
Medical history							
Diabetes	33 (42)	149 (31)	0.047	116 (20)	<0.0001	35 (23)	0.002
Chronic lung disease	26 (33)	117 (24)	0.09	74 (13)	<0.0001	17 (11)	<0.0001
Renal failure	19 (24)	93 (19)	0.39	37 (6)	<0.0001	17 (11)	0.008
Malignancy	13 (17)	97 (20)	0.47	48 (8)	0.02	30 (20)	0.58
Health care–associated risk factors, past 12 mo							
Hospitalization	58 (74)	296 (62)	0.03	148 (26)	<0.0001	58 (38)	<0.0001
Use of invasive device	35 (45)	168 (35)	0.09	66 (12)	<0.0001	35 (23)	0006
Surgery	35 (45)	179 (37)	0.19	79 (14)	<0.0001	33 (22)	0.0002
History of MRSA infection	26 (33)	77 (16)	0.0003	82 (14)	<0.0001	29 (19)	0.015
Stay in long-term care facility	22 (28)	155 (32)	0.47	34 (6)	<0.0001	14 (9)	0.0002
Hemodialysis	15 (19)	47 (10)	0.068	18 (3)	<0.0001	10(7)	0.015
Other	10 (13)	29 (6)	0.03	40 (7)	0.06	7 (5)	0.02
Invasive devices ≤7 d before infection							
Central venous catheter	25 (32)	156 (32)	0.94	61 (11)	<0.0001	32 (21)	0.06
Foley catheter	17 (22)	99 (21)	0.8	55 (10)	0.001	17 (11)	0.03
Hemodialysis	13 (17)	49 (10)	0.02	19 (3)	<0.0001	8 (5)	0.008
Mechanical ventilation	14 (18)	85 (18)	0.95	35 (6)	0.0002	11 (7)	0.012
Drainage tubes	8 (10)	50 (10)	0.97	10 (2)	<0.0001	5 (3)	0.02
Total parenteral nutrition	8 (10)	22 (5)	0.04	4 (1)	<0.0001	0	0.0001
Other	20 (26)	71 (15)	0.016	39 (7)	<0.0001	15 (10)	0.0015
Classification of MRSA infection							
Health care–associated	32 (41)	197 (41)	0.97	63 (11)	<0.0001	37 (24)	0.01
Health care–associated community onset	42 (54)	214 (44)	0.12	113 (20)	<0.0001	49 (32)	0.0012
Community-associated	4 (5)	70 (15)	0.02	398 (69)	<0.0001	67 (44)	<0.0001
Outcome							
Cure	23 (29)	149 (31)	0.81	94 (16)	0.005	38 (25)	0.49
Failure	3 (4)	7 (1.5)	0.14	12 (2)	0.33	5 (3)	0.81
Relapse	4 (5)	7 (1.5)	0.03	2 (1)	0.0001	1 (1)	0.022
Recurrent	11 (14)	45 (9)	0.2	36 (6)	0.012	3 (2)	0.0002
Indeterminate	20 (26)	192 (40)	0.015	408 (71)	0.001	97 (63)	0.0001
Death	17 (22)	81 (17)	0.29	22 (4)	0.001	9 (6)	0.0003
No. patients admitted	74	429	0.005	310	0.0003	111	0.003
Admitting service							
Intensive care unit	14 (19)	58 (14)	0.2	29 (9)	0.019	9 (8)	0.047
Medicine service	31 (42)	238 (55)	0.03	203 (66)	0.0002	75 (68)	0.0007
Surgery service	20 (27)	117 (27)	0.95	59 (19)	0.12	20 (18)	0.126
Other specialty care unit†	9 (12)	16 (4)	0.002	19 (6)	0.073	7 (6)	0.16
Destination after discharge							
Home	20 (27)	146 (34)	0.24	209 (67)	<0.0001	60 (54)	0.0003
Another facility, long-term care center, or rehabilitation center	35 (47)	204 (48)	0.97	79 (24)	<0.0001	40 (36)	0.12
Median duration of hospitalization,‡ d (range)	16 (1–143)	11 (0–136)	0.07	6 (0–169)	<0.0001	9 (1–124)	0.01

*Values are no. (%) unless otherwise indicated. MRSA, methicillin-resistant *Staphylococcus aureus*; ST, sequence type; NA, not applicable. The p-values were generated to assess whether differences in demographic and risk factor history existed between ST239 and other strains (USA100, USA300, all other strains). χ^2 tests were used for categorical variables and *t*-tests were used for continuous variables.

†Other specialty care unit admitting services included bone marrow, cardiology, hematology, surgical oncology, transplant, and obstetrics and gynecology.

‡Duration of hospitalization and days to MRSA culture–positive included only inpatients; outpatient visits such as clinic or emergency department visits were excluded from the analysis. Duration of hospitalization was calculated from the date of the current hospital admission to the date of discharge. Hospitalization days at another institution before transfer were not included.

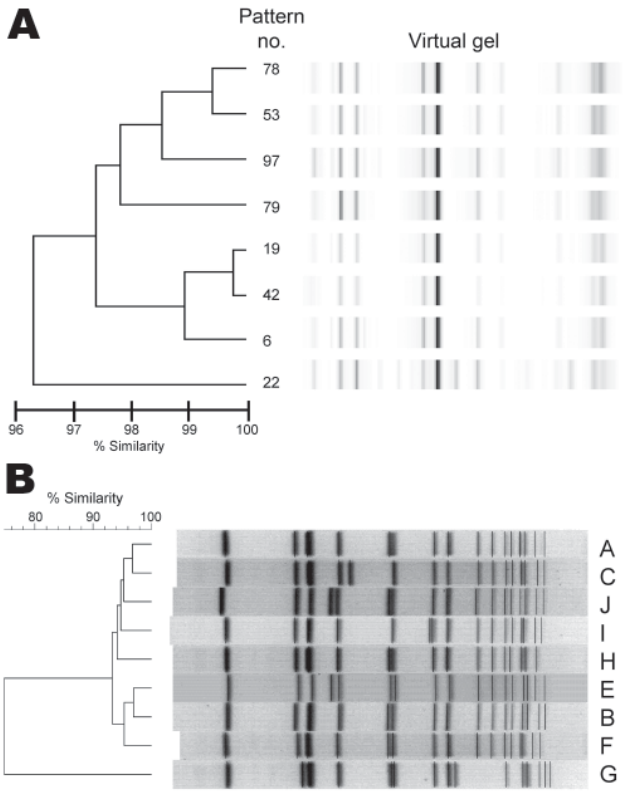

Figure 1. Methicillin-resistant *Staphylococcus aureus* sequence type 239-III isolates, Ohio, USA, 2007–2009, based on A) repetitive element PCR (rep-PCR) and B) pulsed-field gel electrophoresis. Virtual gel results are shown for 8 DiversiLab System (bioMérieux, Durham, NC, USA) rep-PCR patterns. Pattern numbers assigned are unique to the Ohio State University Wexner Medical Center.

resistance to antimicrobial drugs, and demonstrates a capacity for causing invasive disease (*5–7*,*28*,*29*). Comparison of characteristics of patients infected with MRSA ST239-III with those infected with PFGE USA300, PFGE USA100, and other strains showed that MRSA ST239-III is similar to USA100 in terms of health care–associated risk factors and outcomes (Table).

Concern for the invasive potential of MRSA ST239-III was illustrated during a 2-year intensive care unit (ICU) outbreak in London in which patients infected with MRSA ST239-III were more likely than patients with non–ST239 strains to show development of bacteremia (47% vs 13%; p<0.001) and have culture-positive vascular access device infections (59% vs 26%; p<0.001) (*29*). In our study, 19% (14/74) of patients infected with MRSA ST239-III strains were admitted to ICU, compared with 11% (96/850) of patients infected with non–ST239 strains (Table). Among MRSA ST239-III ICU admissions, 50% (7/14) were BSIs and 50% (7/14) were LRTIs. The crude mortality rate for persons with BSIs and LRTIs was 22% (8/37) and 47% (9/19) respectively. In our study population, MRSA ST239-III had a higher case-fatality rate than did USA300 (4%) and USA100 (17%) (Table).

Increased illness and death for pulmonary disease might be caused by an enhanced ability of MRSA ST239-III strains to produce biofilms and adhere to airway epithelial cells, providing a biologically plausible explanation for its potential to cause pneumonia and central line–associated BSIs (*28*,*29*). The association of MRSA ST239-III isolates with pulmonary infections has also been reported in South Korea (*30*). Moreover, in a multicenter study involving 21 hospitals in Beijing, where MRSA ST239-III is especially prevalent, 61% of MRSA LRTI cases were attributed to MRSA ST239-III (*31*). The London outbreak strain of MRSA ST239-III, and most examined MRSA ST239-III strains from the People's Republic of China, belong to the same clade (clade II) as the Ohio strain of MRSA ST239-III, demonstrating the potential for this lineage to disseminate and cause disease (*9*,*27*,*32*). Because of incomplete collection of all MRSA strains causing LRTIs, whether MRSA ST239-III is the prevalent strain in MRSA pneumonias is unknown. A total of 17% (203/1,206) of the study isolates were pulmonary infections and 9% (19/222) of the pneumonias were caused by MRSA ST239-III.

PFGE is the standard for MRSA outbreak investigations. However, we chose a rapid PCR–based method that could serve as a potential screening surveillance tool to identify outbreaks of a particular strain. Although many institutions might be using PFGE for outbreak investigations to identify clonal clusters, methods for PFGE subtype classification are often not standardized between laboratories (*33*,*34*). To maximize uniformity and enable comparisons of PFGE classifications between institutions, the standardized CDC protocols for performing and analyzing PFGE results were used. The initial CDC PFGE analysis classified our isolates as the Brazilian clone. However, SNP typing unambiguously placed the Ohio isolates within a different clade than the Brazilian clone (Figure 3) (*9*). Because PFGE typing cannot define specific lineages for MRSA ST239-III, CDC has changed the reporting of MRSA ST239-III strains from Brazilian subtype to ST239. Laboratories will need to perform additional testing to identify specific clades or clonal groups. Classification of the 78 MRSA ST239-III isolates by the traditional PFGE outbreak method would identify only 2 PFGE subtypes instead of the 8 observed patterns. PFGE subtype 1 would consist of isolates A–E, G, and H, and subtype 2 would consist of isolate F (Figure 1, panel B).

Classification issues also exist with respect to the DiversiLab system. Five of the 8 initial rep-PCR patterns matched isolates in the DiversiLab library, which likewise categorized them as the Brazilian clone. In contrast, 3 other rep-PCR patterns did not match any isolates in

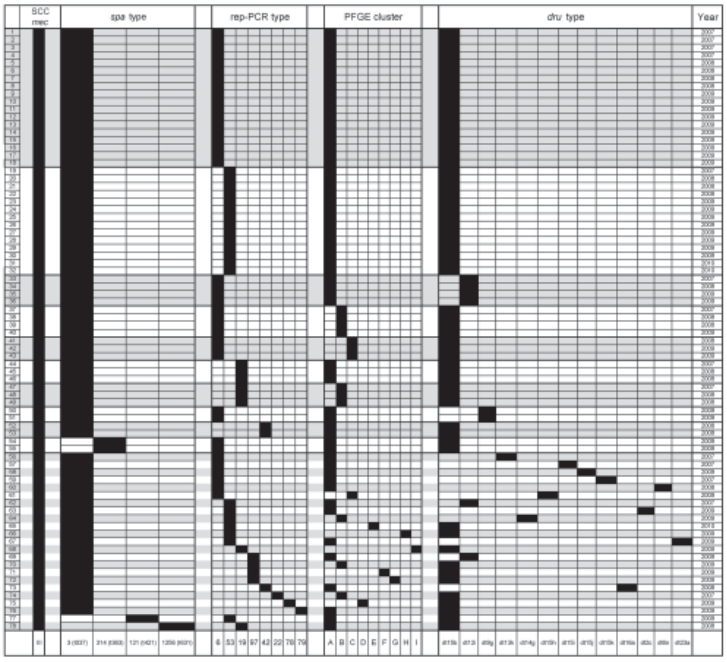

Figure 2. Molecular characteristics of 78 methicillin-resistant *Staphylococcus aureus* sequence type 239-III isolates, Ohio, USA, 2007–2009. SCC*mec*, staphylococcal cassette chromosome *mec*; *spa*, staphylococcal protein A; rep-PCR, repetitive element PCR; PFGE, pulsed-field gel electrophoresis; *dru*, direct repeat unit; dt, *dru* type. The 33 unique genotypic combinations are contrasted by black and gray shading.

the DiversiLab library. Additional molecular tests were performed to identify these isolates as MRSA ST239-III.

Because of the novel identification of MRSA ST239-III strains, we performed a battery of molecular tests to better classify the strains. Although the same *dru* types might be found in unrelated MRSA lineages and different staphylococcal species (*32*), variation at the *dru* locus within the SCC*mec* III element is consistent with MRSA ST239-III phylogeny (*9*). Because use of this typing method is relatively new, further study of its phylogenetic utility is needed (*21*). The feasibility of population-based genome-wide SNP datasets has been demonstrated for MRSA ST239-III by Harris et al. (*27*). Cluster groupings from these PFGE, rep-PCR, and *dru* methods were not identical, suggesting that the molecular tests are not completely interchangeable. Of the various molecular methods used to index genomic variation in MRSA, *dru* typing and rep-PCR appeared to be more discriminatory.

The publication of only 2 recent reports of MRSA ST239-III in the United States (*13*,*14*) might have resulted from inadequate national surveillance or low transmissibility of the strain. Institutions might not be able to perform genotyping on all their isolates because of lack of appropriate laboratory facilities or resources. An alternative method of surveillance for MRSA ST239-III might be evaluation of the drug susceptibility pattern. Most of the MRSA ST239-III strains in our study were resistant to clindamycin, tetracycline, and gentamicin. In a subset analysis, the phenotypic patterns of antimicrobial drug susceptibility and MRSA genotype prediction were determined for 798 MRSA isolates from the OSHN dataset. Ninety-four percent (63/67) of the MRSA ST239-III isolates were resistant to all 3 drugs (clindamycin, tetracycline, and gentamicin) (*35*). Use of phenotypic drug susceptibility patterns might alert infection control practitioners that they are dealing with a MRSA ST239-III clone. Additional molecular testing, such as *spa* typing in conjunction with SCC*mec* typing, can then be performed to confirm the strain type.

We have genotyped and geocoded 1,286 MRSA isolates in the CDC Prevention Epicenter Study by using rep-PCR. The lack of a clear association between MRSA ST239-III molecular genotype patterns could be caused by the retrospective nature of this study, lack of complete population sampling, and the inability to gather social network history. The rep-PCR and PFGE methods might be of limited use in evaluating geographic clustering at the scale studied here unless specific lineages of the reference strains are included in the respective databases. Because of the clonality exhibited by MRSA ST239-III, even a relatively small panel of 43 SNPs is able to identify the same major phylogenetic lineages that are identified by genome-wide SNPs. A more detailed genome-wide SNP analysis might be required to resolve geographic clustering. Additional social networking for determining spatial, temporal, and geographic relationships of patients at the medical institutions studied is under way to identify potential nosocomial interhospital and intrahospital transmission.

Recent identification of a strain of MRSA ST239-III in the midwestern United States is a major public health concern. Globally, this strain has demonstrated increased

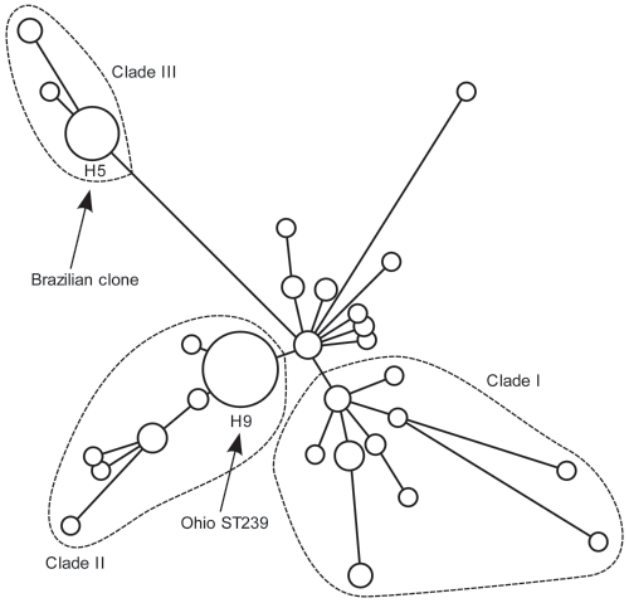

Figure 3. Single-nucleotide polymorphism (SNP) haplotype map showing position of methicillin-resistant *Staphylococcus aureus* sequence type 239-III (MRSA ST239-III) isolates, Ohio, USA, 2007–2009, within the global population structure of the MRSA ST239-III clonal group. Circles indicate distinct haplotypes, as defined by using a panel of 43 SNPs (*9*). Sizes of circles indicate relative frequency of different haplotypes. Arrows indicate haplotype 5 (H5), which includes the Brazilian clone, and haplotype 9 (H9), which includes the 22 MRSA ST239-III isolates from Ohio. Relationships between haplotypes were determined by using maximum-parsimony analysis (*9*).

virulence and widespread dissemination. Because of an inadequate national surveillance system, temporal emergence and dissemination of the strain in the United States is uncertain. The MRSA ST239-III strains in Ohio have molecular heterogeneity and geographic diversity. The SNP-based data suggest that the strains also show clonal diversity. Continued surveillance is warranted because this MRSA ST239-III strain, similar to related strains worldwide, exhibits increased antimicrobial drug resistance, capacity for causing invasive disease, potential for causing outbreaks, and resulting in illnesses and deaths. MRSA ST239-III strains might have the potential to become established locally and disseminate among health care institutions as reported in other regions. Our study underscores the value of molecular surveillance, including traditional fingerprinting methods and newer sequence-based typing methods, as a critical component in understanding the evolving epidemiology of MRSA.

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Dr Wang is an assistant professor of medicine at The Ohio State University in Columbus, Ohio. Her research interests are molecular genotyping, geocoding, and social network analysis for evaluation of transmission of MRSA and *Mycobacterium tuberculosis*.

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Epidemiology of Foodborne Norovirus Outbreaks, United States, 2001–2008

Aron J. Hall, Valerie G. Eisenbart, Amy Lehman Etingüe, L. Hannah Gould, Ben A. Lopman, and Umesh D. Parashar

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Release date: September 17, 2012; Expiration date: September 17, 2013

Learning Objectives

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

- Describe general characteristics and outcomes of US norovirus outbreaks, based on an analysis of data reported during 2001-2008 to the CDC Foodborne Disease Outbreak Surveillance System
- Describe sources of US norovirus outbreaks, based on an analysis of data reported during 2001-2008 to the CDC Foodborne Disease Outbreak Surveillance System
- Describe recommended interventions to reduce the frequency and effects of foodborne norovirus outbreaks, based on an analysis of data reported during 2001-2008 to the CDC Foodborne Disease Outbreak Surveillance System

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Noroviruses are the leading cause of foodborne illness in the United States. To better guide interventions, we analyzed 2,922 foodborne disease outbreaks for which norovirus was the suspected or confirmed cause, which had been reported to the Foodborne Disease Outbreak Surveillance System of the Centers for Disease Control and Prevention during 2001–2008. On average, 365 foodborne norovirus outbreaks were reported annually, resulting in an estimated 10,324 illnesses, 1,247 health care provider visits, 156 hospitalizations, and 1 death. In 364 outbreaks attributed to a single commodity, leafy vegetables (33%), fruits/nuts (16%), and mollusks (13%) were implicated most commonly. Infected food handlers were the source of 53% of outbreaks and may have contributed to 82% of outbreaks. Most foods were likely contaminated during preparation and service, except for mollusks, and occasionally, produce was contaminated during production and processing. Interventions to reduce the frequency of foodborne norovirus outbreaks should focus on food workers and production of produce and shellfish.

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Noroviruses, the leading cause of foodborne illness in the United States, are responsible for an estimated 58% of all domestically acquired foodborne illness from known agents (*1*). The estimated 5.5 million annual foodborne norovirus illnesses in the United States, which constitute those associated with recognized outbreaks and those considered sporadic, result annually in 15,000 hospitalizations and 150 deaths and cost ≈$2 billion in health care expenses and lost productivity (*1*,*2*). Noroviruses are also the leading cause of foodborne disease outbreaks reported in the United States, accounting for about half of all foodborne outbreaks in which an etiologic agent is identified (*3*–*5*). Classified into the genus *Norovirus* within the family *Caliciviridae*, noroviruses are a genetically diverse group of nonenveloped, single-stranded RNA viruses, comprising at least 5 genogroups (GI–GV) and >35 genotypes (*6*). Since 2001, noroviruses within the GII.4 genotype have caused most viral gastroenteritis outbreaks worldwide (*7*).

Noroviruses can be spread through a variety of means, including direct person-to-person transmission through the fecal–oral route; ingestion of aerosolized vomitus; and indirect transmission through contaminated surfaces, food, or water. Norovirus has a low infectious inoculum (≥18 viral particles) and is shed copiously by ill persons (10^5–10^{11} viral copies per gram of feces), which enables its rapid and efficient spread (*8*–*10*). Noroviruses also remain infectious on surfaces for as long as 2 weeks and in water for ≥2 months (*11*,*12*) and are resistant to many common disinfectants (*13*,*14*). Foods can be contaminated with noroviruses at any point along the farm-to-fork continuum, although the most frequent pathways are thought to be through an infected food handler or exposure to water contaminated with fecal matter (e.g., surface water used for produce irrigation or water containing sewage discharge where shellfish grow) (*15*–*17*). Bivalve mollusks, such as oysters, bioaccumulate noroviruses in their body through filtration and selective binding mechanisms and therefore are readily contaminated when they are grown in harvesting areas contaminated with human feces (*18*).

Attribution of norovirus disease to specific foods and increasing understanding of the various contamination pathways that result in disease can help identify potential targets for interventions. Although most foodborne norovirus disease in the United States is not outbreak associated, outbreaks provide the most robust information about the foods that cause illness and the factors contributing to their contamination. The last published description of foodborne norovirus outbreaks in the United States was based on surveillance data from 1991 through 2000, before molecular diagnostic tools were widely available (*19*). Since that time, norovirus diagnostics have become incorporated more routinely into public health outbreak investigations, resulting in markedly increased recognition of norovirus illnesses and outbreaks. Using more recent surveillance data, we therefore sought to robustly describe foodborne norovirus outbreaks, including temporal, geographic, and demographic trends, and attribution to specific foods, settings, and contamination factors.

Methods

Data Source

Since 1973, the Centers for Disease Control and Prevention (CDC) has collected data on foodborne disease outbreaks from state, local, and territorial health departments through the Foodborne Disease Outbreak Surveillance System (FDOSS) (*20*). A foodborne disease outbreak is defined as ≥2 similar illnesses resulting from ingestion of a common food. Data collected for each outbreak included outbreak characteristics (e.g., dates, number of ill persons, locations, etiologic agents), case-patient characteristics (e.g., demographic characteristics, symptoms, health care seeking, and whether the illness resulted in death), setting of food preparation, contributing factors, and the implicated food vehicle(s) (*21*). Outbreaks of norovirus infection are considered laboratory confirmed if stool or vomitus specimens from ≥2 ill persons are positive for norovirus by reverse transcription PCR, enzyme immunoassay, or electron microscopy (*6*). Norovirus may be implicated as the suspected etiologic agent in the absence of laboratory confirmation when reasonable clinical or epidemiologic evidence exists, such as the previously validated Kaplan criteria (*22*). Data were extracted for all foodborne outbreaks in which norovirus was either a suspected or confirmed etiologic agent and the first illness occurred during 2001–2008.

Data Analysis

Frequencies of outbreaks and outbreak-related illnesses, health care provider visits, hospitalizations, and deaths were calculated. Annual variations were analyzed by grouping data into seasonal years from July–June and comparing data with information from the 2 adjacent years. Seasonal trends and variations in reporting by state were also assessed. Because data on health care provider visits, hospitalizations, deaths, age groups, and sex were not always reported (i.e., they were provided for 63%, 66%, 69%, 82%, and 89% of all reported illnesses, respectively), the relative proportions of illnesses by age and outcome from the outbreaks that included such data were extrapolated to all reported outbreak-associated illnesses. For example, among the 68,452 outbreak-associated illnesses that included data on age, 22,301 (32.5%) occurred in persons >50 years of age; this proportion (32.5%) was applied to the total number of outbreak-associated illnesses reported

(82,591) to yield the estimated number of outbreak-associated illnesses among those >50 years of age (26,872). Rates of reported outbreaks and outbreak-associated illnesses per 1,000,000 person-years were calculated by dividing the average annual number of these illnesses by the corresponding US intercensal estimate at the midpoint of the study period, July 2004 (*23*).

Food vehicles implicated in outbreaks were classified on the basis of a categorization hierarchy of 17 mutually exclusive commodity groups (*24*). If a food contained a single contaminated ingredient or all ingredients belonged to a single commodity, it was classified into that commodity. Food vehicles that contained ingredients from multiple commodities were classified as complex. Outbreaks were not attributed to any of the commodities if an implicated food vehicle could not be assigned to one of these commodities, multiple food vehicles were implicated, or no specific food vehicle was implicated.

Factors contributing to contamination and methods of food preparation were reported according to standard categorization schemes (*21*). Contributing factors were not mutually exclusive and were further grouped into the following categories: food handler contact, cross-contamination during preparation, contaminated raw product, and insufficient cooking and/or heating. Outbreak reports also indicated whether a food handler was specifically implicated as the source of contamination (e.g., handled the implicated food while symptomatic) rather than simply being a potential contributor. For implicated foods that could be classified into 1 of the 17 commodities, the likely point of contamination (POC) was assessed on the basis of a combination of contributing factors and whether a food handler was implicated as the source of contamination. POC was categorized to distinguish production or processing (i.e., a raw product contaminated from the environment or obtained from a polluted source) versus preparation or service (i.e., vehicle handled by infected food worker or cross-contamination during preparation). Those outbreaks with insufficient or conflicting information reported regarding the likely POC were classified as unknown.

The settings of food preparation in outbreaks were classified into the following categories: commercial (e.g., restaurant, grocery store, caterer), institutional (e.g., school, nursing home, prison), private (e.g., home, church, picnic), and other (including unknown); multiple settings could be selected in a given outbreak. Differences in median number of illnesses associated with outbreaks in different settings were assessed by using Wilcoxon rank-sum tests. χ^2 tests were used to evaluate trends among categorical variables. Analyses were performed by using SAS v9.2 (SAS Institute Inc., Cary, NC, USA) and Epi Info v3.4.3 (CDC, Atlanta, GA, USA).

Results

Outbreak Characteristics

During 2001–2008, a total of 9,206 foodborne disease outbreaks were reported in the United States. For 2,922 (46%) of the 6,355 outbreaks with a known cause, norovirus was the confirmed or suspected cause, an average of 365 foodborne norovirus outbreaks annually (1.2 outbreaks/1,000,000 person-years). Of these, 1,683 (58%) outbreaks were laboratory confirmed, primarily on the basis of reverse transcription PCR. Genogroup information, reported in most confirmed norovirus outbreaks only since 2006, was available for 648 norovirus outbreaks, among which 111 (17%) were caused by genogroup I (GI) and 518 (80%) were caused by genogroup II (GII); 19 (3%) involved both GI and GII noroviruses. Sixteen (0.5%) norovirus outbreaks also involved other suspected or confirmed etiologic agents, including nontyphoidal *Salmonella* spp. (n = 4), adenovirus (n = 2), *Bacillus cereus* (n = 2), *Campylobacter* spp. (n = 2), *Clostridium perfringens* (n = 2), *Listeria monocytogenes* (n = 1), *Shigella* spp. (n = 1), and *Vibrio parahaemolyticus* (n = 1).

Foodborne norovirus outbreaks were reported from 49 states (all but Vermont) and the District of Columbia, with substantial state-to-state variation in the rate of reported outbreaks (Figure 1). The highest per capita rates of reported outbreaks were in Minnesota (6.2/1,000,000 person-years) and Oregon (6.1/1,000,000 person-years); the lowest reported rates were in Texas (0.25/1,000,000 person-years) and Kentucky (0.27/1,000,000 person-years). The greatest number of outbreaks was reported by California (n = 526, 18%). Residents from multiple states were affected in 138 (5%) outbreaks, of which 8 (6%) involved exposures to contaminated foods distributed to multiple states.

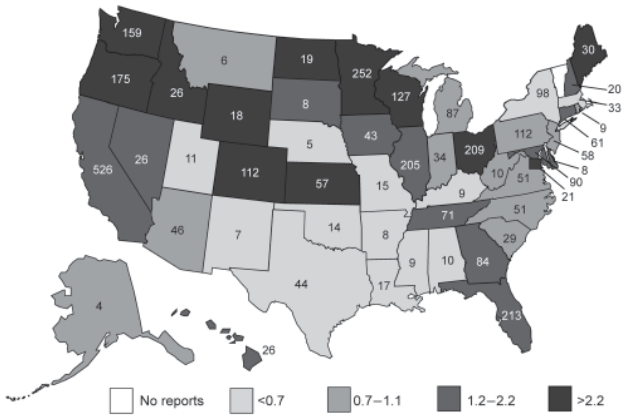

Figure 1. Total number and rate of reported foodborne norovirus outbreaks per 1,000,000 person-years by affected states, United States, 2001–2008. Number given in each state indicates the total number of outbreaks over the 8-year study period; shaded boxes in key indicate the reported rate by quartiles. Multistate outbreaks are assigned as outbreaks to each state involved.

Outbreaks were slightly more frequent in the winter months than in the rest of the year, with 957 (33%) outbreaks occurring during December–February; however, the seasonal pattern varied somewhat from year to year (Figure 2). The temporal pattern of outbreaks in which norovirus was confirmed by laboratory testing was similar to that of outbreaks in which norovirus was suspected as the etiologic agent. Although no consistent secular trend was observed over the 8-year study period, the number of outbreaks in 2006–07 (n = 442) was 24% higher than the average number of outbreaks during the 2 adjacent seasonal years of 2005–06 (n = 359) and 2007–08 (n = 352).

On average, 10,324 reported illnesses were associated with foodborne norovirus outbreaks each year (Table 1). These included an estimated 1,247 (12%) health care provider visits, 156 (1.5%) hospitalizations, and 1 (0.01%) death annually. Most (80%) outbreak-associated illnesses affected adults ≥20 years of age, and 56% of illnesses affected women. Children <5 years of age had a significantly lower rate of foodborne norovirus outbreak–associated illness (11/1,000,000 person-years) than did all other age groups combined (37/1,000,000 person-years) ($p<0.001$). Among outbreaks for which data were available, the median incubation period was 33 hours (n = 2,348), and the median attack rate was 61% (n = 1,099).

Norovirus outbreaks involved foods prepared most often in commercial settings (83%) and less frequently in private (11%), institutional (8%), and other (12%) settings (Table 2). Outbreaks involving institutional settings were significantly larger in terms of total number of illnesses (median 36 illnesses/outbreak) than those in other settings (median 15 illnesses/outbreak) ($p<0.001$). The most commonly reported settings were restaurants or delicatessens (62%), caterers (11%), and private homes (10%).

Food Attribution

At least 1 food vehicle was implicated in 1,298 (44%) outbreaks, of which 534 (41%) involved a complex food, 364 (28%) involved a simple food that could be classified into one of the 17 commodities, 279 (21%) involved multiple foods, and 121 (9%) involved a single food item that could not be classified. The reasons for implicating specific foods included statistical evidence from epidemiologic investigations (55%); laboratory evidence, such as identification of the agent in the food (2%); compelling supportive information (30%); other data (0.1%); and prior experience in the absence of specific evidence (5%). For 8% of implicated foods, no reasons were given. Of the 813 outbreaks that involved complex or multiple foods, 328 (40%) implicated sandwiches, salads, or other foods eaten raw or lightly cooked.

Factors that may have contributed to contamination were provided in 886 (68%) of outbreaks with at least 1 implicated food; among these outbreaks, food handler contact with ready-to-eat food was identified in 725 (82%), consumption of a contaminated raw product in 111 (13%), cross-contamination during preparation in 109 (12%), and inadequate cooking or heating in 28 (3%). A food handler was specifically implicated as the source of contamination in 473 (53%) outbreaks. This determination was reportedly made on the basis of laboratory and epidemiologic evidence (33%), epidemiologic evidence only (45%), laboratory evidence only (4%), and prior experience only (17%); no reason for implicating a food handler was given for 2 of these outbreaks. No significant differences in contributing factors were identified between different settings where foods were prepared.

Among the 364 outbreaks with a single, simple food implicated, the most frequent commodities were leafy vegetables (33%), fruits/nuts (16%), and mollusks (13%), although all commodities except sprouts were implicated in at least 1 outbreak (Figure 3). Information was available to indicate the likely POC in 191 (52%) of these outbreaks, among which contamination during preparation or service was more frequent (85%, n = 162) than was contamination during production or processing (15%, n = 29) ($p<0.001$). All 26 mollusk-associated outbreaks in which the likely POC could be determined were caused by contamination during production or processing. Production or processing contamination was also indicated in 3 of the 109 outbreaks associated with either leafy greens or fruits/nuts in which a POC could be determined. Outbreaks involving all other commodities resulted either from contamination during preparation or service or from an unknown POC. A traceback was performed in 8 outbreaks, all of which involved mollusks; 3 of these resulted in a product recall.

Discussion

This study demonstrates the predominant etiologic role of norovirus in foodborne disease outbreaks in the United States, with an average of 1 foodborne norovirus

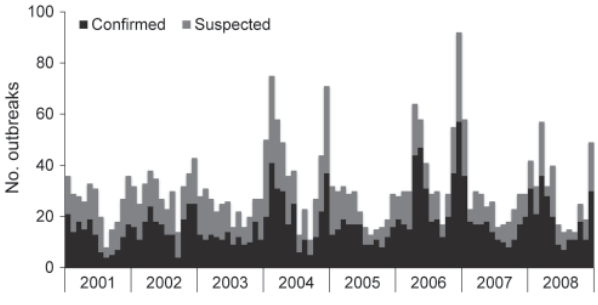

Figure 2. Number of reported foodborne norovirus outbreaks by month of first illness onset, United States, 2001–2008. Outbreaks are confirmed as caused by norovirus if fecal or vomitus specimens from >2 persons are positive for the virus by reverse transcription PCR, electron microscopy, or enzyme immunoassay.

Table 1. Estimated annual number and rate of reported illnesses associated with foodborne norovirus outbreaks by age, sex, and outcome, United States, 2001–2008

Characteristic	Estimated annual outbreak-associated illnesses*	
	No. (%)	Rate†
Age group, y		
<5	215 (2)	10.6
5–19	1,827 (18)	30.3
20–49	4,923 (48)	39.8
≥50	3,359 (33)	37.7
Sex		
F	5,819 (56)	39.0
M	4,505 (44)	31.3
Outcome		
Health care provider visit	1,247 (12)	4.3
Hospitalization	156 (1.5)	0.5
Death	1 (0.01)	0.002
Total Illnesses	10,324 (100)	35.2

*Proportions of illnesses by age, sex, and outcomes among outbreaks for which such data were reported were extrapolated to all reported foodborne norovirus outbreak–associated illnesses.
†Reported rate per 1,000,000 person-years calculated by dividing the number of illnesses by the corresponding US intercensal estimate at the study period midpoint (July 2004) (*23*).

outbreak reported every day, and highlights the frequency of norovirus contamination of raw and other ready-to-eat foods. Fresh produce, primarily leafy vegetables and fruits, was implicated in over half of all outbreaks that could be classified into a single commodity; ready-to-eat foods that contain fresh produce, such as sandwiches and salads, were frequently implicated complex vehicles. Mollusks, which are also commonly served raw or undercooked, were also implicated frequently. Food handler contact with raw and ready-to-eat foods was identified as the most common scenario resulting in foodborne norovirus outbreaks, underscoring the need to better understand and control endemic norovirus disease as a means of foodborne disease prevention. Contact with food handlers during preparation was cited in 82% of outbreaks as a possible contributor to contamination and was specifically implicated as the source of contamination in 53% of outbreaks. These proportions likely reflect lack of positive evidence and therefore may be underestimated because of disincentives for reporting illness and asymptomatic infections among food handlers (*25*,*26*). Most foods implicated in norovirus outbreaks were prepared in restaurants, delicatessens, and other commercial settings, which suggests that these are key locations for intervention. Steps to curtail contamination of ready-to-eat foods by food handlers in these settings include adherence to appropriate recommendations for hand washing and use of gloves; compliance with policies to prevent ill staff from working; and presence of a certified kitchen manager, as recommended by the Food Code of the US Food and Drug Administration (*27*).

With the exception of mollusk-associated outbreaks, we rarely identified contamination during production or processing in this analysis, although it is likely underrecognized in norovirus outbreaks. Indeed, the point of contamination could not be determined in ≈50% of single-commodity outbreaks, many of which may be additional instances of contamination before preparation. Oysters are well documented as food vehicles that are prone to norovirus contamination during production (*17*,*28*), but examples of contaminated produce have also been reported, including raspberries and lettuce, which are likely contaminated from irrigation waters (*16*,*29*). In a recent multicountry study,

Table 2. Settings of food preparation in foodborne norovirus outbreaks, United States, 2001–2008*

Setting†	No. (%) outbreaks	Median no. cases/outbreak (IQR)
Commercial	2,432 (83)	14 (7–26)
Restaurant or delicatessen	1,824 (62)	12 (6–23)
Caterer	313 (11)	25 (16–40)
Banquet facility	110 (4)	30 (19–59)
Grocery store	105 (4)	13 (9–18)
Commercial product served without further preparation	42 (1)	20 (11–30)
Wedding reception	22 (1)	25 (18–35)
Fair, festival, other temporary/mobile service	16 (1)	21 (6–50)
Institutional	248 (8)	36 (19–71)
School	91 (3)	43 (29–92)
Nursing home	66 (2)	35 (19–60)
Camp	29 (1)	34 (18–53)
Noncafeteria workplace	18 (1)	23 (13–56)
Prison or jail	15 (1)	58 (23–137)
Workplace cafeteria	12 (0.4)	22 (18–78)
Hospital	11 (0.4)	53 (16–72)
Day care center	4 (0.1)	31 (19–65)
Office setting	2 (0.1)	18 (15–21)
Private	336 (11)	16 (10–26)
Private home	296 (10)	15 (9–25)
Church, temple, or other place of worship	31 (1)	23 (12–40)
Picnic	9 (0.3)	19 (12–32)
Other/Unknown	267 (9)	28 (14–49)
All outbreaks	2,922 (100)	14 (8–30)

*IQR, interquartile range.
†Multiple locations may be implicated in a given outbreak.

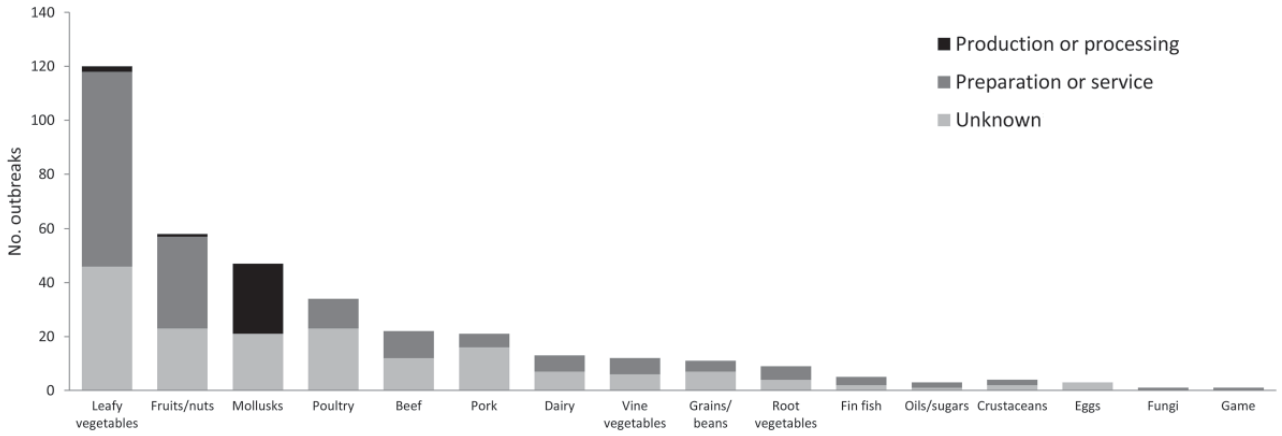

Figure 3. Commodity and point of contamination implicated in reported norovirus outbreaks involving simple foods (consisting of a single commodity; n = 364), United States, 2001–2008. Point of contamination was classified as unknown if insufficient or conflicting information was provided in outbreak report.

norovirus RNA was detected in 28%–50% of samples from leafy green vegetables and in 7%–34% of samples from soft red fruit (e.g., strawberries and raspberries) obtained from retail markets or directly from processing companies (*30*). These findings demonstrate that fresh produce often comes in contact with norovirus during production. However, because the available detection methods cannot distinguish infectious virus from noninfectious genomic material, the specific risks posed to public health from this potential contamination of produce remain unclear. Outbreak investigations can provide learning opportunities to possibly link such contamination with human illness through tracebacks and root-cause analyses to determine when, where, and how foods became contaminated. Unfortunately, no tracebacks were performed during produce-associated outbreaks included in this analysis, despite evidence in some of these outbreaks that suggested possible contamination before preparation.

No specific food vehicle was implicated in 56% of the foodborne norovirus outbreaks included in this analysis, a substantially higher proportion than that for foodborne outbreaks with bacterial etiologic agents (*3*–*5*). This discrepancy underscores the challenges in food attribution of norovirus outbreaks, given the potential for multiple transmission pathways, contamination of multiple vehicles by an ill food handler, and time lags in reporting by citizen complaint. However, the discrepancy may also reflect the relative deprioritization of investigating suspected norovirus outbreaks. Misperceptions may exist that foodborne norovirus outbreaks result only from local contamination events and afford little opportunity for further prevention and/or control. Such decisions regarding investigations are often made in the face of scant public health resources, inadequate staffing, and competing priorities (*31*). Overcoming these misperceptions, prioritizations, and limited resources are key challenges to improving the knowledge base for effective prevention and control of foodborne norovirus disease.

Although overall reporting of foodborne norovirus outbreaks has vastly improved since the 1990s (*19*), great disparity remains among states. A 25-fold difference in population-based rates of foodborne norovirus outbreaks was identified between the highest and lowest reporting states. Although these differences may be due in part to true variations in the incidence of foodborne norovirus outbreaks, they also suggest varying degrees of underreporting. Thus, rates of outbreaks and outbreak-associated illnesses captured through surveillance represent reporting rates, not true incidence, and likely underestimate the true incidence. Capacity of state and local health departments to investigate foodborne disease outbreaks varies widely, with the most notable limitations being lack of dedicated personnel and delayed notification of outbreaks (*31*). Efforts to better understand the gaps in foodborne outbreak response, including laboratory, epidemiologic, and environmental health capacity, may ultimately inform strategies to overcome the challenges of limited public health resources (*32*).

The increasing trend in the 1990s in the number of foodborne norovirus outbreaks reported appears to have leveled off, likely reflecting the availability of diagnostic testing for norovirus at almost all public health laboratories across US states. However, a substantial increase was observed during 2006–07, contemporaneous with the emergence of 2 new GII type 4 (GII.4) norovirus variants (*7*). Interestingly, the surge in foodborne outbreaks during the 2006–07 epidemic season appeared less pronounced than that observed among norovirus outbreaks in general (*7*,*33*), most of which result from direct person-to-person spread (*34*), as well sporadic norovirus-associated hospitalizations and

deaths in the United States (*35*,*36*). Furthermore, foodborne norovirus outbreaks did not increase in association with emergence of a new GII.4 variant in the 2002–03 seasonal year, which has been previously correlated with other indicators of increased norovirus activity (*7*,*35*,*36*). These observations coupled with the paucity of genotype information reported directly through FDOSS underscores the need for and importance of CaliciNet, the recently implemented national laboratory surveillance network for norovirus outbreaks (*37*). Integration of sequence-based genotyping data with epidemiologic data will enable timely recognition of the impact of emergent noroviruses on foodborne disease and potentially identify links between outbreaks due to widely distributed food vehicles. Additionally, beginning in 2009, US data on enteric disease outbreaks associated with any mode of transmission, i.e., foodborne and nonfoodborne, are systematically collected through a single comprehensive system, the National Outbreak Reporting System (NORS), allowing for attribution of all norovirus outbreaks by mode of transmission and setting (*38*).

Despite the demonstrated prevalence and effects of these infections, relatively few evidence-based interventions exist for preventing and controlling foodborne norovirus disease. This study provides a comprehensive analysis of US foodborne norovirus outbreaks, highlighting both potential targets for interventions and key remaining data gaps. Fresh produce, mollusks, and ready-to-eat foods were identified as the primary vehicles of foodborne norovirus disease. Continued focus on food handlers in retail settings is warranted, although the role and mechanisms of contamination during production, particularly as they relate to fresh produce, remain unclear. To that end, further development and validation of analytic methods for the detection of norovirus in foods, particularly those that can quantitatively distinguish contamination levels correlating with infection risk and distinguish infectious from noninfectious virus, are critically needed (*32*). Likewise, there is ongoing need to define the duration of infectiousness of ill food handlers and to elucidate the role of asymptomatic food handlers in norovirus transmission, issues that have been hampered by the inability to culture human noroviruses in vitro. Other areas needing attention are improving understanding of the roles of various norovirus genotypes in different foods, identifying the environmental antecedents leading to food contamination, and the role of other transmission modes in propagating norovirus spread. Initiatives to improve outbreak surveillance and response can help address some of these data gaps (*35*,*37*–*39*), but only if public health agencies have adequate resources. Finally, as the development of candidate norovirus vaccines continues to progress (*40*), their possible public health utility in specific populations, such as food handlers, should be assessed.

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Constant Transmission Properties of Variant Creutzfeldt-Jakob Disease in 5 Countries

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Variant Creutzfeldt-Jakob disease (vCJD) has been reported in 12 countries. We hypothesized that a common strain of agent is responsible for all vCJD cases, regardless of geographic origin. To test this hypothesis, we inoculated strain-typing panels of wild-type mice with brain material from human vCJD case-patients from France, the Netherlands, Italy, and the United States. Mice were assessed for clinical disease, neuropathologic changes, and glycoform profile; results were compared with those for 2 reference vCJD cases from the United Kingdom. Transmission to mice occurred from each sample tested, and data were similar between non-UK and UK cases, with the exception of the ranking of mean clinical incubation times of mouse lines. These findings support the hypothesis that a single strain of infectious agent is responsible for all vCJD infections. However, differences in incubation times require further subpassage in mice to establish any true differences in strain properties between cases.

Variant Creutzfeldt-Jakob disease (vCJD) is an acquired transmissible spongiform encephalopathy (TSE), or prion disease, that results in a fatal neurodegenerative condition in humans. vCJD was first reported in the United Kingdom in 1996 (*1*) and was most likely caused by dietary exposure to contaminated products from cattle that had bovine spongiform encephalopathy (BSE). The similarity between BSE and vCJD was shown by experimental transmission of the 2 diseases into standard panels of inbred wild-type mouse lines (RIII, C57BL, and VM [*1*,*2*]) and into FVB mice (*3*).

The strain properties of vCJD and BSE have been extensively characterized in these sets of mice by using a combination of the order in which each strain of mouse dies of disease (incubation period rankings), distribution of brain vacuolation at the terminal stage of disease (lesion profiles), distribution pattern of abnormal prion protein (PrP^{Sc}) in the brain, and glycosylation pattern of PrP^{Sc} as assessed by Western blot analysis. BSE and vCJD produce highly reproducible and similar incubation period rankings and neuropathology, which indicates that they are the same strain in the RIII, C57BL, and VM mice, whereas in the FVB mice, both diseases exhibit the same pattern of PrP^{Sc} deposition (*3*–*5*).

During 1996–2011, a total of 176 cases of definite or probable vCJD were reported in the United Kingdom; the number of deaths peaked at 28 in 2000. Since 2006, deaths from vCJD have leveled off to 2–5 per year (*6*). Although vCJD was originally restricted to the United Kingdom, 49 cases have been reported in 11 other countries, including some outside Europe, bringing the worldwide total number of cases to 225. Most cases outside the United Kingdom have occurred in France, which is believed to be related to the large volume of beef imports; ≈60% of UK beef exports to Europe were destined for France (*7*). This possible relationship is borne out by a peak in vCJD

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cases in France 5 years after a similar peak in cases in the United Kingdom, which fits in well with the timing of an increase in beef imports from the United Kingdom during 1985–1995 (*7*).

More recently, comparative studies of vCJD cases from France and the United Kingdom have shown evidence from clinical, epidemiologic, pathologic, and biochemical analyses that a common strain of agent may be responsible for vCJD infection in both countries (*8*). In Europe, active surveillance to monitor BSE cases was implemented in 2001 (*6*,*9*). Although it appears that exports of meat, cattle, or both from the United Kingdom may have played a major role in the incidence of vCJD cases in other countries (*10*), indigenous BSE from before 2001 or another unidentified source may have caused some vCJD infections.

To determine whether vCJD cases in different countries have been caused by the same infectious agent, samples from 4 vCJD case-patients from the Netherlands, Italy, France, and the United States were made available for strain typing analysis using a standard panel of wild-type mouse lines. Each sample was methionine homozygous at codon 129 of the prion gene (Table). None of the patients had received blood or organ donations. Two patients (from Italy and the United States) were treated with quinacrine. All case-patients showed classic clinical characteristics of vCJD; postmortem examination confirmed the diagnosis. We conducted transmission studies in mice using brain samples from these 4 vCJD case-patients and compared transmission characteristics of all 4 cases to those of 2 historical cases of vCJD in the United Kingdom.

Materials and Methods

CJD Inocula

Frozen brain tissue consisting of ≈3 g of frontal cortex from each of 4 vCJD case-patients originating from the Netherlands, Italy, France, and the United States was available for transmission studies (Table). An additional similar sample of cerebellum from the vCJD case-patient from Italy was also studied to assess any differences in transmission characteristics between different regions of the brain. Tissue samples were homogenized at 10% (wt/vol) concentration in sterile physiologic saline and stored at –20°C until use. Ethical consent for the use of these materials for research was obtained and approved by the Lothian National Health Service Board Research Ethics Committee (reference: 2000/4/157).

Experimental Animals

Two lines of mice expressing *Prn-p*^{a} (RIII, C57BL) and 1 line expressing *Prn-p*^{b} (VM) were used for the transmission experiments. The *Prn-p*^{a} and *Prn-p*^{b} alleles have a major influence on the incubation period of disease, with each TSE strain having a distinct and reproducible incubation period ranking with each of the *Prn-p* genotypes (*11*). Mice were anesthetized with halothane and inoculated with brain homogenate by a combination of intracerebral (i.c.) (0.02 mL) and intraperitoneal (0.1 mL) routes to ensure efficient uptake of the agent. Because the quantity of material available was limited, mice received only i.c. inoculations (0.02 mL) from the case-patient from the United States.

Mice were scored weekly for signs of clinical neurologic disease from 100 days as described by

Table. Demographic and clinical features of case-patients with variant CJD from the Netherlands, France, Italy, and United States and 2 reference case-patients from the United Kingdom*

Characteristic	The Netherlands	France	Italy	United States	United Kingdom 1	United Kingdom 2
Case-patient sex	F	F	F	F	M	M
Case-patient age at illness onset, y	24	36	25	22	24	35
Case-patient age at death, y	26	37	27	24	25	36
Disease duration, mo	19	14	27	32	14	12
Early psychiatric symptoms	Yes	Yes	Yes	Yes	Yes	Yes
Persistent painful sensory symptoms	Yes	No	Yes	No	Yes	No
Ataxia	Yes	Yes	Yes	Yes	Yes	Yes
Myoclonus, dystonia, or chorea	Yes	Yes	Yes	Yes	Yes	Yes
Dementia	Yes	Yes	Yes	Yes	Yes	Yes
No typical appearance of sporadic CJD on EEG	Yes	Yes	No	Yes	Yes	Yes
Bilateral symmetric pulvinar high signal on MRI scan of brain	Yes	No	Yes	Yes	Yes	No
Positive tonsil biopsy result	ND	Yes	Yes	Yes	ND	ND
Treatment	No specific treatment	No specific treatment	Quinacrine	Quinacrine	No specific treatment	No specific treatment
History of travel to or residence in the United Kingdom	No	No	No	Yes†	Yes	Yes
Codon 129MM	Yes	Yes	Yes	Yes	Yes	Yes
Type 2B PrP	Yes	Yes	Yes	Yes	Yes	Yes

*CJD, Creutzfeldt-Jakob disease; EEG, electroencephalogram; MRI, magnetic resonance imaging; ND, not done; PrP, prion protein.
†Born in United Kingdom in 1979, moved to United States in 1992.

Fraser and Dickinson (*12*). Mice were killed by cervical dislocation whether due to TSE or other nonspecific disease and the brain removed at postmortem. Brains were cut sagitally; half was snap-frozen for biochemical analysis, and the remaining half was fixed in 10% formol saline for histologic analysis. Experiments were approved by The Roslin Institute's Ethical Review committee and were conducted according to the regulations of the UK Home Office Animals (Scientific Procedures) Act 1986.

Scoring of Vacuolation

After fixation, mouse brains were treated for 1.5 hours in 98% formic acid to reduce the infective titer for safety reasons. Brains were then cut transversely into 4 sections (hind brain, midbrain, forebrain, and hippocampus/thalamus) and embedded in paraffin. Samples underwent hematoxylin and eosin staining, and each mouse was scored for degree of vacuolation (ranked 1–5, with 1 the least affected) found in 9 standard gray matter regions and 3 white matter regions. Scores for >6 mice were averaged to produce a mean lesion profile for each of these areas (*12*).

PrP Immunohistochemical Analysis

Paraffin-embedded tissue sections were pretreated to aid antigen retrieval by autoclaving for 10 min at 121°C. Sections were then immersed in formic acid for 10 min to enhance PrP labeling. PrP immunostaining was performed by using the monoclonal anti-PrP antibody 6H4 (Prionics, Schlieren-Zurich, Switzerland) at a dilution of 1:10,000 overnight at room temperature. The secondary antibody was biotinylated rabbit anti-mouse (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) used at 1:400 for 1 h. The Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) was used, and visualization of antibody binding was through deposition of 3,3′-diaminobenzidine chromogen.

Western Blot Analysis of PrP^{Sc}

Frozen brain samples were homogenized in 0.9% saline solution to yield a 10% suspension. This material was cleared by centrifugation and the supernatant treated with 50 μg/mL proteinase K for 1 h at 37°C (for detailed methods, see Head et al. [*13*]). The digested product was denatured and then loaded onto a 10% Bis/Tris NuPAGE Novex gel (Invitrogen, Paisley, UK). After electrophoresis, the gel was blotted onto polyvinylidene fluoride membranes. Detection of prion protein used the enhanced chemiluminescence ECL+ technique (Amersham Biosciences, Little Chalfont, UK) with primary antibody 6H4 at 1:30,000 and an anti-mouse IgG peroxidase-linked secondary antibody (Amersham Biosciences) at 1:60,000. Images were captured on radiographic film.

Results

All 5 vCJD brain isolates transmitted successfully to the wild-type mouse panel with the appearance of clinical and pathologic signs associated with prion disease. Mean incubation periods and mouse line ranking order were calculated and compared with reference data from the United Kingdom. The inocula from the Netherlands, Italy (cortex and cerebellum), and the United States showed variation in the temporal order of appearance of clinical symptoms in each of the mouse strains when compared with the UK reference cases (Figure 1). These 3 brain inocula showed the shortest incubation periods in the RIII mice, occurring at ≈365 days postinoculation (dpi), followed by VM at ≈450 dpi; the longest was found in the C57BL mice (≈465 dpi). The ranking of incubation time differed with brain inocula from France, with the C57BL mice dying of disease before the VM mouse line, similar to the pattern observed in the UK reference cases. The exact timing of the appearance of clinical symptoms varied, with wide ranges observed for each line of mouse within and between the different inocula; incubation periods were 301–463 days in RIII mice, 361–553 days in VM mice, and 335–553 days in C57BL mice.

Lesion Profiles

Sufficient numbers of wild-type mice scored positive for the presence of TSE-associated vacuolation to enable lesion profiles to be generated from their mean scores. Similar patterns of vacuolation distribution were seen for all vCJD isolates, including those taken from either the cortex or cerebellum (case-patient from Italy) (Figure 2). Mouse strains of the same PrP genotype showed close similarities in lesion profiles with *Prn-p*^{a} mice (RIII and C57BL), showing moderate gray matter vacuolation of the medulla, hypothalamus, and septum, with C57BL mice additionally exhibiting mild white matter vacuolation of the basal cerebellar peduncle. *Prn-p*^{b} mice (VM) showed mild to moderate gray matter vacuolation of the medulla, superior colliculus, thalamus, and septum. Some variability was found in the intensity of vacuolation dependent on the vCJD isolate, most notably in the VM mice and particularly in the superior colliculus, hypothalamus, and thalamic regions.

Immunohistochemical Analysis of PrP

A total of 9 mice per inoculum, corresponding to 3 animals per genotype, were analyzed in a blinded experiment design. Animals from each group showed variability in the amount of PrP accumulation (Figure 3). Fine punctate deposits were the most consistently observed pattern of PrP accumulation in mice from all genotypes. However, in numerous instances, PrP plaques were also seen in the corpus callosum and subventricular area and, eventually, in

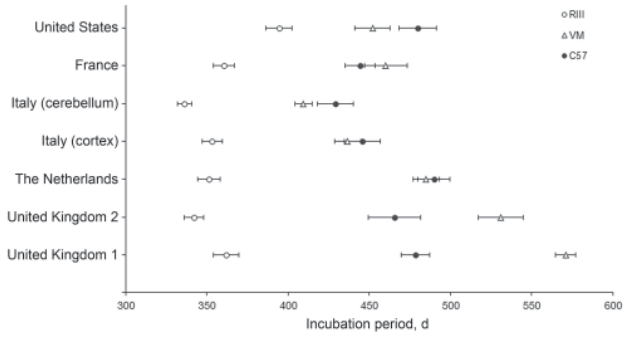

Figure 1. Comparison of variant Creutzfeldt-Jakob disease incubation periods from 5 sources in wild-type mice. Data show mean incubation period ± SEM. i.c., intracerebral; i.p., intraperitoneal; UK, United Kingdom.

the brain parenchyma of the cerebrum and cerebellum. VM mice showed a similar pattern of PrP deposition with each inoculum and consistently showed PrP plaques. RIII mice appeared to show less staining with the accumulation of PrP forming fine punctate, coarse, pericellular, and plaque deposits. In the C57BL line, PrP deposition appeared more variable within and between inocula.

Biochemical Analysis of PrP

Biochemical analysis of PrP^{Sc} by Western blot produced a type 2B–like gel mobility and glycosylation profile in all mouse strains (Figure 4). This profile is characterized by the predominance of the diglycosylated form of PrP^{Sc} and an ≈19-kDa unglycosylated fragment, similar to that seen in all vCJD human brain tissue examined to date. The unglycosylated fragment appeared as a doublet with a distinct upper and lower band and the relative intensity of the 2 bands varying with mouse *Prn-p* genotype; the lower band appeared more intense in *Prn-p*^{a} mice and the higher band more intense in *Prn-p*^{b} mice. The biochemical profile was identical for all 5 brain isolates and identical to the pattern observed in the UK cases.

Discussion

We used transmission studies with wild-type mice to define the strain characteristics of geographically distinct cases of vCJD to establish whether a common strain of agent is responsible for vCJD cases from 5 different countries. Transmission properties such as TSE-associated vacuolation, PrP^{Sc} deposition, glycosylation profile, and mobility of PrP all show strong similarities between vCJD cases from the Netherlands, Italy, France, and the United States and with reference cases from the United Kingdom.

The distribution of TSE vacuolation and PrP^{Sc} deposition in the brains of the RIII and VM mice was similar for each brain isolate examined and indicates that the same strain of agent is present in each inoculate. C57BL mice showed more variability in PrP^{Sc} deposition, which may be a result of the transmission of the vCJD agent across a species barrier. Each brain isolate produced a type 2B–like glycoform profile on biochemical analysis; similar to results from previous studies, the unglycosylated fragment appeared as a doublet (*14*).

We observed differences in the timing of the onset of clinical signs of disease for each of the non-UK cases, which may be because of differences in the infectious titer of the different vCJD isolates. We also identified a change in the

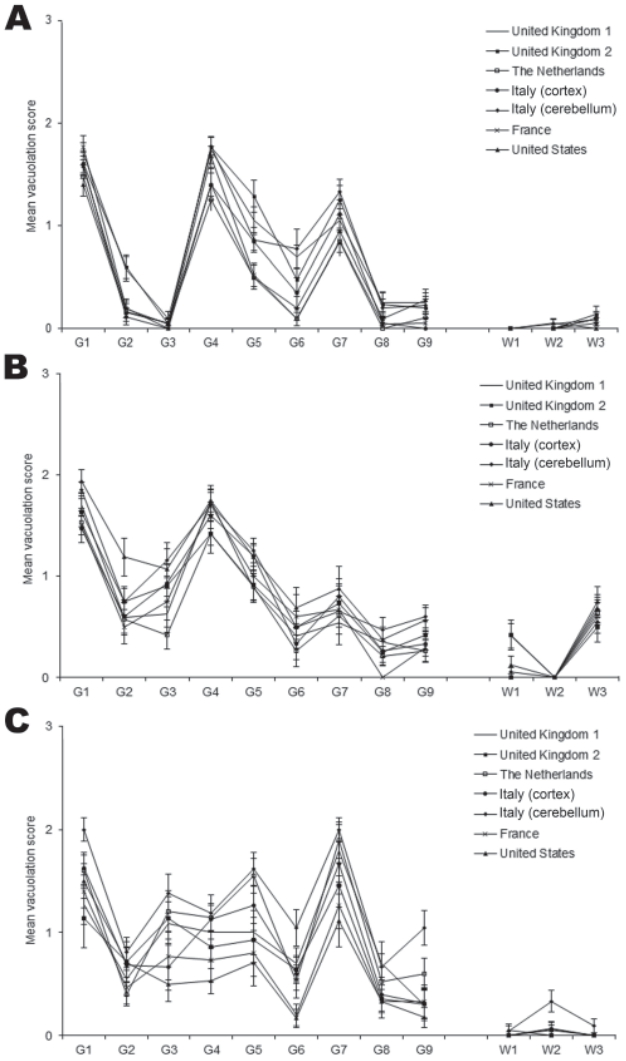

Figure 2. Lesion profile comparison of variant Creutzfeldt-Jakob disease cases show similarities in vacuolar pathology levels and regional distribution in mouse brains. Wild-type mouse lines RIII (A), C57 (B), and VM (C) are shown. Data show mean lesion profile ± SEM (n>6). G1–G9, gray matter scoring regions: G1, dorsal medulla; G2, cerebellar cortex; G3, superior colliculus; G4, hypothalamus; G5, thalamus; G6, hippocampus; G7, septum; G8, retrosplenial and adjacent motor cortex; G9, cingulate and adjacent motor cortex. W1–W3, white matter scoring regions: W1, cerebellar white matter; W2, mesencephalic tegmentum; W3, pyramidal tract.

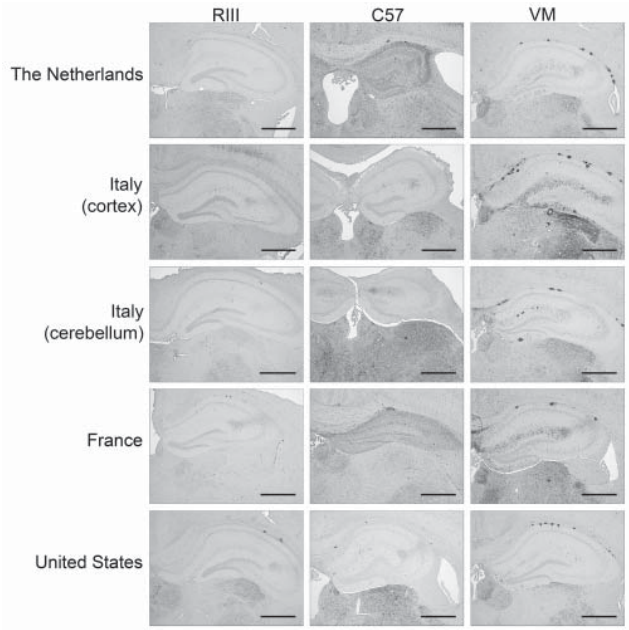

Figure 3. Immunohistochemical detection of abnormal prion protein (PrP^{Sc}) in the hippocampus and thalamus of RIII, C57, and VM wild-type mice after inoculation with variant Creutzfeldt-Jakob disease brain tissue. Scale bars = 500 µm. The anti–prion protein detection antibody used was 6H4.

incubation period ranking of the mouse strains between the inoculum from France and other locations (Figure 1). For each brain isolate, the RIII mice were the first line to die of disease, consistent with previous transmissions from vCJD cases in the United Kingdom.

Furthermore, our study has shown that the ≈100-day difference in incubation period between the RIII and C57BL mice (both *Prn-p*^{a}), which is characteristic of the BSE strain, has been maintained in the experimental transmission of the vCJD worldwide cases (*15*). The C57BL and VM mice have resulted in close incubation period ranges that can overlap, as was demonstrated previously (*14*,*15*). For inocula from the non-UK cases, incubation periods appeared in the order RIII, VM, C57BL, whereas the inocula from France showed incubation periods in the order RIII, C57BL, VM, as do the historic UK cases. This change in the order could be attributed to genetic drift in the mouse lines used over the course of the historical and current studies or variations in the strain of TSE agent.

The VM mice used in this study have shown substantially shortened incubation periods compared with those from in earlier studies, which may have caused the alteration in ranking. This alteration in incubation time ranking was also identified recently in a comparable transmission study involving a more recent UK vCJD case (M. Bishop et al., unpub. data). Moreover, the difference in time ranking is unlikely to be attributable to variation in brain area; we have established similar transmission characteristics between brain regions and have aimed in this study to use identical brain regions for each non-UK case. Differences in inoculation route are also not an explanation for the alteration in ranking. Differences were not found between the case from the United States, which was inoculated i.c. only, and the cases from the Netherlands and Italy, which were inoculated i.c. and intraperitoneally.

The infectivity titer of the inoculum may have played a role in alteration of the incubation periods. However, Ritchie et al. (*14*) suggested that changes in titer would affect all mouse strains and would not result in a change in the ranking of the mouse strains. Experimental transmission of vCJD from the case from the United States, in which lower volumes were inoculated, shows the same temporal pattern as the cases from the Netherlands and Italy.

Variability in incubation time is often associated with the primary transmission of a TSE agent between species but often stabilizes on subsequent mouse-to-mouse passage, while variability within groups decreases. Therefore, variations observed in this study do not necessarily point to strain variation between the cases of vCJD. However, to rule out this possibility, further transmission studies will be conducted. Moreover, passage of cases of vCJD occurring during the past 15 years will be undertaken in a comparative study to establish whether genetic drift in mouse lines or strain variation in vCJD underlies the differences observed in this study. Two of the brain isolates

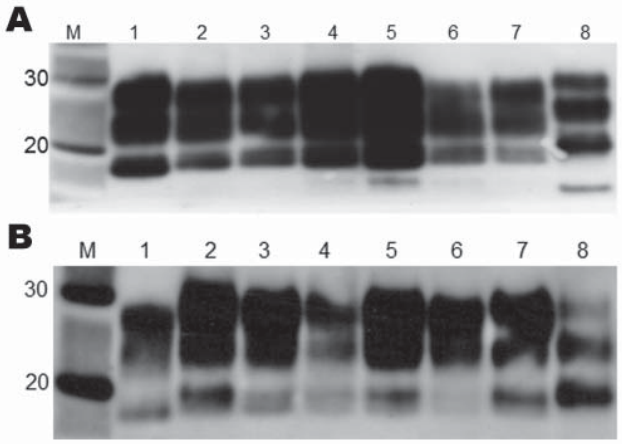

Figure 4. Western blot analysis of brain extracts from RIII (A) and VM (B) wild-type mice inoculated with variant Creutzfeldt-Jakob disease (vCJD) brain tissue. Lane M, positive control; lane 1, human vCJD brain homogenate (UK origin) showing the typical abnormal prion protein (PrP^{Sc}) type 2B; lane 2, United Kingdom; lane 3, The Netherlands; lane 4: Italy (cortex); lane 5, Italy (cerebellum); lane 6, France; lane 7, United States; lane 8, human sporadic Creutzfeldt-Jakob disease brain homogenate showing the typical PrP^{Sc} type 1. Type 2B and 1 differ in mobility of the unglycosylated band (≈19 kDa and ≈20 kDa, respectively). All samples were treated with proteinase K. The anti–prion protein detection antibody used was 6H4.

inoculated (from Italy and the United States) were from patients who had been treated with quinacrine. However, this treatment appears to have had no effect on incubation periods, vacuolation profiles, PrP^{Sc} deposition patterns, or the glycosylation and mobility of PrP.

The similarities in lesion profiles, biochemistry, and immunohistochemistry between this series of vCJD transmission studies support the hypothesis that a single strain of infectious agent is responsible for all vCJD cases, regardless of geographic origin, which would suggest that current diagnostic criteria for vCJD are sufficient to detect cases in all countries at this time. Still to be determined is whether the differences in incubation period rankings in some cases represent changes in strain phenotype over time, which could affect future diagnosis.

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WU and KI Polyomaviruses in Respiratory Samples from Allogeneic Hematopoietic Cell Transplant Recipients

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Data are limited regarding 2 new human polyomaviruses, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), in immunocompromised patients. We used real-time PCR to test for these and 12 respiratory viruses in 2,732 nasal wash samples collected during the first year after allogeneic hematopoietic cell transplantation from 222 patients. Specimens were collected weekly until day 100; then at least every 3 months. One year after hematopoietic cell transplantation, the cumulative incidence estimate was 26% for KIPyV and 8% for WUPyV. Age <20 years predicted detection of KIPyV (hazard ratio [HR] 4.6) and WUPyV (HR 4.4), and detection of a respiratory virus in the previous 2 weeks predicted KIPyV detection (HR 3.4). Sputum production and wheezing were associated with detection of KIPyV in the past week and WUPyV in the past month. There were no associations with polyomavirus detection and acute graft versus host disease, cytomegalovirus reactivation, neutropenia, lymphopenia, hospitalization, or death.

In 2007, two new human polyomaviruses, KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV), were identified in respiratory specimens from patients with respiratory illness (*1*,*2*). Since then, KIPyV and WUPyV have been frequently detected in respiratory specimens, especially those from children with respiratory symptoms and from patients co-infected with a respiratory virus (*3*–*11*). However, KIPyV and WUPyV were detected at similar rates in specimens from symptomatic patients and in persons without respiratory symptoms (*6*,*8*,*12*,*13*), suggesting that these viruses might not cause respiratory illness in immunocompetent children.

Two other human polyomaviruses, BK and JC, cause mild or asymptomatic primary infections early in life, followed by persistent, subclinical infections in healthy persons (*14*–*16*). However, these viruses can reactivate, primarily from the kidney, bone marrow, and lymphoid tissue, and cause serious disease in immunocompromised patients (*14*–*16*). Similarly, reactivation of KIPyV and WUPyV from lymphoid tissue was described among immunosuppressed persons with AIDS, although clinical consequences of reactivation were not examined (*17*).

Because KIPyV and WUPyV are frequently detected in association with respiratory symptoms, inhalation is suspected as a potential route of transmission. If KIPyV and WUPyV are respiratory pathogens, they may be more likely to cause respiratory illness in immunocompromised persons, either during primary infection or reactivation. Few, mostly retrospective, studies on the prevalence of KIPyV and WUPyV in respiratory specimens have included large numbers of immunocompromised patients (*18*–*21*). No prospective data are available that comprehensively describe the incidence, symptoms, risk factors, and outcomes associated with detection of KIPyV and WUPyV in respiratory specimens from hematopoietic cell transplant (HCT) recipients. This question is particularly relevant with the increasing use of multiplex PCR panels for detection of re-

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spiratory viruses, especially in samples from immunocompromised patients.

To investigate whether respiratory detection of these new polyomaviruses is associated with specific outcomes in patients after HCT, a real-time PCR specific for KIPyV and WUPyV DNA was developed and used to examine nasal wash specimens collected prospectively from HCT recipients with and without respiratory symptoms for 1 year after transplantation. Clinical data and standardized symptom surveys obtained at each specimen collection were analyzed to determine associations between respiratory KIPyV and WUPyV detection and illness.

Methods

Patients and Collection of Specimens

Combined nasopharyngeal wash (or swab) and oropharyngeal swab samples were collected weekly beginning 1–2 weeks before transplantation until day 100; then every 1–3 months for ≤1-year after transplantation from allogeneic HCT recipients enrolled in a prospective surveillance study approved by the Institutional Review Board at Fred Hutchinson Cancer Research Center (Seattle, WA, USA) (*22*). Participants provided written informed consent. Additional specimens were collected when respiratory symptoms were reported. Patients had ≥1 specimen collected during January 2006–December 2007. Participants completed surveys weekly for 1 year and reported any of 11 respiratory or 4 systemic symptoms. Clinical and laboratory data were obtained from medical records.

Detection of KIPyV and WUPyV DNA and PCR Validation

An in-house, duplex, real-time TaqMan PCR was developed, which was specific for viral protein 2–3 and viral protein 1 genes of KIPyV and WUPyV, respectively. Ten microliters of extracted sample were added to the PCR master mixture containing KIPyV and WUPyV primers and probes (Table 1). Samples with PCR cycle threshold values >40 were considered negative. Viral copies per milliliter were determined by using standard curves generated by PCR amplification of 10-fold dilutions of plasmids containing amplicon sequences ranging in concentration from 10 to 1 $\times 10^7$ copies/reaction. To validate this PCR, a subset of KIPyV-positive, WUPyV-positive, KIPyV-negative, and WUPyV-negative samples was blindly retested by using published assays for detection of KIPyV (*23*) and WUPyV (*4*).

The in-house PCR had a sensitivity of 5–10 DNA copies/PCR, which provided a sensitivity of 500–1,000 copies/mL. This PCR did not detect JC or BK virus DNA. A subset of 397 samples, including 31 positive for KIPyV and 27 positive for WUPyV by the PCR, was retested by using published real-time PCRs. Samples with discordant KIPyV results included 3 positive by the in-house PCR and negative by the alternate PCR (*23*) and 1 negative by the in-house PCR and positive by the alternate PCR. Samples with discordant WUPyV results included 4 positive by the in-house PCR and negative by the alternate PCR (*4*) and 1 negative by the in-house PCR and positive by the alternate PCR. Samples with discordant results had <5,000 copies/mL, indicating low levels of DNA near the assay limits of detection.

Detection of Respiratory Virus

Samples were tested for 12 respiratory viruses by using a multiplexed panel of real-time, TaqMan reverse transcription PCRs. Assays to detect respiratory syncytial virus; human metapneumovirus; influenza virus A; parainfluenza viruses 1, 2, and 3; adenoviruses; coronaviruses; rhinoviruses; and bocavirus were performed as described (*24–30*). Assays to detect influenza virus B and parainfluenza virus 4 were performed by using the same reagents and thermocycling conditions as the other respiratory virus assays but by substituting the specific primer and probe sets (Table 1). The sensitivity of each assay was 1,000 viral copies/mL. Throughout this report, respiratory virus refers to any of these 12 viruses.

Table 1. Primers and probes used for detecting KIPyV, WUPyV, and 2 other viruses by real-time RT-PCR and PCR in HCT recipients*

Virus	Target gene	Amplicon size, bp	Function	Sequence/label, 5′→3′	PCR concentration, nmol/L
KIPyV	VP2–3	74	Forward primer	CTATCCCTGAATACCAGTTGGAAAC	425
			Reverse primer	GTATGACGCGACAAGGTTGAAG	425
			Probe	FAM-TTCCGGGCATCCCAGACTGGC-BHQ1	125
WUPyV	VP1	75	Forward primer	AACCAGGAAGGTCACCAAGAAG	300
			Reverse primer	TCTACCCCTCCTTTTCTGACTTGT	300
			Probe	HEX-CAACCCACAAGAGTGCAAAGCCTTCC-BHQ1	75
Influenza B	Matrix	76	Forward primer	CACAATTGCCTACCTGCTTTCA	250
			Reverse primer	CCAACAGTGTAATTTTTCTGCTAGTTCT	250
			Probe	VIC-CTTTGCCTTCTCCATCTT-MGBNFQ	100
Parainfluenza type 4	NP	94	Forward primer	TGCCAAATCGGCAATAAACA	250
			Reverse primer	GGCTCTGGCAGCAATCATAAG	250
			Probe	VIC-TGATTCTGCATTGATGTGG-MGBNFQ	100

*KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; RT-PCR, reverse transcription PCR; HCT, hematopoietic cell transplantation; VP, viral protein; NP, nucleoprotein.

Statistical Analysis

The probability of detecting KIPyV or WUPyV DNA in ≥1 nasal wash sample from transplantation to 1-year after transplantation was estimated by using cumulative incidence curves. Patient records were censored at 14 days past the time of last eligible respiratory sample, death, or 1 year after transplantation, whichever occurred first. Death before 1 year and within 14 days after the last sample of a patient was treated as a competing risk for detection.

The association of KIPyV and WUPyV detection with respiratory virus positivity was estimated by using a logistic regression model. Differences in KIPyV and WUPyV copies/mL according to patient age at transplantation and co-occurrence of a respiratory virus were assessed by using linear regression. For both model types, robust standard errors were calculated to account for correlation within repeated measures for the same person.

Cox regression models were fit to evaluate potential risk factors for detection of KIPyV or WUPyV in the first year after transplantation, including age, sex, disease risk (standard or high) (*29*,*31*), stem cell source, donor type, conditioning regimen (myeloablative versus nonmyeloablative), donor and recipient cytomegalovirus (CMV) serostatus, grades 2–4 graft versus host disease (GvHD), and respiratory virus detection. Donor CMV serostatus was defined as negative for cord blood recipients. Acute GvHD and respiratory virus detection were treated as time-dependent covariates; respiratory virus detection was set to 1 when ≥1 respiratory virus had been detected in the previous 2 weeks and 0 otherwise. Cox regression models were fit to evaluate risk factors for KIPyV or WUPyV detection in the first 100 days after transplantation.

CMV reactivation was modeled as a time-dependent indicator defined as any antigenemia or positive PCR value, or antigenemia >10 cells per slide or >100 copies/mL by PCR. Neutropenia was modeled as a time-dependent covariate set to 1 when the absolute neutrophil count was <500 cells/mm^3. When values were missing, the indicator for the previous day was carried forward. Lymphopenia was modeled similarly with 2 thresholds, 100 and 300 cells/mm^3.

We evaluated detection of KIPyV or WUPyV as predictors of clinical outcomes, including diagnosis of grades 2–4 acute GvHD, CMV reactivation, neutropenia, lymphopenia, and hospitalization within 100 days; and viral symptoms, increased liver transaminase and total bilirubin levels, and death within 1 year. For time-to-event outcomes, patient records were censored at date of last contact or death, and KIPyV and WUPyV detection were treated as time-dependent covariates in Cox regression models. Potential confounders for these models included age, sex, donor type, stem cell source, and conditioning regimen.

Outcomes with multiple occurrences over time were analyzed as functions of recent KIPyV or WUPyV detection by using logistic and linear regression models. Virus detection within the last week was defined as a positive sample at the current or last study contact and a lag time ≤14 days. Virus detection within the last month was analyzed for wheezing and cough because these symptoms may persist, and was defined as a positive sample at the current or any of the last 4 study contacts, as long as those contacts occurred within 45 days. Models were adjusted for detection of respiratory virus within the last week, day relative to transplantation, age, conditioning regimen, donor type, and acute GvHD. Measurements based on multiple patient contacts were entered as repeated measures and adjusted for possible correlation between values within a person by using generalized estimating equations.

For regression models, p values were obtained by using the Wald test; no adjustments were made for multiple comparisons. Two-sided p values <0.05 were considered significant. Analyses were performed by using SAS version 9.0 (SAS Institute, Cary, NC, USA).

Results

Patient Characteristics and Detection of KIPyV and WUPyV

A total of 2,732 nasal wash specimens were collected from 222 eligible patients at weekly or longer intervals (median 7 days, range 4–155 days). The cohort had a median of 13 specimens per patient (range 1–30 specimens). Patients ranged in age from 9.6 months to 75.2 years (median 51.5 years) and remained in the study a median of 111 days (range 1–365 days) after HCT. A respiratory virus was detected in 17% of specimens; 51% of patients had ≥1 sample positive for any respiratory virus during their time in the study. Clinical characteristics of the cohort are provided in Table 2.

KIPyV and WUPyV DNA was detected in 203 (7%) specimens from 49 (22%) patients and in 35 (1%) specimens from 15 (7%) patients, respectively. Two patients were positive for both viruses; 1 patient was positive concurrently in the same specimen, and an additional patient was positive for 1 virus in different specimens. Among the 62 WUPyV-positive or KIPyV-positive patients, 1 patient provided only 1 specimen during the study. At 1 year after HCT, cumulative incidence estimates were 26% (95% CI 20%–33%) for KIPyV and 8% (95% CI 4%–12%) for WUPyV (Figure 1). KIPyV and WUPyV were first detected a median of 45 days (range 1–187 days) and 43 days (range 4–46 days) after transplantation, respectively. KIPyV-positive or WUPyV-positive specimens were detected in every month, and there was no apparent seasonality (Figure 2). A respiratory virus was detected in 32% of KIPyV-positive and 14% of WUPyV-positive specimens. Rhinoviruses and

Table 2. Characteristics of 222 HCT recipients tested for KIPyV or WUPyV*

Characteristic	No. (%) recipients		
	All, n = 222	Infected with either virus, n = 62	Not infected, n = 160
Age, y			
<20	23 (10)	18 (29)	5 (3)
20–39	37 (17)	14 (23)	23 (14)
40–59	99 (45)	17 (27)	82 (51)
>60	63 (28)	13 (21)	50 (31)
Sex			
F	87 (39)	23 (37)	64 (40)
M	135 (61)	39 (63)	96 (60)
Underlying disease risk†			
Standard	142 (64)	40 (65)	102 (64)
High	80 (36)	22 (35)	58 (36)
Stem cell source			
Bone marrow	28 (13)	12 (19)	16 (10)
Peripheral blood	179 (81)	47 (76)	132 (83)
Cord blood	15 (7)	3 (5)	12 (8)
CMV serostatus			
D+/R+	56 (25)	17 (27)	39 (24)
D+/R–	81 (36)	22 (35)	59 (37)
D–/R+	16 (7)	4 (6)	12 (8)
D–/R–	69 (31)	19 (31)	50 (31)
Donor match			
Related–matched	77 (35)	23 (37)	54 (34)
Related–mismatched	9 (4)	6 (10)	3 (2)
Unrelated–cord blood	15 (7)	3 (5)	12 (8)
Unrelated–matched	97 (44)	24 (39)	73 (46)
Unrelated–mismatched	24 (11)	6 (10)	18 (11)
Conditioning regimen			
Myeloablative	128 (58)	41 (66)	87 (54)
Nonmyeloablative	94 (42)	21 (34)	73 (46)
Acute graft-versus-host disease			
Grade 0 or 1	78 (35)	30 (48)	48 (30)
Grade 2–4	144 (65)	32 (52)	112 (70)

*HCT, hematopoietic cell transplantation; KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; CMV, cytomegalovirus; D, donor; R, recipient.
†The underlying disease stage of a patient was categorized as low, intermediate, or high risk (*29*,*31*).

coronaviruses accounted for 78% of respiratory virus co-detections. Respiratory and KIPyV viruses tended to co-occur (odds ratio [OR] 2.4, 95% CI 1.2–5.1, p = 0.02).

Twenty-one (43%) KIPyV-positive and 9 (60%) WUPyV-positive patients had 1 positive specimen. Positive episodes with detection in ≥4 consecutive specimens were seen in 18 (37%) KIPyV-positive patients, including 6 with 9–19 consistently positive specimens (Figure 3, panel A) and in 2 (13%) WUPyV-positive patients, including 1 with 11 consecutive specimens (Figure 3, panel B). The maximum number ($\log_{10}$ copies/mL) of KIPyV and WUPyV virus per positive episode ranged from 2.55 to 10.58 (median 5.31) and 2.57–9.02 (median 3.08), respectively. Patients with >1 KIPyV-positive specimen had an average of >3 logs higher maximum viral $\log_{10}$ copies/mL (median 6.56) than KIPyV-positive patients with 1 positive specimen (median 2.86, p<0.001).

If we considered all positive samples, the number of KIPyV copies/mL was significantly higher in specimens positive for KIPyV and a respiratory virus than in specimens in which KIPyV was the only virus detected (medians 6.35 vs. 4.25, respectively, p<0.001). In contrast, WUPyV-positive specimens with a respiratory virus co-pathogen had significantly lower viral copy numbers than specimens positive for WUPyV virus alone (medians 2.84 vs. 3.62, respectively, p = 0.01). Patient age at transplantation was not correlated with KIPyV or WUPyV copies/mL.

Risk Factors for Detection of KIPyV and WUPyV

Time in study and number of specimens collected did not differ among patients according to polyomavirus detection. Age <20 years was a significant predictor of KIPyV and WUPyV detection in multivariable models (hazard ratio [HR] 4.6, 95% CI 2.5–8.6, p<0.001; HR 4.4, 95% CI 1.5–12.8, p = 0.007; respectively). All 10 patients <12 years of age and 18 (78%) of 23 patients <20 years of age were positive for KIPyV or WUPyV compared with 44 (22%) of 199 patients ≥20 years of age (Figure 4). Detection of a respiratory virus within the last 2 weeks was a significant predictor of KIPyV detection (HR 3.4, 95% CI 1.8–6.4, p<0.001) in a model adjusted for age, transplantation type, and donor type. CMV reactivation, neutropenia, and lymphopenia were not associated with detection of KIPyV and WUPyV within the first 100 days after transplantation.

Associations between KIPyV or WUPyV Detection and Symptoms

Detection of KIPyV within the past week was significantly associated with sputum production (OR 1.7, 95% CI 1.0–2.9, p = 0.04) (Table 3). WUPyV detection within the past month was significantly associated with wheezing (OR 3.1, 95% CI 1.2–8.1, p = 0.02). Limiting the analysis to 20 patients with high levels of KIPyV detection within the past week (>5 $\log_{10}$ copies/mL) showed a significant

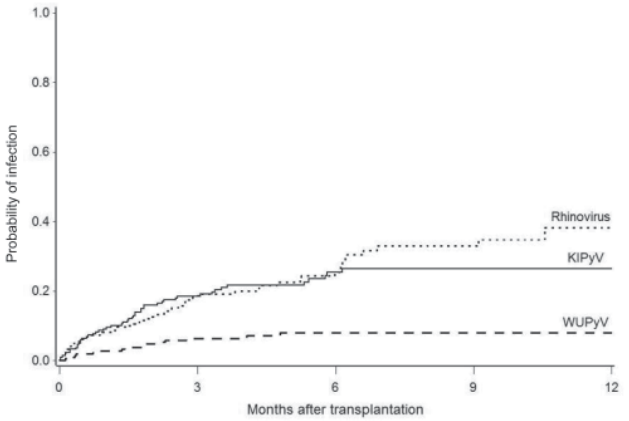

Figure 1. Cumulative incidence of KI polyomavirus (KIPyV) and WU polyomavirus (WUPyV) detection after transplantation in 222 hematopoietic cell transplantation recipients. Cumulative incidence of human rhinovirus is shown for comparison.

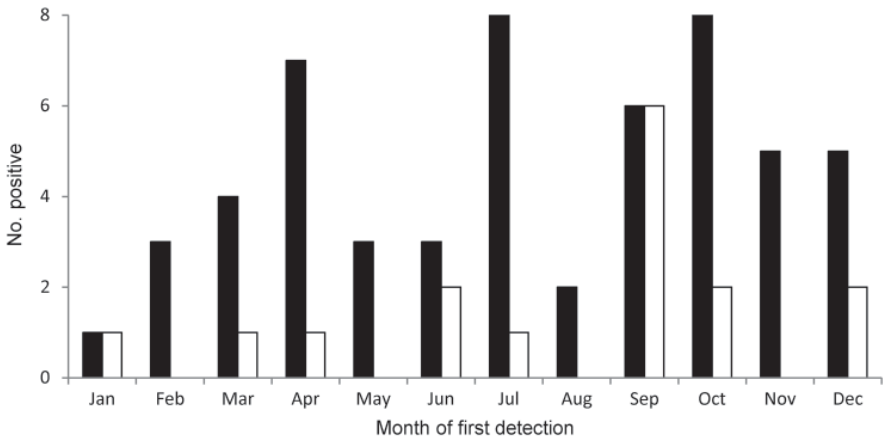

Figure 2. Number of samples positive for KI polyomavirus (black bars) and WU polyomavirus (white bars) by month of first detection in hematopoietic cell transplantation recipients.

association with sputum production (OR 2.0, 95% CI 1.1–3.8, p = 0.03). Analysis of symptoms as a function of KIPyV positivity among 17 patients with a respiratory virus detected within the past week showed a similar association between sputum production and KIPyV detection (OR 2.7, 95% CI 1.1–6.6, p = 0.03).

Associations between KIPyV or WUPyV Detection and Clinical Outcomes

Longitudinal analyses showed significant relationships between WUPyV detection and a lower risk for lymphopenia defined by <300 cells/mm^3 (OR 0.3, 95% CI 0.1–0.6, p = 0.001) and grades 2–4 acute GvHD (HR 3.1, 95% CI 1.3–7.7, p = 0.01) in a model adjusted for donor type and stem cell source among 136, 27, and 6 patients given diagnoses of grades 2, 3, and 4 acute GvHD, respectively. However, this association was based on few cases; only 5 patients had WUPyV detected before the GvHD diagnosis. No relationships between detection of KIPyV or WUPyV and CMV reactivation, risk for hospitalization ≤100 days after transplantation, and mean values for alanine aminotransferase, aspartate aminotransferase, and total bilirubin levels were found in multivariable models.

Nineteen bronchoalveolar lavage (BAL) samples from 13 KIPyV-positive patients and 57 BAL samples from 37 KIPyV-negative and WUPyV-negative patients were tested for KIPyV and WUPyV. Collection of BAL samples occurred within 2 weeks of a positive nasal wash sample in only 4 of the KIPyV-positive patients, including 3 with collection on the same day. Only 1 of 76 BAL samples was positive for KIPyV (6.3 $\log_{10}$ copies/mL). This sample was collected from a 2-year-old child on the same day as the KIPyV-positive nasal wash sample (165 days after transplantation); CMV was also isolated in culture and the patient received treatment for CMV pneumonia. Of 66 deaths within 1 year of transplantation, 12 occurred in patients positive for KIPyV, 4 in patients positive for WUPyV, and 1 in a patient positive for KIPyV and WUPyV. Neither virus was associated with the 1-year mortality rate in in an adjusted model.

Discussion

Our large longitudinal surveillance study provides a rigorous evaluation of KIPyV and WUPyV detection in upper respiratory tract specimens from HCT recipients. One-year cumulative incidence estimates were high, 26% and 8% for KIPyV and WUPyV, respectively, and there were prolonged episodes of detection for ≥4 weeks in 37% of patients positive for KIPyV and 13% of patients positive for WUPyV. These numbers are comparable to those of our report of rhinoviruses and coronaviruses, the most common respiratory virus types detected in the same patient population, in which we found day 100 estimates of 22% and 11%, respectively, and detection for ≥1 month in 44% and 37%, respectively (*29*). In comparison, cross-sectional studies testing specimens from immunocompetent children with acute respiratory tract illnesses found the prevalence of KIPyV and WUPyV ranged from 0% to 2.8% and from 2% to 7.1% (*1–13*,*21*).

A recent study of pediatric hematology/oncology patients and immunocompetent persons found a higher KIPyV mean viral load in respiratory tract specimens from the immunocompromised group, suggesting potential for increased pathogenicity in this population (*21*). A study in adult HCT recipients tested sequential nasopharyngeal aspirates from 31 asymptomatic patients, in which KIPyV and WUPyV were detected in 1 each of 126 samples (*19*). A cross-sectional study that tested specimens from 45 HCT recipients with respiratory illness detected KIPyV in 8 (17.8%), similar to our findings, but with no WUPyV detected (*19*,*20*). Respiratory viruses were often co-detected in our cohort, 32% with KIPyV and 14% with WUPyV, consistent with high rates of co-infection reported by others (*1–3*,*6*,*10*,*21*,*32*).

In our study, young age was a risk factor for detection of KIPyV or WUPyV, which has been reported (*4*,*7*,*32*,*33*).

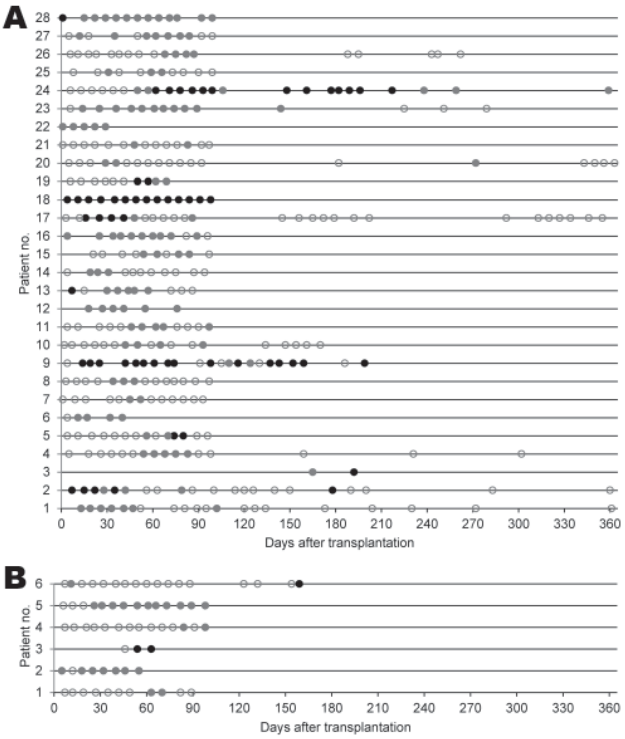

Figure 3. Detection of A) KI polyomavirus (KIPyV) DNA in 28 hematopoietic cell transplantation (HCT) recipients with ≥2 KIPyV-positive specimens and B) WU polyomavirus (WUPyV) DNA in 6 HCT recipients with ≥2 WUPyV-positive specimens, with and without detection of a respiratory virus by day after transplantation. Each line represents 1 patient in order of age (KIPyV-positive patients 1–10 and WUPyV-positive patients 1 and 2 are <20 years of age). Circles indicate specimen collection. Gray indicates detection of only KIPyV or WUPyV, black indicates detection of KIPyV or WUPyV and a respiratory virus, and white indicates negative results for KIPyV or WUPyV.

The seroprevalence of antibodies to KIPyV and WUPyV for children 5–20 years of age was similar to that for adults (*34*,*35*), suggesting that primary exposure occurs in childhood. We also found that detection of a respiratory virus within the previous 2 weeks was an independent risk factor for detection of KIPyV. Compared with samples in which only KIPyV DNA was detected, higher viral copy numbers of KIPyV were detected in samples also positive for a respiratory virus, perhaps because of stimulation of viral replication during a respiratory virus infection. Our current data do not provide evidence that this is a biologically meaningful difference.

Prolonged detection and younger age associated with a higher prevalence of KIPyV and WUPyV were also reported in a longitudinal study of KIPyV and WUPyV in children (*33*). As with BK and JC polyomaviruses, KIPyV and WUPyV might persist for life after primary infection in undetermined sites and become reactivated during periods of immune suppression. Prolonged detection episodes may represent long-term viral shedding after new acquisition of virus, especially in young patients, or reactivation or stimulation of replication of persistent virus in the respiratory tract because of immune suppression or a recent or concomitant respiratory virus infection. A site of persistence for these new polyomaviruses has not yet been identified, although human tonsils have been postulated as a reservoir and reactivation has been demonstrated in lymph nodes and spleen (*17*,*36*,*37*).

The longitudinal design of our study and specimen collection during symptomatic and asymptomatic periods enabled us to thoroughly evaluate symptoms associated with KIPyV and WUPyV detection in HCT recipients. We found that KIPyV detection in the past week was associated with sputum production and WUPyV detection in the past month was associated with wheezing. Limiting the analysis to patients with high viral copy numbers of KIPyV in their nasal wash sample did not provide additional associations. Likewise, co-detection of KIPyV and a respiratory virus was not associated with symptoms other than sputum production, suggesting that infection with KIPyV did not exacerbate symptoms caused by the respiratory virus. Of 50 patients who underwent bronchoscopy for workup of possible lower respiratory illness and had a BAL specimen available for testing, we detected KIPyV in 1 sample from a child who also had CMV pneumonia. Overall, our analyses provide some support for these viruses as respiratory pathogens. However, we did not observe severe respiratory illness or death that could be conclusively attributed to polyomavirus detection.

In seeking other biologically plausible and clinically relevant associations for these new viruses, we found no association of KIPyV and WUPyV detection in nasal wash-

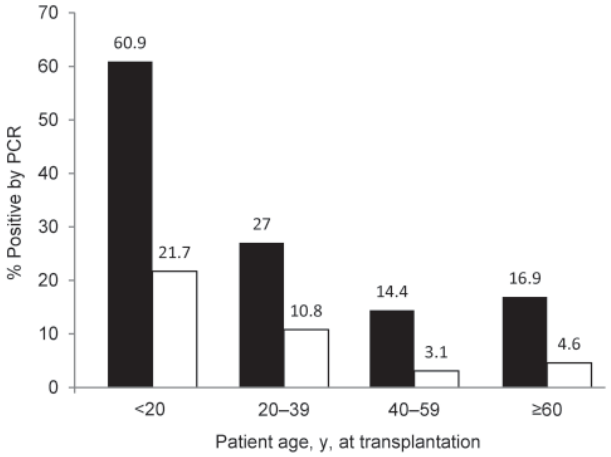

Figure 4. Proportion of hematopoietic cell transplantation recipients in each age group with samples positive for KI polyomavirus (black bars) and WU polyomavirus (white bars). Values above bars are percentages.

Table 3. Respiratory and systemic symptoms associated with KIPyV or WUPyV detection within the prior week or month in HCT recipients*

	KIPyV†		WUPyV†	
Symptom	OR (95% CI)	p value	OR (95% CI)	p value
Within prior week				
Runny nose	1.3 (0.7–2.4)	0.39	1.2 (0.5–3.2)	0.66
Sinus congestion	0.8 (0.5–1.3)	0.34	1.4 (0.6–3.5)	0.46
Postnasal drip	0.8 (0.4–1.8)	0.65	1.2 (0.5–3.0)	0.72
Shortness of breath	0.9 (0.4–1.8)	0.73	0.9 (0.3–2.9)	0.83
Sputum production	1.7 (1.0–2.9)	0.04	1.5 (0.5–4.8)	0.47
Pharyngitis	1.0 (0.6–1.7)	0.99	2.1 (0.8–5.4)	0.12
Sneezing	1.2 (0.7–2.0)	0.54	1.3 (0.6–2.9)	0.48
Watery eyes	1.5 (0.8–2.6)	0.17	1.6 (0.4–6.2)	0.51
Cough	1.1 (0.6–2.0)	0.68	1.4 (0.6–3.5)	0.47
Wheezing	1.1 (0.5–2.5)	0.82	2.1 (0.8–5.9)	0.15
Fever	0.9 (0.5–1.5)	0.59	1.3 (0.4–3.7)	0.64
Headache	0.6 (0.4–1.1)	0.08	0.7 (0.2–2.8)	0.58
Myalgia	0.6 (0.3–1.1)	0.08	1.1 (0.3–3.5)	0.92
Diarrhea	0.8 (0.5–1.3)	0.40	0.7 (0.3–1.8)	0.47
Within prior month				
Cough	1.0 (0.6–1.8)	0.91	1.6 (0.7–3.5)	0.26
Wheezing	1.0 (0.5–2.2)	0.99	3.1 (1.2–8.1)	0.02

*Values are for 211 of 222 patients with symptom survey data. Ear pain was too rare for analysis. KIPyV, KI polyomavirus; WUPyV, WU polyomavirus; HCT, hematopoietic cell transplantation. OR, odds ratio.
†Each result was adjusted for the following covariates: detection of the other polyomavirus (KIPyV or WUPyV), detection of a respiratory virus, day relative to transplantation, age at transplantation, stem cell source, donor type, and grades 2–4 acute graft-versus-host-disease.

es with CMV reactivation, increased liver enzyme levels, hospitalization, or neutropenia in the first 100 days after transplantation. Detection of WUPyV was associated with a lower risk for lymphopenia.

We did not test other samples, such as blood, urine, or feces, for KIPyV and WUPyV DNA. Previous studies reported detection of these viruses in plasma, serum, and peripheral blood samples from patients infected with HIV-1, healthy blood donors, and children and in urine samples from children (*1*,*2*,*38*) and immunocompetent and immunocompromised adults (*18*,*38*). WUPyV was detected in serum and feces of children whose nasopharyngeal aspirate samples contained high viral loads (*39*). Both viruses were detected in fecal samples from HCT recipients (*18*,*40*), and an association was found between KIPyV and diarrhea (*40*). However, we did not find an association between virus and diarrhea. Another limitation is that although we report prolonged, uninterrupted KIPyV and WUPyV detection, we did not perform genetic analyses to confirm whether these detections represent the same or different viral subtypes.

In conclusion, we detected KIPyV or WUPyV in one third of allogeneic HCT recipients during the first year after transplantation. Prolonged detection episodes and high viral copy numbers in respiratory specimens were also observed. However, we did not observe many associations with acute respiratory symptoms. We found that detection of a respiratory virus was a risk factor for KIPyV detection and that concurrent detection of the polyomaviruses and respiratory viruses was common. Although it has been suggested that KIPyV and WUPyV might be major pathogens in immunosuppressed patients, we did not find a clear role for these viruses as respiratory pathogens with classic upper and lower respiratory tract symptoms. At this time, we do not recommend routine testing for these viruses in immunocompromised patients or inclusion in multiplexed respiratory virus PCR panels. Further investigations examining other specimen types, such as BAL, blood, and urine, from a larger patient cohort over a longer period of time may be necessary to elucidate the role of these viruses in highly immunocompromised patients.

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Wild Birds and Urban Ecology of Ticks and Tick-borne Pathogens, Chicago, Illinois, USA, 2005–2010

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Bird-facilitated introduction of ticks and associated pathogens is postulated to promote invasion of tick-borne zoonotic diseases into urban areas. Results of a longitudinal study conducted in suburban Chicago, Illinois, USA, during 2005–2010 show that 1.6% of 6,180 wild birds captured in mist nets harbored ticks. Tick species in order of abundance were *Haemaphysalis leporispalustris*, *Ixodes dentatus*, and *I. scapularis*, but 2 neotropical tick species of the genus *Amblyomma* were sampled during the spring migration. *I. scapularis* ticks were absent at the beginning of the study but constituted the majority of ticks by study end and were found predominantly on birds captured in areas designated as urban green spaces. Of 120 ticks, 5 were infected with *Borrelia burgdorferi*, spanning 3 ribotypes, but none were infected with *Anaplasma phagocytophilum*. Results allow inferences about propagule pressure for introduction of tick-borne diseases and emphasize the large sample sizes required to estimate this pressure.

Wild birds can affect zoonotic disease risk to humans, wildlife, and domestic animals through their mobility and influence on the distribution and abundance of pathogens and vectors. Most notably, avian migration allows for rapid transcontinental transportation of novel pathogens and vectors that may seed new disease foci in receptive environments. For example, the spread of highly pathogenic avian influenza into and throughout most countries in Europe most likely occurred through the movement of migratory birds (*1*). Infected wild birds also contributed to the spread of West Nile virus (WNV) across North America (*2*). Thus, models of interseasonal connectivity among areas used by migratory birds can be used to forecast disease spread (*3*).

Over finer spatial scales, the patterns of bird use by blood-feeding vectors affect the prevalence of vector-borne pathogens. Host variation impacts the survival of vectors that feed on birds rather than on other vertebrates (*4*), and avian species exhibit differential reservoir competency for vector-borne pathogens (*5*). In combination, these factors influence disease risk; for example, just a few avian species that are heavily fed upon by mosquitoes and highly competent for WNV apparently drive most WNV transmission (*6*). Furthermore, host association of strains might help maintain pathogen diversity in some vector-borne diseases systems for which birds play critical roles (*7*).

Urban environments may promote pathogen transmission through increased host contact rates, high rates of pathogen introduction (i.e., propagule pressure), and warmer microclimates that are favorable to pathogens and vectors (*8*). These effects, in turn, may elevate disease risk to high-density urban human populations. Across gradients of urbanization, the incidence of some zoonotic pathogens has been found to be highest in urban cores (*9*). Reduced species richness in urban areas may contribute to elevated risk for diseases that are caused by multihost pathogens with generalist vectors (*10*), although the associations between biodiversity and disease risk are variable (*11*).

In humans, Lyme disease and anaplasmosis caused by infection with the bacteria *Borrelia burgdorferi* and *Anaplasma phagocytophilum*, respectively, are the 2 most common tick-borne diseases in the midwestern and northeastern United States, and both are emerging among human and canine populations (*12*,*13*). In eastern North America,

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both pathogens are maintained in blacklegged tick (*Ixodes scapularis*)–rodent cycles (*14*,*15*). We investigated the role of birds in the urban ecology of tick-borne zoonotic diseases. Our objectives were to 1) ascertain the prevalence of tick parasitism of birds in residential and urban green spaces in southwestern suburban Chicago, Illinois, USA, during a 6-year period; 2) estimate the infection prevalence of *Borrelia* spp. and *A. phagocytophilum* in ticks removed from birds; and 3) characterize the diversity of pathogens in ticks removed from birds by using genetic methods.

Materials and Methods

Bird Capture

During May–October 2005–2010, birds were captured at 20 field sites in southwestern suburban Chicago (Cook County; 87°44′ W, 41°42′ N; Figure). Field sites were categorized as residential sites (n = 14) or urban green spaces (n = 6) and have been described in detail (*6*). We used 8–10 mist nets (Avinet, Dryden, NY, USA) to capture birds at 7–15 sites per year ≈1 morning per site every 1.5 weeks (2005–2007) or every 3 weeks (2008–2010). For each captured bird, we recorded species, sex, age class (hatch year and after hatch year), and weight, and we attached a numbered leg band before release. All birds were checked for ticks by blowing apart feathers and inspecting the skin, especially around the ears, head, and vent. Ticks were removed and preserved in 70% ethanol. Migratory status of each avian species was assigned (*16*). Fieldwork was carried out with approvals from animal care review boards at Michigan State University and University of Illinois.

Detection and Typing of *Borrelia* spp. and *A. phagocytophilum*

Ticks were identified morphologically to species and stage; a subset was subjected to PCR and sequencing for confirmation (*17*). All ticks were tested for pathogens, except for 2 specimens that were deposited in the US National Tick Collection (housed at Georgia Southern University, Statesboro, GA, USA) for molecular identification and vouchering. Total DNA from ticks was extracted by using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA, USA) with modifications as described (*18*). Nymphal ticks were extracted individually, whereas same-species larvae from the same individual animal were pooled. All ticks were tested for the presence of *B. burgdorferi* sensu stricto and *A. phagocytophilum* by using a quantitative PCR targeting the 16S rRNA gene (*19*) and PCR targeting the *p44* gene (*20*), respectively.

B. burgdorferi–positive tick samples were typed by DNA sequencing of both strands of the 16S–23S rRNA intergenic spacer (IGS) region (*21*); strains were identified, using updated nomenclature (*22*), to ribosomal spacer type 1, 2, or 3 (*23*) and IGS subtype by comparing them with the 25 major *B. burgdorferi* IGS subtypes (*21*,*24*). The outer surface protein C (*ospC)* genotype was inferred on the basis of the linkage disequilibrium between IGS locus and *ospC* locus (*21*,*22*).

Statistical Analyses

Logistic regression was used to assess the variation in tick infestations among years. We used 2- and 3-sample tests for equality of proportions to assess the effects of site category, sex, and age on the prevalence of tick infestations. The Wilson interval with continuity correction was used to estimate the 95% binomial CIs for infection prevalence data. Minimum infection prevalence (i.e., assuming 1 positive larva/pool) was used for tests conducted on pooled larvae. Statistical analyses were performed by using Program R (R Foundation for Statistical Computing, Vienna, Austria).

Results

Bird Captures

We recorded 6,180 total captures, comprising 5,506 individual birds (10.9% recaptures) and 78 species (Table 1).

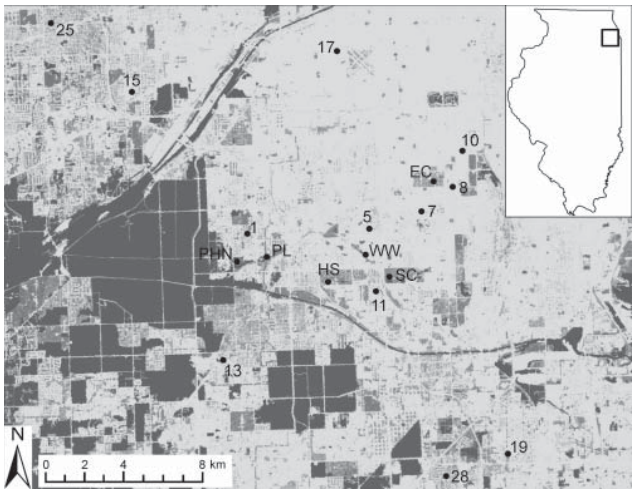

Figure. Field sites used for sampling birds in southwest suburban Chicago, Illinois, USA, 2005–2010. Sites consist of residential areas (numbered sites) and urban green spaces (lettered sites). Two residential sites not shown on the map (21 and 22) are ≈20 km north of this region. Box in inset map indicates location of sampling area. Main map shows the landscape gradient of impervious surfaces (National Land Cover Database 2001, US Geological Survey, Sioux Falls, SD, USA): dark gray areas are those with a low proportion of impervious cover (urban green spaces, e.g., forest preserves, parks, cemeteries, riparian buffers); light gray areas and white areas are those with a high proportion of impervious cover (areas with high density of buildings, residential housing, and roads). EC, Evergreen Cemetery; PHN, Palos Hills Natural; PL, Pleasure Lake; WW, Wolfe Wildlife Refuge; HS, Holy Sepulchre Cemetery; SC, Saint Casimir Cemetery.

Table 1. Birds sampled for presence of ticks in southwestern suburban Chicago, Illinois, USA, 2005–2010*

Bird	Migratory status	Total no. examined	Proportion infested	No. birds infested with *Haemaphysalis leporispalustris* Larvae	*H. leporispalustris* Nymphs	*Ixodes dentatus* Larvae	*I. scapularis* Larvae	*I. scapularis* Nymphs
American goldfinch	B, M	363						
American redstart†	B, M	38	0.03					
American robin	B, M	1,049	0.01	2	4	1	4	2
Baltimore oriole	B, M	31						
Barn swallow	B, M	7						
Black and white warbler	NB, M	9						
Black-capped chickadee	B, NM	25						
Blue jay	B, M	22	0.09					2
Brown-headed cowbird	B, M	65						
Brown thrasher	B, M	12						
Cedar waxwing	B, M	16						
Chipping sparrow	B, M	24						
Common grackle	B, M	105	0.03		2	1		
Common yellowthroat	B, M	8						
Dark-eyed junco	NB, M	8						
Downy woodpecker	B, M	50						
Eastern wood-pewee	B, M	5						
Empidonax spp. flycatchers	B, M	27						
European starling	B, M	141	0.01	1				
Fox sparrow	NB, M	5						
Gray catbird	B, M	429	0.01	3	3			
Gray-cheeked thrush	NB, M	18	0.11	1				1
Hermit thrush	B, M	5						
House finch	B, M	157						
House sparrow	B, NM	2,097	0.01	25	4			
House wren	B, M	57	0.02	1				
Indigo bunting	B, M	19						
Least flycatcher	B, M	5						
Lincoln's sparrow	NB, M	5						
Magnolia warbler	NB, M	19						
Mourning dove	B, M	63						
Mourning warbler	NB, M	5						
Nashville warbler	NB, M	7						
Northern cardinal	B, NM	311	0.04	9	3		1	
Northern flicker	B, M	10						
Northern waterthrush	NB, M	44						
Orchard oriole	B, M	4						
Ovenbird	B, M	41	0.10	4				
Palm warbler	NB, M	6						
Red-eyed vireo	B, M	11						
Red-winged blackbird	B, M	191	0.01	1	2			
Song sparrow	B, M	228	0.07	13	6	1		
Swainson's thrush‡	NB, M	131	0.08	4	4	1	1	
Tennessee warbler	NB, M	9						
Tree swallow	B, M	14						
Veery	B, M	8						
Warbling vireo	B, M	35						
White-crowned sparrow	NB, M	11						
White-throated sparrow	NB, M	61	0.02			1		
Willow flycatcher	B, M	63						
Wilson's warbler	NB, M	8						
Yellow warbler	B, M	34						
Yellow-bellied flycatcher	NB, M	6	0.17			1		
Yellow-rumped warbler	NB, M	26						
All		6,197§	0.02	64	28	6	6	5

*_Empidonax_ spp. flycatchers that could not be identified are considered at the genus level. Numbers of birds infested by larvae and nymphs of 3 tick species are indicated. Common names conform to species as specified by the American Ornithologist Union. B, confirmed breeding in Chicago region; M, migratory; NB, non-breeder in Chicago region; NM, non-migratory. Blank spaces mean none infested.

†One American redstart infested with a single *Amblyomma longirostre* nymph.

‡One Swainson's thrush infested with a single *A. nodosum* larva.

§This total includes 49 unlisted captured birds from the following species: American woodcock, American tree sparrow, black-billed cuckoo, black-throated blue warbler, blackpoll warbler, brown creeper, Carolina wren, Canada warbler, Eastern towhee, Eurasian collared–dove, great crested flycatcher, golden-crowned kinglet, hairy woodpecker, killdeer, marsh wren, olive-sided flycatcher, red-breasted nuthatch, rose-breasted grosbeak, ruby-crowned kinglet, savannah sparrow, scarlet tanager, swamp sparrow, white-breasted nuthatch, and wood thrush. The sample size for each of these species was <5, and none of the birds harbored ticks.

Five species comprised 67% of all captures: *Passer domesticus* (house sparrow), *Turdus migratorius* (American robin), *Dumetella carolinensis* (gray catbird), *Spinus tristis* (American goldfinch), and *Cardinalis cardinalis* (northern cardinal). Among all captured birds, 27.3% were known males, 21.3% known females, and 51.3% of unknown sex. The age class was after hatch year for 53.1%, hatch year for 41.8%, and unknown for 5.1% of the birds. Similar numbers of birds were captured from residential sites (3,326, 53.8%) and urban green spaces (2,854, 46.2%). Approximately 2× the number of birds were captured per year in 2005–2007 (1,455 ± 45) as in 2008–2010 (605 ± 159) due to higher mist netting efforts in the initial 3 years of the study.

Tick Prevalence

We removed 357 ticks from 97 individual birds (1 bird with ticks was caught twice), yielding an overall tick infestation prevalence of 1.6% (Table 1). Ticks were usually located beneath the auricular feathers within the skin of the ear canal and second most commonly located in the rictus of the bill and in the skin of the orbital region. Infested birds were collected at 17 of the 20 field sites (11/14 residential sites, 6/6 urban green spaces). Birds with the highest prevalence of infestation (>7% of captures infested) were song sparrows (*Melospiza melodia*), Swainson's thrushes (*Catharus ustulatus*), blue jays (*Cyanocitta cristata*), ovenbirds (*Seiurus aurocapilla*), gray-cheeked thrushes (*Catharus minimus*), and yellow-bellied flycatchers (*Empidonax flaviventris*) (Table 1).

Most ticks were of 3 species: *Haemaphysalis leporispalustris* (87.4% of all ticks), *Ixodes dentatus* (4.8%), and *I. scapularis* (7.8%). Morphologic and molecular identifications were congruent for all 21 birds subjected to both methods of identification (GenBank accession nos. JQ868565–JQ868585). Overall, 1.3%, 0.1%, and 0.2% of birds were infested with *H. leporispalustris*, *I. dentatus*, and *I. scapularis*, respectively (Table 1). In addition, a single *Amblyomma nodosum* larva was removed from an after–hatch year Swainson's thrush on May 17, 2005, and a single *A. longirostre* nymph was removed from an after–hatch year American redstart (*Setophaga ruticilla*) on May 18, 2005. The 2 ticks were found on birds captured at site HS (see Figure) during the spring migration. They were identified genetically and vouchered at the US National Tick Collection but not tested for pathogens.

The number of ticks on infested birds ranged from 1 to 23 (median 2 ticks). Of the infested birds, 47% harbored 1 tick and 20% harbored ≥5 ticks. *H. leporispalustris* larvae accounted for the greatest tick loads (average 4.3 ticks/bird). Of 98 parasitized birds, 11 (11.2%) were infested with >1 life stage of tick or >1 tick species. Although the overall prevalence of infested birds did not change over the 6-year study (z value = -1.6, df = 6178, p = 0.109), the proportion of infested birds that harbored *I. scapularis* increased significantly from 0 to 80% (z value = 3.873, df = 96, p = 0.0001), and *I. scapularis* comprised >90% of ticks removed from birds in the final year of the study. Of the 10 *I. scapularis*–infested birds, the majority (8) came from urban green spaces (0.28% *I. scapularis* infestation prevalence across all green spaces), and the minority (2) came from residential sites (0.06% prevalence; z value = 2.2, p = 0.03). Information about the timing of *I. scapularis* infestation combined with the species and age of the avian host provides evidence for local (Chicago area) acquisition of ticks and for migratory importation of ticks from the north and the south (Table 2).

Tick Infection with *B. burgdorferi* and *A. phagocytophilum*

A total of 120 tick samples were tested for pathogens. No ticks tested positive for *A. phagocytophilum* infection. Five samples tested positive for *B. burgdorferi* infection: 3 of 6 *I. scapularis* nymphs (50%, 95% CI 14.0%–86.1%), 1 of 22 *I. scapularis* larval pools (minimum infection prevalence 4.5%), and 1 of 34 *H. leporispalustris* nymphs (2.9%, 95% CI 0.2%–17.1%) (Table 3). All 5 positive tick samples were from unique after–hatch year birds of 4 species (American robin, blue jay, red-winged blackbird [*Agelaius phoeniceus*], Swainson's thrush) at 4 field sites, including urban green spaces and residential sites. *B. burgdorferi* 16S–23S rRNA IGS sequences were obtained from all 3 *I. scapularis* nymphs and represented 3 IGS ribotypes (2, 28, and 14; GenBank accession nos. JQ868562–JQ868564) within ribosomal spacer type 2 and 3; inferred *ospC* genotypes were H, T, and A3, respectively (Table 3).

Discussion

The presence of *B. burgdorferi*–infected *I. scapularis* ticks on migratory and residential birds in the Chicago region reflects the continued invasion and establishment of this tick and pathogen across the Midwest. In Illinois, as in many other areas of North America (*25*), there is growing public health concern over the emergence of Lyme disease (*26*); although, the statewide incidence in Illinois over the study period (1.1 cases/100,000 persons) was an order of magnitude lower than that which characterizes the Lyme disease–endemic regions in the northeastern United States (*27*). Our study provides evidence of established local populations of *I. scapularis* ticks in Chicago that may be supplemented by importation of *I. scapularis* ticks from other populations to the north or south by migratory birds. The Chicago region is a natural corridor for migratory birds, and the risk for tick and pathogen introduction is likely to be elevated on migratory flyways because of seasonal concentrations of birds.

Table 2. Demographic information about 10 avian hosts infested with *Ixodes scapularis* ticks in southwestern suburban Chicago, Illinois, USA, 2005–2010*

Bird	Date of capture	Age	Site, category	*I. scapularis* stage (quantity)	Presumed *I. scapularis* acquisition
American robin	2007 Jul 18	AHY	1, residential	L (9); N (1)	Local
American robin	2009 Aug 18	HY	PL, green space	L(2)	Local
American robin	2010 Jun 22	AHY	PHN, green space	N(2)	Local
American robin	2010 Jul 13	AHY	PL, green space	L (1)	Local
American robin	2010 Jul 26	HY	PL, green space	L (8)	Local
Blue jay	2009 Jun 15	AHY	PHN, green space	N(1)	Local
Blue jay	2009 Jun 15	AHY	PHN, green space	N (1)	Local
Gray-cheeked thrush	2010 Sep 16	HY	PHN, green space	N(1)	Migratory (from north)
Northern cardinal	2007 Aug 16	HY	13, residential	L(1)	Local
Swainson's thrush	2006 May 23	AHY	WW, green space	L (1)	Migratory (from south)

*AHY, after hatch year; L, larva; N, nymph. HY, hatch year; PL, Pleasure Lake; PHN, Palos Hills Natural; WW, Wolfe Wildlife Refuge.

We detected a *B. burgdorferi*–positive *I. scapularis* larval pool from a Swainson's thrush. Given the absence of transovarial transmission in the *I. scapularis* tick, this finding demonstrates that the Swainson's thrush can be an infectious reservoir host. On the basis of a limited sample (n = 6), we determined that birds in Chicago harbored *B. burgdorferi*–infected *I. scapularis* nymphs at a prevalence (14.0%–86.1%) consistent with that reported for questing nymphs and ticks from birds in Michigan (*18*), Minnesota (*28*), and Canada (*29*). All 3 *B. burgdorferi* IGS ribotypes present within nymphs in this study have been associated with host-seeking nymphs in Lyme disease–endemic areas of the midwestern and northeastern United States; 2 of the 3 ribotypes were previously detected in larvae removed from birds (*30*). Two of the *ospC* types (H and A) presumed present in the collected ticks were among the 4 most invasive genotypes (I, A, H, B) from a study of *B. burgdorferi* isolates from humans in New York (*31*). The presence of avian reservoirs and *I. scapularis* nymphs infected with *B. burgdorferi* strains capable of causing disseminated human disease supports the possibility that reported cases of human Lyme disease in Chicago residents may result from local exposure to infected *I. scapularis* ticks. Although none of the ticks removed from birds were positive for *A. phagocytophilum*, the growing *I. scapularis* tick population in the region raises the possibility that infection with this pathogen could become an emerging health concern.

Other ticks commonly found on birds in Chicago are *I. dentatus* and *H. leporispalustris* ticks, both of which feed almost exclusively on rabbits and birds. *I. dentatus* ticks are enzootic vectors of *B. burgdorferi* in regions where *I. scapularis* ticks do not occur (*24*). *H. leporispalustris* ticks transmit *Francisella tularensis* and spotted-fever group rickettsiae among wildlife (*32*). In our study, *H. leporispalustris* ticks had a wide geographic presence across most residential sites and were most commonly found on house sparrows, including 7 hatch-year birds, implying local acquisition in the residential neighborhoods. Neither *I. dentatus* nor *H. leporispalustris* ticks regularly infest humans.

We document the presence of 2 neotropical tick species, *A. longirostre* and *A. nodosum*, on birds migrating north through Chicago. We note that other species of neotropical *Amblyomma* ticks have been recovered in the spring on migrant birds in southern Canada (*33*). *A. longirostre* and *A. nodosum* ticks are widely distributed in the neotropical region, and are vectors of *Rickettsia amblyommii* (*34*) (which may cause rickettsiosis in humans in North America) (*35*), *R. bellii*, and *R. parkeri* (*36*). In the United States, *R. parkeri* is a newly recognized cause of human disease, and a high prevalence of infection (>40% in adults) has been associated with growing populations of Gulf Coast ticks (*A. maculatum*) (*37*). Migrant birds from the neotropics likely account for many imports of engorged neotropical ticks and associated pathogens in

Table 3. Prevalence of *Borrelia burgdorferi* infection in ticks removed from birds, by site of origin and date of capture, southwest suburban Chicago, Illinois, USA, 2005–2010*

	Larva			Nymph				
Tick species	No. pools (no. larvae)	% Infected (MIP)	Birds with infected larvae, site, date	No. tested	% Infected (95% CI)	Birds with infected nymphs, site, date	IGS strain (RST group)	*ospC* strain
Haemaphysalis leporispalustris	65 (277)	0	NA	34	2.9 (0.2–17.1)	RWBL, SC site, 2007 Jun 6	NA	NA
Ixodes dentatus	6 (17)	0	NA	0	NA	NA	NA	NA
I. scapularis	6 (22)	16.7 (4.5)	SWTH, WW site, 2006 May 23	6	50 (14.0–86.1)	AMRO, 1 site, 2007 Jul 18; AMRO, PHN site, 2010 Jun 22; BLJA, PHN site, 2009 Jun 15	2 (2); 28 (3); 14 (2)	H, T, A3

*MIP, minimum infection prevalence; IGS, *B. burgdorferi* 16S-23S rRNA intergenic spacer ribotype; RST, ribosomal spacer type 1, 2, or 3; *ospC*, inferred outer surface protein C allele based on linkages reported by Travinsky et al. (*23*); NA, not applicable; RWBL, Red-winged blackbird; SC, Saint Casimir Cemetery; SWTH, Swainson's thrush; WW, Wolfe Wildlife Refuge; AMRO, American robin; PHN, Palos Hills Natural; BLJA, Blue jay.

North America each spring, but a lack of environmental receptivity (host or climatic limitations) has likely prevented establishment.

Data from our large sampling effort show that the dispersal of *I. scapularis* ticks, *B. burgdorferi*, and neotropical vector ticks is a rare but detectable event. We sampled several thousand birds and detected *I. scapularis* ticks on <0.2% and neotropical ticks on <0.05%. However, the rarity of infestations does not mean that infestation is biologically insignificant. Despite the positive relationship between propagule pressure and invasion success, some successful species invasions, especially those of arthropods, can be initiated by a very small number of individuals (*38*). Low propagule pressure but successful invasion may occur when the environment is receptive to the particular species of ticks and pathogens being dispersed. Indeed, during our study, other researchers showed an increase in the occurrence of *B. burgdorferi*–infected adult *I. scapularis* ticks in northwestern Chicago, confirming our prediction (*26*). Such scenarios of rare introduction but successful establishment of ticks and pathogens pose a major risk for the health of humans, wildlife, and domestic animals in urban environments worldwide.

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Spread of Influenza Virus A (H5N1) Clade 2.3.2.1 to Bulgaria in Common Buzzards

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On March 15, 2010, a highly pathogenic avian influenza virus was isolated from the carcass of a common buzzard (*Buteo buteo*) in Bulgaria. Phylogenetic analyses of the virus showed a close genetic relationship with influenza virus A (H5N1) clade 2.3.2.1 viruses isolated from wild birds in the Tyva Republic and Mongolia during 2009–2010. Designated A/common buzzard/Bulgaria/38WB/2010, this strain was highly pathogenic in chickens but had low pathogenicity in mice and ferrets and no molecular markers of increased pathogenicity in mammals. The establishment of clade 2.3.2.1 highly pathogenic avian influenza viruses of the H5N1 subtype in wild birds in Europe would increase the likelihood of health threats to humans and poultry in the region.

Wild aquatic birds are considered natural reservoirs of all known influenza virus subtypes (*1*). Highly pathogenic avian influenza viruses (HPAIVs) usually cause asymptomatic infections in waterfowl. Compared with that for poultry, the number of reported outbreaks of HPAIVs in wild birds (aquatic or terrestrial) before 2002 was low; 1 influenza A (H5N3) outbreak occurred in wild common terns (*Sterna hirundo*) in South Africa in 1961 (*2*), and H7 subtype HPAIV was isolated from a Saker falcon (*Falco cherrug*) in Italy in 2000 (*3*). In December 2002, a die-off of aquatic waterfowl caused by an HPAIV (H5N1) occurred in Penfold Park in Hong Kong. That event was followed by a second outbreak a week later in Kowloon Park, also in Hong Kong (*4*). Since then, cases of HPAIVs (H5 and H7 subtypes) in wild birds have been reported often.

In May 2005, a massive HPAIV (H5N1) outbreak occurred in wild aquatic birds in Qinghai Lake, western People's Republic of China; 6,184 gulls, geese, great cormorants, and ruddy shelducks died (*5*). The lake is a staging area for migratory waterfowl, and the scientific community's fear that the virus would spread during migration (*6*) was realized when the so-called Qinghai-like influenza virus A (H5N1) clade 2.2 spread to western Siberia in Russia and then to many countries in Asia, the Middle East, Europe, and Africa at the end of 2005 and during 2006, killing poultry flocks and wild birds.

The 2008 classification system used to describe the evolution and diversification of the HPAIVs (H5N1) that emerged from the A/goose/Guangdong/96 lineage (*7*) was updated in 2011. Phylogenetic analysis of all isolated influenza (H5N1) viruses showed that some of the 10 first-order clades (0–9) had stopped circulating in 2008 or earlier (clades 0, 3, 4, 5, 6, 8, 9), as had some second- and third-order groups of clade 2. Meanwhile, clades 1, 2.1.3, 2.2, 2.2.1, 2.3.2, 2.3.4, and 7 continued to evolve rapidly (*8*). Clade 2.3.2 is widely distributed in Asia, particularly in China, Hong Kong, Korea, Vietnam, Laos, Bangladesh, Nepal, Mongolia, and the Tyva Republic; it is also distributed in eastern Europe, mainly in Romania and Bulgaria (*8*,*9*). Tyva is part of the Siberian Federal District of Russia, which is located north of Mongolia. The remaining circulating influenza (H5N1) clades have specific geographic locations: clade 1 circulates in southern Vietnam and Cambodia; clade 2.1.3 in Indonesia; clade 2.2 in India and Bangladesh; clade 2.2.1 in Egypt; clade 2.3.4 in China, Hong Kong, Vietnam, Thailand, and Laos; and clade 7 in China and Vietnam.

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Before 2006, no avian influenza outbreaks in poultry had been reported in Bulgaria; 4 cases of HPAIV (H5N1) clade 2.2 were confirmed in dead swans found in 4 regions of the country early that year (*10*). On March 15, 2010, the carcass of a common buzzard (*Buteo buteo*) containing HPAIV (H5N1) was found at St. Konstantin and Helena Black Sea Resort in Bulgaria and submitted to the Regional Diagnostic Laboratory on Avian Influenza (Varna, Bulgaria). The virus was characterized as clade 2.3.2.1.

Materials and Methods

Virus Isolation and Initial Characterization

Pooled lung, trachea, liver, cecal tonsil, and gizzard tissue from a common buzzard were injected into embryonated chicken eggs, and an avian influenza virus (A/common buzzard/Bulgaria/38WB/2010) was isolated. The isolate was subjected to hemagglutination inhibition (HI) assays, as specified in the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011 (*11*). The 50% egg infectious dose (EID_{50}) was assayed in 10-day-old embryonated chicken eggs after a 40-hour incubation at 35°C and calculated according to Reed and Muench (*12*).

Sequence Analysis

Viral RNA was extracted from virus-containing allantoic fluid by using the MagMax-96 AI/ND RNA extraction kit (Applied Biosystems/Ambion, Austin, TX, USA). Reverse transcription PCR was performed by using a universal set of primers (*13*,*14*); whole-genome sequencing was performed by using an Illumina Genome Analyzer (Illumina, San Diego, CA, USA), as described by Ducatez et al. (*15*).

Phylogenetic Analysis

We performed a phylogenetic analysis of all gene segments of A/common buzzard/Bulgaria/38WB/2010. Sequences were retrieved from the National Center for Biotechnology Information influenza virus database (*16*) and aligned by using the ClustalW tool in MEGA4 (*17*). Phylogenetic relationships were estimated by using the MrBayes program (*18*) to apply a Bayesian method. The model of nucleotide substitution that best fits our data was selected by using the Modeltest 3.7 program (*19*).

Antigenic Characterization and Pathogenicity Studies

We assessed the antigenic relationship of A/common buzzard/Bulgaria/38WB/2010 with other influenza (H5N1) viruses by performing HI assays with a panel of postinfection ferret antiserum against viruses from clades 2.3.2, 2.3.2.1, and 2.3.4; the panel was produced at St. Jude Children's Research Hospital (Memphis, TN, USA; Table). All animal studies were conducted in United States Department of Agriculture–approved biosafety level 3 enhanced facilities at St. Jude Children's Research Hospital.

Pathogenicity Tests in Chickens

The intravenous pathogenicity index test was conducted according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2011 (*11*) to determine whether A/common buzzard/Bulgaria/38WB/2010 is pathogenic in chickens. A natural route of infection study was performed in five 8-week-old specific pathogen–free chickens. A/common buzzard/Bulgaria/38WB/2010 (10^6 EID_{50} in 0.5 mL) was administered to each bird as follows: 0.1 mL in the nares, 0.1 mL in the trachea, 0.2 mL in the throat, and 1 drop in each eye. The chickens were examined daily for clinical signs of disease.

Pathogenicity Tests in Mice and Ferrets

The 50% mouse lethal dose was determined to assess the pathogenicity of the buzzard influenza (H5N1) virus in mammals. The experiment was conducted as described by Boon et al. (*20*), and the titer was calculated according to Reed and Muench (*12*).

We also performed a pathogenicity and transmissibility study in a ferret model (*21*). We collected nasal washes from all ferrets (i.e., inoculated ferrets, naive direct-contact ferrets, and naive respiratory droplet–contact ferrets) every other day and nasal swabs from only the donors (i.e., inoculated ferrets) on day 4 postinfection. EID_{50} was calculated according to Reed and Muench (*12*).

Table. Hemagglutination inhibition titers used to compare the antigenicity of influenza A(H5N1) virus strain A/common buzzard/Bulgaria/38WB/2010 with other common strains*

	Strain (clade)			
Strain	A/common magpie/Hong Kong/ 5052/2007 (2.3.2.1)	A/Muscovy duck/Vietnam/ 1455/2006 (2.3.2)	A/duck/Laos/ 3295/2006 (2.3.4)	A/Japanese white-eye/Hong Kong/1038/2006 (2.3.4)
A/common magpie/Hong Kong/5052/2007	160	160	<40	<40
A/duck/Laos/3295/2006	<40	<40	160	80
A/Japanese white-eye/Hong Kong/1038/2006	<40	40	160	320
A/Muscovy duck/Vietnam/1455/2006	80	80	<40	<40
A/common buzzard/Bulgaria/38WB/2010	80	80	<40	<40

*Values represent titers that are the reciprocal of the lowest dilution of ferret antisera that inhibited hemagglutination caused by 4 hemagglutination units of the virus.

Results

Gross Pathologic Findings

The fresh common buzzard carcass that was initially found was in good condition, without any discharge or other clinical signs of disease. Necropsy revealed a sufficient quantity of yellow subcutaneous and abdominal fat tissue. Gross pathologic changes were not observed in the lungs, trachea, heart, liver, spleen, gizzard, stomach, pancreas, or intestines.

Antigenic Characterization and Pathogenicity in Chickens

In the HI test, A/common buzzard/Bulgaria/38WB/2010 had a titer of 80 to antiserum from influenza (H5N1) clades 2.3.2 and 2.3.2.1 (Table). The intravenous pathogenicity index of A/common buzzard/Bulgaria/38WB/2010 was scored 3.0 because all 10 birds were found dead within 24 hours after inoculation. The natural route of infection study resulted in 2 chickens being found dead on day 2 postinfection, and the other 3 were sick. On day 3 postinfection, the remaining 3 chickens were found dead. The clinical signs observed before death were cloudy eyes, cyanosis of the exposed skin and wattles, edema of the face, and diarrhea.

Pathogenicity in Mice and Ferrets

The 50% mouse lethal dose of A/common buzzard/Bulgaria/38WB/2010 was 30 EID_{50} (EID_{50} of the virus was 10^9/mL). All 3 donor ferrets were shedding virus by day 5 postinfection; however, only 1 was still shedding virus by day 7 postinfection (Figure). Virus titers were not detected in the nasal washes of contact ferrets, indicating that A/common buzzard/Bulgaria/38WB/2010 is not transmissible by direct contact or respiratory droplets. All ferrets used in this study were healthy during the experiment and showed no clinical signs of disease; they were alert, playful, and eating and drinking normally.

Molecular Characterization of A/common buzzard/Bulgaria/38WB/2010

Genomic mutations identified in A/common buzzard/Bulgaria/38WB/2010 are listed in the online Technical Appendix Table (wwwnc.cdc.gov/EID/pdfs/12-0357-Techapp.pdf). The genome sequences of A/common buzzard/Bulgaria/38WB/2010 (H5N1) have been deposited in GenBank (accession nos. CY110851–CY110858).

Hemagglutinin

The virus's hemagglutinin (HA) cleavage site had the polybasic amino acid sequence PQRERRRKRGLF, which is characteristic of HPAIVs (*22*). The cleavage site also had a K329 deletion (H5 numbering used throughout).

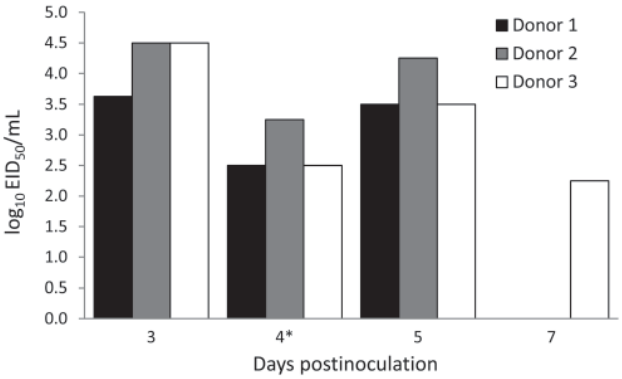

Figure. Virus titers in nasal washes or nasal swabs (*) of individual donor ferrets inoculated with A/common buzzard/38WB/2010. EID_{50}, 50% egg infectious dose.

However, none of the antigenic sites in HA1 of the H5 HA were mutated in comparison with A/whooper swan/Mongolia/2/2009, A/great crested grebe/Qinghai/1/2009, A/grebe/Tyva/3/2009, A/bar-headed goose/Qinghai/1/2010, and other closely related strains from clade 2.3.2.1 available in GenBank (site 1: amino acids 136–141; site 2: 152–153; site 3: 124–129, H5 numbering).

The receptor-binding pocket of HA1 retained residues E186, Q222, and G224, which preferentially bind to avian α-2,3-NeuAcGal receptors (*23*). All conserved residues within the HA receptor–binding domains of A/common buzzard/Bulgaria/38WB/2010 were identical to those of other clade 2.3.2 HA sequences available in GenBank (online Technical Appendix Figure).

Neuraminidase

Compared with that of A/goose/Guangdong/1/96, the amino acid sequence of A/common buzzard/Bulgaria/38WB/2010s neuraminidase had a 20-residue deletion in the stalk region (residues 49–68), which was thought to be required for influenza viruses to adapt from wild aquatic birds to domestic chickens (*24*). This deletion causes a loss of the N terminal NQS glycosylation site (positions 50–52). Residues E119, H275, or N295 (N1 numbering) were not mutated, which suggests sensitivity to oseltamivir and zanamivir (*25*,*26*).

Polybasic Proteins

The A/common buzzard/Bulgaria/38WB/2010s *PB1* gene sequence contained 3 synonymous mutations, G204A, A750G, and G2052A; G204A in PB1's open reading frame (ORF) caused a unique R37Q mutation in the polybasic (PB) 1-F2 protein. The PB1-F2 protein generated by the ORF plus 1 of the virus's *PB1* genes is a 90-aa polypeptide. According to Zell et al. (*27*), PB1-F2 proteins

consisting of >78 aa are intact and functional. The PB1-F2 N66S mutation, which is characteristic of increased viral pathogenicity and contributed to the high lethality of the 1918 pandemic influenza virus (*28*), was not observed in A/common buzzard/Bulgaria/38WB/2010. However, several PB2 mutations were present (online Technical Appendix Table).

Nonstructural Proteins

The nonstructural (NS) 1 protein of A/common buzzard/Bulgaria/38WB/2010 is encoded by 230 aa. This sequence differs from other closely related NS sequences in the National Center for Biotechnology Information database in that it lacks the 5-aa deletion (residues 80–84) that became common in HPAIV (H5N1) NS sequences after they were first observed during 2001 in poultry in Hong Kong.

Matrix Proteins

The *M* gene–encoded ORF of matrix (M) 1 protein consists of 252 aa, and that of M2 consists of 97 aa. Residues 26L, 27V, 30A, 31S, and 34G of M2's transmembrane region indicate that A/common buzzard/Bulgaria/38WB/2010 is an amantadine-sensitive strain (*29*).

Nucleoprotein

This virus's nucleoprotein (NP) contained 4 aa substitutions, V33I, R293K, N395T and S450N. Normally, equine and avian influenza NPs have V33 (*30*). I33 is characteristic of human and swine influenza NPs (*30*,*31*), and V33I is a common amino acid substitution found in the pandemic viruses from 1918, 1957, 1968, 1977, and 2009 (*32*). K293 is also unique to human viruses (*32*,*33*). The NP of A/common buzzard/Bulgaria/38WB/2010 and that of the closely related A/whooper swan/Mongolia/1/2010 virus contain N450, a substitution found in North American bird strains; however, Eurasian strains typically contain S450.

Phylogenetic Analysis

To determine the place of A/common buzzard/Bulgaria/38WB/2010 in the modern classification of influenza (H5N1) viruses, we phylogenetically analyzed HA nucleotide sequences of viruses representing all clades that had been reported as of the end of 2010. The HA of the Bulgarian isolate clustered with subtypes from clade 2.3.2.1 that were isolated in Mongolia and Tyva in 2009 and 2010 and originated from the A/black headed gull/Tyva/115/2009 and A/great crested grebe/Qinghai/1/2009 strains (online Technical Appendix Figure).

To assess possible reassortment in the A/common buzzard/Bulgaria/38WB/2010 genome, we performed phylogenetic analysis of the remaining genes using the same group of viruses that had been used to make the HA tree. In the N1, PB1, PB2, PA, NS, and NP phylogenetic trees, A/common buzzard/Bulgaria/38WB/2010 clustered with the other subtype H5N1 viruses from clade 2.3.2.1 (data not shown). In the *M* gene tree, the Bulgarian subtype H5N1 virus clustered with the clade 2.3.4 subtypes from Guangxi, Hunan, Fujian, Shantou, and Hong Kong that were isolated in 2005, 2006, and 2008; all other subtype H5N1 viruses from clade 2.3.2.1 clustered in a separate group (online Technical Appendix Figure). The evolutionary distance between the 2 groups of isolates in the M tree is long, indicating that the *M* gene of A/common buzzard/Bulgaria/38WB/2010 originates from a non–clade 2.3.2 ancestor.

Discussion

The day after we received the HPAIV (H5N1)–containing common buzzard carcass, officials in Romania notified the OIE of an outbreak of an HPAIV (H5N1) in Letea Village (Danube Delta); 47 backyard chickens were found dead. These reports are considered to be the introduction of HPAIV (H5N1) clade 2.3.2.1 into Europe. Furthermore, the European Reference Laboratory on Avian Influenza and Newcastle Disease (Weybridge, UK) found that the HA gene sequence of the Bulgarian isolate is 99.9% similar to that of the Romanian isolates from Letea Village (*34*), confirming that both viruses are derived from a common source, which is most likely wild birds.

St. Konstantin and Helena Black Sea Resort, where the common buzzard carcass was found, is located on the border of Batova (43°21′11″N, 27°57′33″E), which is a complex of habitats typical for woodland bird species, waterfowl, and poultry. This area is defined as a bottleneck migration site of global importance because 3 flows of migratory birds meet over the Batova River Valley (*35*). Each spring (March–April), migratory waterfowl from Africa, Bosphorus, and the Dardanelle Straits stop at the lakes along the coast of the Black Sea in Bulgaria on their way north (*36*). Their next resting spot is the Danube Delta, where the Romanian outbreak occurred and <250 km from where the Bulgarian buzzard carcass was found. Thus, if we are correct in our hypothesis that birds from Via Pontica are the hosts that carried the HPAIV (H5N1) to Bulgaria and Romania, the virus most likely came from Tyva and Mongolia, where its ancestral viruses had been isolated, to Via Pontica by using ≥2 other overlapping flyways. Although this is the most likely scenario, it is still unconfirmed. To confirm this hypothesis, the pathogenicity of A/common buzzard/Bulgaria/38WB/2010 in ducks and its possible transmission among them must be defined, which will require additional biologic studies.

To trace the possible routes of introduction of HPAIVs (H5N1) into Bulgaria, the veterinary authorities

and ornithologists from the Bulgarian Society for the Protection of Birds organized continuous monitoring of the bird areas along the Black Sea coast, near the Danube River and around the Ogosta Dam and Lom River. From January 1, 2010, through April 30, 2010, a total of 812 cloacal, fecal, and tissue samples from wild birds collected from these areas were tested for avian influenza virus; 269 samples were collected after March 15, 2010. All samples tested were negative for HPAIV (H5N1). Five carcasses of common buzzards found in different areas were submitted to the Regional Diagnostic Lab on Avian Influenza after March 15, 2010; only 1 was carrying HPAIV (H5N1).

Common buzzards are considered territorial birds that usually do not migrate long distances. A 3-year study conducted in southern England showed that local radio-tagged common buzzards forage within 1 km of their nests during their first winter; most of the birds that do not disperse make only brief excursions before they opt for a stay-at-home strategy, and most of those that disperse return to their natal area during the following breeding season (*37*). Long-term ornithologic studies conducted during 1979–2005 in Bulgaria, however, showed unambiguously that extensive autumn and spring migrations of common buzzards (as many as 42,100 birds) occur on the western Black Sea Via Pontica flyway (*35*,*38*). Kostadinova et al. reported ≈6 pairs of common buzzards breeding in the Batova habitat, but during autumn and spring migrations, ornithologists counted as many as 19,712 individuals of the species in the area (*35*). In most cases, common buzzards migrate singly or in loose flocks with other raptors, lesser spotted eagles, white pelicans, or black storks (*38*).

Migration of common buzzards in different parts of Europe appears to depend on the local climate; buzzards from northern Europe fly to the western Black Sea area during the winter season, whereas buzzards from Bulgaria fly south. Tracking bands belonging to common buzzards from Finland, Romania, and Israel have been found in Bulgaria (*39*). The buzzard infected with HPAIV (H5N1) in Bulgaria was not banded; however, even if it had been a migrant, the habitat nearest to St. Konstantin and Helena Black Sea Resort that provides different food sources is Batova, with an area of 38,132.8 ha (*35*). The migration of the common buzzards suggests that these birds are capable of spreading pathogens over long distances.

Our results show that chickens are highly susceptible to influenza virus A/common buzzard/Bulgaria/38WB/2010 (H5N1) and that the virus is highly pathogenic in them. Mammals appear not to be susceptible. Although buzzards can serve as intermediate hosts of HPAIV (H5N1) between migratory birds and poultry, the lack of gross pathologic findings in the buzzard carcass we examined indicates that the bird died shortly after infection. Thus, in this case, the buzzard could not have served as a reservoir of infection to spread the virus over a long distance. Additionally, the lack of poultry farms within 10 km of the area where the buzzard carcass was found may partially explain why no outbreak occurred. As part of a regular avian influenza surveillance plan, we tested 1,709 cloacal and fecal samples from mule ducks that were collected monthly during January 1, 2010–April 30, 2010, from 64 farms in 5 regions of Bulgaria (Plovdiv, Pazardjik, Stara Zagora, Haskovo, and Dobrich). No notifiable avian influenza viruses were isolated from any sample.

Since clade 2.3.2 was first isolated from a dead Chinese pond heron in Hong Kong in 2004, it has spread geographically and evolved genetically. A new fourth-order clade, 2.3.2.1, was recently identified, and A/common buzzard/Bulgaria/38WB/2010 was classified in this clade (*8*). The question that arises now that clade 2.3.2.1 has spread from Asia to Europe is whether it can cause a scenario similar to that caused by clade 2.2 from 2005–2006, when HPAIV (H5N1) killed millions of birds in Asia, Europe, and Africa. Although no new HPAIV (H5N1)–related events have been reported in Europe since March 2010, some of the aspects of the 2.3.2.1 clade make it difficult to predict the consequences of the clade's arrival on the continent. For example, this clade is already widely distributed in Asia and is being perpetuated in many wild bird species, which is a prerequisite for long-distance distribution through migration. Wild bird species infected with HPAIV (H5N1) from clade 2.3.2.1 include gray herons, peregrine falcons, and great egrets in Hong Kong; whooper swans, ruby shelducks, and bar-headed geese in Mongolia; and grebes and black-headed gulls in Tyva (*8*).

The potential of clade 2.3.2.1 HPAIV (H5N1) to cause an outbreak is heightened because vaccines currently in use do not efficiently protect poultry flocks from a strain of this clade that was recently identified in Vietnam (*40*). Now that clade 2.3.2.1 has spread to Europe, implementing active surveillance plans in all high-risk areas and monitoring the wild birds in the region will play key roles in early detection of incidences of HPAIV (H5N1) infection and in prevention of outbreaks. The expansion of the geographic distribution of HPAIV (H5N1) in wild birds and poultry and the virus's repeated interspecies transmission to humans make this virus a substantial pandemic threat.

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Dengue Outbreaks in High-Income Area, Kaohsiung City, Taiwan, 2003–2009

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Kaohsiung City, a modern metropolis of 1.5 million persons, has been the focus of dengue virus activity in Taiwan for several decades. The aim of this study was to provide a temporal and spatial description of dengue virus epidemiology in Kaohsiung City by using data for all laboratory-confirmed dengue cases during 2003–2009. We investigated age- and sex-dependent incidence rates and the spatiotemporal patterns of all cases confirmed through passive or active surveillance. Elderly persons were at particularly high risk for dengue virus–related sickness and death. Of all confirmed cases, ≈75% were detected through passive surveillance activities; case-patients detected through active surveillance included immediate family members, neighbors, and colleagues of confirmed case-patients. Changing patterns of case clustering could be due to the effect of unmeasured environmental and demographic factors.

Dengue virus disease or dengue virus–like disease has circulated in southern Taiwan since the late nineteenth century (*1*); transmission initially occurred as intermittent epidemics with intervals of several years to decades (*2*–*6*). However, for the past decade, dengue virus epidemics have occurred annually in Taiwan, and the main focus of activity has been in Kaohsiung City, a modern metropolis of 1.5 million persons. Kaohsiung City was the epicenter of the 2002 dengue virus epidemic, which with 2,820 confirmed cases and several hundred cases of dengue hemorrhagic fever (DHF), was one of the largest ever recorded in Taiwan (*3*). During 2002–2011, Kaohsiung City has had annual outbreaks of variable scales, resulting in ≈6,800 confirmed cases (*3*).

Cocirculation of >2 of the 4 dengue virus serotypes (DENV-1–4) has been reported in Kaohsiung City, and the molecular characteristics of the serotypes have been well documented for several epidemics, indicating the possible origin and transmission dynamics of the causative strains (*2*–*9*). The spatiotemporal patterns of disease transmission during the 2002 DENV-2 epidemic also have been investigated, and findings indicate several possible mechanisms by which the virus might have dispersed after being introduced into the population (*10*,*11*). Furthermore, Lin et al. (*12*) examined the relationship between disease-related illness and death and the distribution of primary and secondary infections for dengue virus cases reported across Taiwan during 2002–2007.

We provide a detailed description of the epidemiology of dengue virus infection in Kaohsiung City during 2003–2009; the description is based on routine disease and vector surveillance data provided by the Department of Health, Kaohsiung City Government. The temporal case distribution is compared with available climate data and the index of peridomestic adult vectors, *Aedes aegypti* and *Ae. albopictus* mosquitoes, and case characteristics are examined across the age and sex of patients and across the surveillance method (active vs. passive). In addition, the study provides an assessment of spatiotemporal case clusters, identifying possible hot spots for dengue virus transmission in Kaohsiung City during the 7-year study period.

Materials and Methods

Study Area

Kaohsiung City, located on the southwestern coast of Taiwan (22°38′N, 120°16′E), has a tropical monsoon climate (*13*) (Figure 1). During the study period, January

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Figure 1. Kaohsiung City, Taiwan (22°38′N, 120°16′E), indicating the 11 districts and 463 administrative units (Li) of the city and the main entry points for international travel and commerce. Insets show location of Taiwan in Southeast Asia (box) and of Kaohsiung City in Taiwan (gray shading).

2003–December 2009, the highest annual temperatures were during June–August (monthly mean temperature range 28.7°C–30.5°C), and the lowest temperatures were recorded during January and February (mean temperature 18.4°C and 20.4°C, respectively). The highest monthly accumulative precipitations occurred during June–August (range 901.5–1,229.3 mm), whereas there was almost no precipitation during November–February (*14*) (Figure 2).

Kaohsiung City is a major port and industrial metropolis and the most densely populated urban center in Taiwan (1.5 million persons within a total area of 150 km^2). The city is divided into 11 districts; a Li is the smallest administrative unit within these districts. The overall number of Lis decreased from 463 to 459 in 2006. On average, the population density of a Li is 150–65,000 persons/km^2 (Figure 1). Piped water is available for 99% of the city households, and household waste is removed daily throughout the city by the Environmental Protection Bureau, Kaohsiung City Government. The city has an international airport, which is a major access point for tourists and foreign workers, many of whom are employed in the commercial port and the industrial zones of the city. Most of the 15,000 foreign workers who arrive in the city each year are citizens from neighboring countries, such as the Philippines, Indonesia, Vietnam, and Thailand (*15*).

Dengue Surveillance and Laboratory Diagnosis

The disease surveillance system in use during the study period was introduced under the Communicable Disease Prevention Act in 1999 by the Taiwan Center for Disease Control (Taiwan CDC). The system ensured reporting and laboratory confirmation of all suspected cases of dengue virus infection identified through passive or active surveillance activities, using the probable case definition of the World Health Organization (WHO) (*16*). Passive surveillance involved mandatory reporting of probable dengue virus infection cases to Taiwan CDC within 24 hours after a patient sought medical assistance at any of the city's 1,747 health facilities, including public health centers, general practitioner clinics, and public and private hospitals. Passive surveillance activities also involved school-based reporting of febrile students and self-reporting to health authorities by citizens with probable dengue virus infection (*3*). Active surveillance included fever checkpoints at the airport and screening by the district public health nurse of immediate contacts (e.g., family members, colleagues, and neighbors) of persons with confirmed dengue virus infection (*3*,*17*). Basic patient information was collected and blood samples were obtained from all suspected case-patients and sent to the Fifth Branch Office of Taiwan CDC in Kaohsiung City.

A patient was confirmed to have dengue virus infection if 1) dengue virus RNA was detected in a serum sample by real-time reverse transcription PCR, 2) dengue virus–specific antibodies were detected in single serum samples by IgM- or IgG-capture ELISA, or 3) a >4-fold increase in IgG ELISA titers was detected in paired acute- and convalescent-phase serum samples (*3*). In August 2008, detection of dengue virus nonstructural protein 1 by use of a rapid diagnostic test (Platelia Dengue NS1 Ag assay; Bio-Rad, Marnes la Coquette, France) was included as a fourth diagnostic method (*3*). DHF, including dengue shock syndrome (DSS), was distinguished from dengue fever by the presence of 1) hemorrhagic tendencies, including a positive tourniquet test result and bleeding from the

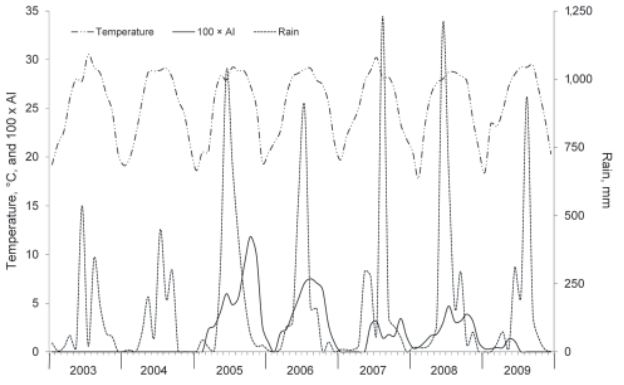

Figure 2. Monthly average temperature, rainfall, and adult index (AI) for *Aedes aegypti* and *Ae. albopictus* mosquitoes, Kaohsiung City, Taiwan, 2003–2009. AI was calculated as number of adult female mosquitoes captured per number of inspected premises.

mucosa, gastrointestinal tract (hematemesis, hematuria, or melena) or other locations; 2) thrombocytopenia (<100,000 cells/mm^3); or 3) plasma leakage (*3*). The severity of DHF was not further classified. All laboratory tests and most of the incurred medical expenses were covered by the National Health Insurance.

Patient Data

During the study period, January 2003–December 2009, patient data for all laboratory-confirmed cases were provided by the Department of Health, Kaohsiung City Government. The data included the registered home address, sex, date of birth, date of manifestation onset, surveillance methods (active or passive), and reported clinical manifestations (fever, anorexia, headache, arthralgia, rash, myalgia, thirst, diarrhea, nausea, pruritus, vomiting, retro-orbital pain, and hemorrhagic manifestations).

Vector Index

Vector surveillance activities by the Department of Health, Kaohsiung City Government, were initiated in 2005 by using specially trained personnel. The Li was used as the surveying unit in which 50–100 households were randomly selected for inspection of *Ae. aegypti* and *Ae. albopictus* mosquito infestation (*3*). Adult *Aedes* mosquitoes were captured indoors and outdoors with hand-nets at 8:30–11:30 AM or 1:30–4:30 PM (*3*). Capture activities were completed for all rooms, including the basement, within a maximum of 10 minutes for each inspected premise. The adult index was calculated as the number of adult female mosquitoes captured divided by number of inspected premises.

Epidemiologic Analysis

Incidence rates and clinical manifestations were calculated for age-specific groups and sex by using the year-end population data for each study year as the denominator. The *z* test was applied for incidence rate comparison. The 2-sample *t* test was used for the comparison of the average number of clinical manifestations in patients detected in the passive versus the active surveillance system. The threshold of statistical significance was 0.05.

Spatial Analysis

Spatial patterns of dengue incidence in each Li were assessed by use of global and local indices. The global spatial pattern was measured by using Moran's I, an index of spatial autocorrelation coefficient, yielding only 1 summary statistic for the whole study area. The theoretical range of Moran's I was from –1 to 1; the value around 0 provided the indication of spatial random distribution. Higher positive values implied a stronger clustering pattern, and lower negative values represented a stronger dispersion tendency (*18*). We determined partial autocorrelation by using global statistics and actual cluster location by using the local indicator of spatial association (LISA). Anselin's LISA provided the local version of Moran's I, used here to compare mean incidence rates for each Li and its neighboring Lis (*19*). The mapped LISA results indicated how spatial autocorrelation varied over the study region according to 5 categories: 1) hot spot, denoting a high-incidence Li surrounded by high-incidence Lis; 2) high-value outlier, denoting a high-incidence Li surrounded by low-incidence Lis; 3) low-value outlier, denoting a low-incidence Li surrounded by high-incidence Lis; 4) cold spot, denoting a low-incidence Li surrounded by low-incidence Lis; 5) not significant, denoting no spatial autocorrelation presented.

All epidemiologic and temporal analyses were performed by using Excel 2002 (Microsoft, Redmond, WA, USA) and R-2.7.2 for Windows (http://cran.r-project.org/bin/windows/base/old/2.7.2/). Spatial analyses were done by using ArcGIS 9.2 (ESRI, Redlands, California, USA).

Results

During January 2003–December 2009, Taiwan CDC recorded 2,087 laboratory-confirmed cases of dengue virus infection in Kaohsiung City. The cases were detected by passive and active surveillance activities. Of the confirmed cases, 98.7% (2,060) were classified as dengue fever and 1.3% (27) as DHF/DSS. The 7-year fatality rate for patients with DHF/DSS was 25.9% (7/27).

Temporal Case Distribution

Most (96.9%) of the confirmed cases of dengue virus infection were recorded during epidemics occurring during July–December of each year. The interannual variations in outbreak scale were considerable, ranging from 45 confirmed patients in 2004 to 766 in 2006. A dominant serotype was evident during each epidemic, representing >80.0% of cases confirmed by virus detection (real-time reverse transcription PCR) in a given year (Figure 3).

The annual onset of epidemic activity generally coincided with the peak in monsoon rainfall and temperature levels (Figures 2 and 3). The epidemic peaked within 1–3 months after the onset of the epidemic, and all activity ceased at the end of the monsoon season. Vector data for 2005–2009 showed that the peak of the adult mosquito population followed the peak of the monsoon rainfall, with a lag period of 1–2 months that corresponded to disease activity (Figures 2 and 3).

Characteristics of Case-Patients

Age

The median age of patients with confirmed dengue virus infection was 46 years (range 4 months–95 years).

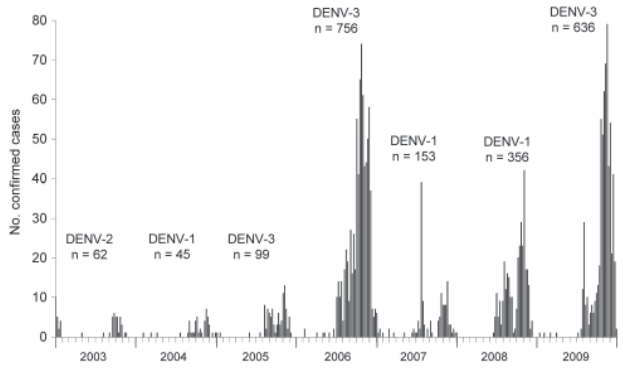

Figure 3. Epidemic curve of confirmed cases of dengue virus (DENV) infection (N = 2,087), by week of onset, Kaohsiung City, Taiwan, 2003–2009. Predominant serotypes (DENV-1–3) and numbers of confirmed cases are shown.

The average age-specific incidence rate was lowest among persons <5 years of age (4.5/100,000 persons) and increased steadily by age group (12.0, 14.3, 15.3, 17.9, and 25.3/100,000 persons for case-patients 5–14, 15–24, 25–34, 35–44, and 45–54 years of age, respectively) until peaking in persons 55–64 years of age (37.9/100,000 persons) (Table 1). The pattern of observed age-specific incidence rates was uniform across all epidemics during the study period (data not shown).

Among 27 patients with confirmed cases of DHF, fatality was highest among those >74 years of age (3/5, 60.0%) followed by those 65–74 years of age (3/9, 33.3%) and 55–64 years of age (1/5, 20.0%). No fatalities occurred among other age groups (Table 1).

Sex

For both sexes, persons 55–64 and 65–74 years of age had the highest and second highest incidence rates of confirmed dengue virus infection (Table 1). Overall, the incidence rate for the female population was slightly, but not significantly, higher than that for the male population (19.9 vs. 19.4/ 100,000 persons; p = 0.624). Among persons 15–24 years of age, the incidence rate for the male population was significantly higher than that for the female population (17.1 vs. 11.1/100,000 persons; p = 0.002).

Case Detection by Active and Passive Surveillance

The active surveillance system detected 538 (25.8%) of the confirmed cases during 2003–2009. Of these cases, 520 (96.7%) were in household members, neighbors, or colleagues of confirmed case-patients, and 18 were detected by the fever-screening system at the airport. The highest proportions of cases detected through active surveillance were among children 0–4 and 5–14 years of age (Figure 4).

The average number of reported disease manifestations was significantly higher among cases detected through passive than through active surveillance (n = 5.1 vs. 3.7; p<0.001), and each clinical manifestation was reported more frequently through the passive than the active system, with the exception of pruritus (Table 2). In both surveillance systems, the number of reported manifestations was lowest for persons 0–4, 65–74, and >74 years of age: average of 2.2, 1.9, and 1.2 manifestations, respectively, when detected through active surveillance (Table 3). These numbers of reported clinical manifestations are lower than the number required to meet the WHO criterion for probable dengue virus infection.

Spatial Analysis

The global level of spatial autocorrelation for the dengue virus infection incidence rates across the Lis of Kaohsiung City was significant for each epidemic (range 0.03–0.14, Moran's I; p<0.001) (Table 4), indicating a significant positive spatial autocorrelation within the city for each epidemic year. The type and area of local clustering, as determined by LISA, were identified for each epidemic. In general, hot-spot Lis with a high incidence of infection did not overlap for consecutive years; however, certain hot spots recurred or were adjacent to other hot spots for the epidemics of 2004, 2006, 2008, and 2009. These hot spots overlapped with clusters of high residential density. Some dengue hot spots were also observed for areas of low population density throughout the study period, except for 2004 and 2007. Half of the high-value outliers were observed in low population clusters (Figure 5).

Discussion

Evidence from Kaohsiung City shows that dengue virus infection occurs in persons of all ages; however, the incidence of infection increases notably in persons of increasing age, and elderly persons are at especially high risk for DHF/DSS and death. Most case-patients detected by the active surveillance system were contacts of case-patients detected by the passive surveillance system. Changes in disease hot spots were noted between successive years of the study.

The findings in this study support previous reports of a clear correlation between precipitation, temperature, and the occurrence of dengue virus epidemics in Taiwan (*20*,*21*). For Kaohsiung City, the study shows that the annual onset of dengue virus epidemics coincides with the time of peak rainfall and temperature. Dengue virus outbreaks coincide with the seasonal increase of adult vectors in the peridomestic environment, but there seems to be no correlation between the level of vector abundance and the scale of outbreaks. The same can be concluded for the meteorologic variables assessed in this study. This

Table 1. Age- and sex-specific incidence rates of confirmed cases of dengue virus infection, Kaohsiung City, Taiwan, 2003–2009*

Age group, y, sex	No. cases	Incidence rate/100,000 persons	p value†	No. DHF cases	No. fatal cases	Fatality rate, %
0–4						
M	14	5.7	0.184	2	0	0
F	7	3.1		0	0	0
All	21	4.5		2	0	0
5–14						
M	90	12.7	0.497	0	0	0
F	74	11.3		1	0	0
All	164	12.0		1	0	0
15–24						
M	134	17.1	0.002‡	2	0	0
F	82	11.1		2	0	0
All	216	14.3		4	0	0
25–34						
M	144	15.9	0.582	0	0	0
F	135	14.7		0	0	0
All	279	15.3		0	0	0
35–44						
M	157	18.2	0.968	1	0	0
F	161	17.7		0	0	0
All	318	17.9		1	0	0
45–54						
M	189	23.4	0.097	0	0	0
F	234	27.1		0	0	0
All	423	25.3		0	0	0
55–64						
M	167	35.0	0.142	1	0	0
F	210	41.1		4	1	25.0
All	377	37.9		5	1	20.0
65–74						
M	96	35.0	0.352	5	2	40.0
F	118	39.4		4	1	25.0
All	214	37.3		9	3	33.3
>74						
M	44	21.9	0.418	5	3	60.0
F	31	18.1		0	0	0
All	75	20.2		5	3	60.0
Total						
M	1,035	19.9	0.624	16	5	31.3
F	1,052	19.4		11	2	18.2
All	2,087	19.6		27	7	25.9

*DHF, dengue hemorrhagic fever.
†Difference in incidence between male and female population for the age group given.
‡Level of significance p<0.05.

observation is in line with the general understanding that the extent of dengue virus epidemics may be influenced by a variety of factors, including the level of herd immunity to the circulating serotype(s); the virulence of the circulating strain(s); and the effect of human-vector contact exerted by human behavior, specific climatic phenomena, and prevention and control operations.

All 4 dengue virus serotypes were identified in Kaohsiung City, and at least 2 serotypes cocirculated during each outbreak of the study period. DENV-1 and DENV-3 were clearly predominant during 3 outbreaks each; DENV-2 detection was limited, and DENV-4 detection was negligible. Specific information on the circulating genotypes or strains was not available for assessment. However, recent studies suggest that most dengue virus outbreaks in Taiwan can be attributed to the importation of novel dengue virus strains from neighboring Southeast Asian countries, in particular those with which substantial immigration, tourism, and trade relations are maintained (*4*,*7*,*8*).

The age distribution of confirmed dengue case-patients in Kaohsiung City was consistent throughout the study period. Children <5 years of age had the lowest disease incidence, and persons 55–64 and 65–74 years of age had the highest incidence of confirmed cases, including cases of DHF and dengue virus–related deaths.

The low incidence of dengue virus infection among the youngest age group fits well with descriptions of mild or mainly asymptomatic dengue virus infection in younger children (*22*). However, it is often suggested that the lack of vocal ability among small children plays a factor in the health-seeking behavior of their caretakers. In either case, one would expect a relatively larger group of 0- to 4-year-old children than older persons to be identified through the active surveillance system. In fact, the 0- to 4-year-old age

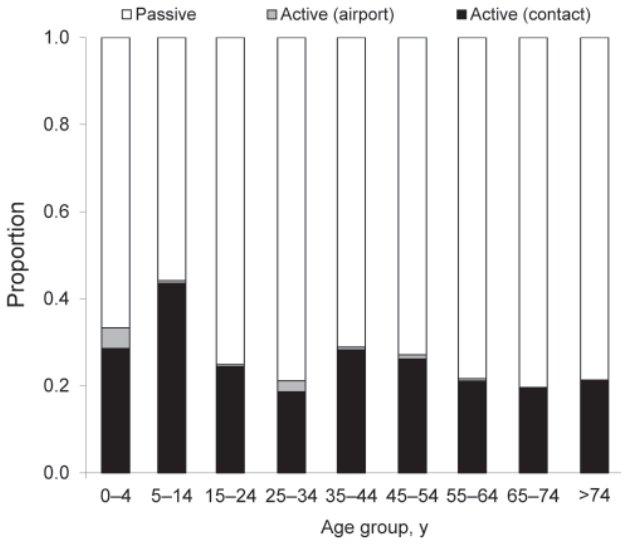

Figure 4. Age-specific distribution of case-patients with confirmed dengue virus infection (N = 2,087) detected by passive and active surveillance systems, Kaohsiung City, Taiwan, 2003–2009. Cases detected through passive surveillance were suspected dengue virus infections reported by health care facilities to Taiwan Center for Disease Control; cases detected through active surveillance were reported from airport screenings and by community contacts of case-patients.

group had the second highest proportion of cases detected through active surveillance (Figure 4); however, the overall detection rate remained far below that for all other age groups, suggesting generally lower rates of dengue virus exposure. This finding could be attributed to specific behavioral aspects of urban living in a high-income setting, such as Kaohsiung City, where, young children spend most of their day in enclosed air-conditioned environments at their home, day care center, or preschool. Hence, vector exposure may be substantially lower for young Taiwanese children than for most of their peers in other Southeast Asian countries.

The case-fatality rate of 26% for older age groups in this study is far greater than the expected average of <1% reported by WHO (*23*). However, our findings correspond with those in previous reports from Taiwan (*12*,*24*–*26*), where underlying chronic diseases more commonly observed among older persons (e.g., hypertension, chronic renal insufficiency [uremia], or diabetes mellitus) have been identified as possible risk factors for severe and fatal DHF (*27*,*28*). The association between age, the presence of underlying disease, and severe dengue virus infection and related death has also been reported from Cuba (*29*,*30*). However, these findings have been disputed by findings from Singapore, where elderly patients did not exhibit more signs or symptoms of dengue virus infection or have higher death rates despite having a greater incidence of underlying diseases (*31*). Information about the presence of underlying diseases was not available for our study.

Overall in Kaohsiung City, the incidence of dengue virus infection in the female population was slightly higher than that in the male population. This finding was not statistically significant, but it was in agreement with findings from previous studies conducted in Taiwan and contrary to findings from several other Asian countries where dengue is reported more frequently among the male population (*32*–*35*). These data suggest that the risk for exposure to dengue virus in Kaohsiung City is shared between sexes. It may also suggest that the combination of passive and active surveillance activities eliminates potential differences in health care–seeking behavior or health care access, as has been suggested for other Asian countries with lower reported rates of cases among the female population (*34*).

More than 25% of the confirmed dengue virus cases were detected through the active surveillance system. Most (96.7%) of these cases involved household members, neighbors, or colleagues of case-patients detected through the passive surveillance system. The 0- to 4-year-old and 5- to 14-year-old age groups had the highest proportion of cases (33.3% and 44.2%, respectively) detected through

Table 2. Comparison of reported clinical manifestations in persons with confirmed dengue virus infection (N = 2,087) detected through passive or active surveillance, Kaohsiung City, Taiwan, 2003–2009

Manifestation	No. passive (%), n = 1,549	No. active (%), n = 538	p value*
Fever	1,509 (97.4)	392 (72.9)	<0.001
Anorexia	916 (59.1)	218 (40.5)	<0.001
Headache	829 (53.5)	224 (41.6)	<0.001
Arthralgia	765 (49.4)	166 (30.9)	<0.001
Rash	760 (49.1)	209 (38.8)	<0.001
Myalgia	754 (48.7)	175 (32.5)	<0.001
Thirst	694 (44.8)	168 (31.2)	<0.001
Diarrhea	486 (31.4)	121 (22.5)	<0.001
Nausea	447(28.9)	95 (17.7)	<0.001
Pruritus	324 (20.9)	120 (22.3)	0.4941
Vomiting	296 (19.1)	53 (9.9)	<0.001
Retro-orbital pain	182 (11.7)	43 (8.0)	<0.001
Hemorrhagic manifestations	96 (6.2)	11 (2.0)	<0.001

*Proportional difference in reporting of specific manifestation by passive and active surveillance.

Table 3. Age-specific frequencies of reported clinical manifestations and average reported number of manifestations for confirmed dengue cases (N = 2,087) detected through passive or active surveillance, Kaohsiung City, Taiwan, 2003–2009

Variable	Age group, y								
	0–4	5–14	15–24	25–34	35–44	45–54	55–64	65–74	>74
Passive surveillance system									
No. cases	14	92	161	220	226	310	297	172	57
Average no. manifestations	3.4	5.0	5.7	5.9	5.5	5.1	4.9	4.5	3.6
Manifestation, no.									
Fever	1.0	1.0	1.0	1.0	1.0	1.0	1.0	0.9	0.9
Anorexia	0.4	0.7	0.6	0.6	0.6	0.6	0.6	0.6	0.6
Headache	0.1	0.5	0.7	0.6	0.6	0.6	0.5	0.4	0.3
Arthralgia	0.0	0.2	0.5	0.6	0.6	0.5	0.5	0.5	0.3
Rash	0.6	0.7	0.6	0.6	0.5	0.4	0.4	0.3	0.3
Myalgia	0.1	0.3	0.5	0.6	0.6	0.5	0.5	0.4	0.3
Thirst	0.3	0.3	0.4	0.5	0.5	0.5	0.5	0.4	0.2
Diarrhea	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Nausea	0.0	0.3	0.4	0.4	0.3	0.3	0.2	0.3	0.2
Pruritus	0.1	0.3	0.3	0.3	0.3	0.2	0.2	0.1	0.0
Vomiting	0.4	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
Retro-orbital pain	0.0	0.1	0.2	0.2	0.2	0.1	0.1	0.0	0.0
Hemorrhagic manifestations	0.0	0.0	0.1	0.1	0.0	0.1	0.1	0.1	0.1
Active surveillance system									
No. cases	7	72	55	59	92	113	80	42	18
Average no. manifestations	2.2	3.5	4.4	4.6	4.6	3.8	3.1	1.9	1.2
Manifestation, no.									
Fever	1.0	0.8	0.9	0.9	0.8	0.8	0.6	0.4	0.4
Anorexia	0.1	0.3	0.5	0.4	0.5	0.4	0.4	0.3	0.2
Headache	0.0	0.5	0.5	0.6	0.5	0.4	0.3	0.2	0.3
Arthralgia	0.0	0.2	0.2	0.4	0.5	0.4	0.3	0.2	0.2
Rash	0.4	0.5	0.5	0.5	0.4	0.4	0.3	0.1	0.0
Myalgia	0.0	0.2	0.3	0.5	0.5	0.4	0.3	0.1	0.1
Thirst	0.1	0.2	0.3	0.4	0.4	0.4	0.3	0.2	0.1
Diarrhea	0.3	0.2	0.2	0.3	0.3	0.2	0.2	0.1	0.1
Nausea	0.0	0.1	0.3	0.2	0.2	0.2	0.2	0.1	0.0
Pruritus	0.1	0.3	0.4	0.3	0.3	0.2	0.2	0.1	0.0
Vomiting	0.0	0.1	0.2	0.1	0.1	0.1	0.1	0.1	0.0
Retro-orbital pain	0.0	0.1	0.1	0.1	0.1	0.1	0.0	0.0	0.0
Hemorrhagic manifestations	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0

active surveillance, a finding in line with the lower number of reported symptoms (milder disease) and the possibly greater likelihood of being at home when the active surveillance nurse visited.

The inherent underreporting of dengue virus infections by passive surveillance, caused by mild and asymptomatic infections, is counterbalanced by the addition of the active surveillance component, although the level of impact remains unknown. High retrieval rates for convalescent-phase 2 and 3 samples (obtained for 455 [21.8%] of the 2,087 cases) ensure that all reported cases were thoroughly analyzed by molecular and serologic testing. However, the sensitivity and specificity of the surveillance and laboratory diagnostic systems and the overall cost-effectiveness of the surveillance and control program in Kaohsiung City must be assessed (*36*).

Elderly case-patients in both surveillance systems reported fewer symptoms, indicating the urgent need for improved diagnosis and treatment of severe dengue virus infection in this high-risk population. However, improved diagnosis and treatment would require better detection of cases that do not fit the currently used criteria for probable dengue virus infection. In addition, consideration would need to be given to the potential influence of underlying disease in treatment for severe dengue virus infection.

The incidence rates of dengue virus cases occurred nonrandomly throughout Kaohsiung City, implying that risk factors for dengue virus infection were spatially heterogeneous (Table 4). Hot-spot Lis were detected in different locations during consecutive years (Figure 5), although some hot spots recurred or were adjacent to other hot spots for the epidemics of 2004, 2006, 2008, and 2009. These hot spots were all shown to overlap with areas of high human residential density. Other hot spots and high-value outliers were detected in areas of low and high population density.

Table 4. Spatial autocorrelation of dengue incidence rates in Kaohsiung City, Taiwan, 2003–2009

Year	No. cases	Moran's I*	p value†
2003	62	0.09	<0.001
2004	45	0.08	<0.001
2005	99	0.04	<0.001
2006	766	0.14	<0.001
2007	153	0.03	<0.001
2008	326	0.10	<0.001
2009	636	0.09	<0.001

*An index of spatial autocorrelation coefficient.
†p<0.05 indicated that the spatial pattern was nonrandom.

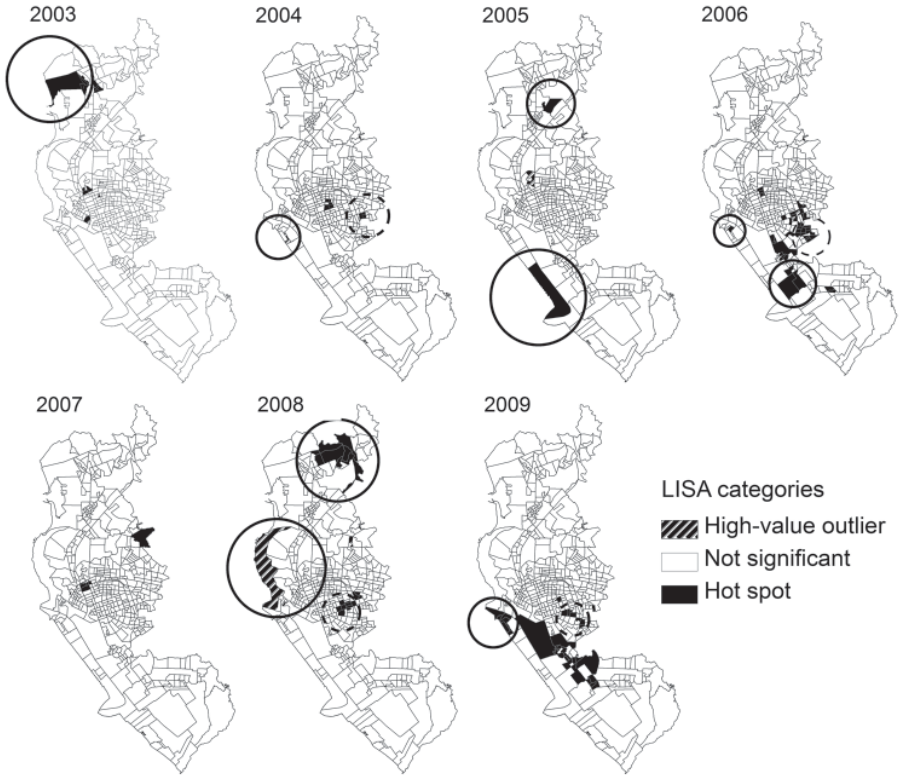

Figure 5. Local indicator of spatial association (LISA) cluster maps of incidence rates for dengue virus infection during each epidemic period, Kaohsiung City, Taiwan, 2003–2009. High-value outlier, high-incidence Li (smallest administrative unit within each of 11 districts in Kaohsiung City) surrounded by low-incidence Lis; not significant, 0 spatial autocorrelation presented; Hot spot, high-incidence Li surrounded by high-incidence Lis. Hot-spot Lis circled with dashed lines are those that overlap with clusters of high residential density; hot-spot or high-value outlier Lis circled with solid lines are those that overlap with clusters of low residential density.

The ambiguous correlation between the incidence of dengue virus infections and population density was also reported by Lin and Wen for the 2002 DENV-2 epidemic in Kaohsiung City (*37*), indicating that variations in population density are insufficient for explaining spatial variations in dengue virus outbreak intensity at the local level. To understand the observed variations and to predict local occurrence of future outbreaks, it is therefore necessary to account for additional spatial factors of potential importance.

Our findings on dengue virus outbreaks and high-risk population groups suggest the need for further research on demographic parameters, such as age distribution and age-dependent behavior at the local level. Variations in socioeconomic status, housing standards, and housing density should also be investigated for a potential role in disease clustering (*38–40*). In addition, the distinct seasonal transmission pattern of dengue virus shown in this study suggests further research into local environmental factors and control activities that influence vector survival and availability and productivity of vector breeding sites. Refined spatial analysis combining data for all spatial factors of identified importance could help identify imminent hot spots and guide improved prevention and control efforts.

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Nontuberculous Mycobacteria in Household Plumbing as Possible Cause of Chronic Rhinosinusitis

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Symptoms of chronic rhinosinusitis (CRS) often persist despite treatment. Because nontuberculous mycobacteria (NTM) are resistant to commonly used antimicrobial drugs and are found in drinking water that patients may use for sinus irrigation, we investigated whether some CRS patients were infected with NTM in New York, New York, USA, during 2001–2011. Two approaches were chosen: 1) records of NTM-infected CRS patients were reviewed to identify common features of infection and *Mycobacterium* species; 2) samples from plumbing in households of 8 NTM-infected patients were cultured for NTM presence. In 3 households sampled, *M. avium* sharing *rep*-PCR and pulsed field gel electrophoresis fingerprints identified *M. avium* isolates clonally related to the patients' isolates. We conclude that patients with treatment-resistant CRS may be infected with NTM and should have cultures performed for NTM so appropriate therapy can be instituted. In addition, the results suggest that CRS patients can be infected by NTM in their household plumbing.

A subset of patients with chronic rhinosinusitis (CRS) often experience persistent symptoms, despite undergoing many medical and surgical modes of treatment. Current theories regarding the cause of CRS include immunologic reactions to microorganisms (*1*,*2*). Even though they receive various treatments, including antimicrobial drugs and sinus irrigation, many patients continue to be symptomatic (*2*). One possible reason for the persistence of symptoms is the presence of microorganisms that are resistant to typically prescribed antimicrobial drugs, for example, nontuberculous mycobacteria (NTM).

Recovery of NTM from the sinus cavity has been documented in 19 patients, including those with cystic fibrosis (*3*), HIV infection (*4*–*10*), and diabetes (*11*). NTM isolation from the sinus cavity has been rarely reported in immunocompetent, nondiabetic patients who do not have cystic fibrosis (*12*–*15*). One case of infection with NTM is documented in a study by Spring and Miller (*14*). The patient had a 21-year history of rhinosinusitis and exhibited left maxillary facial pain, nasal discharge, and congestion. *Mycobacterium chelonae*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* were recovered from sinus cultures. Successful treatment ultimately required a 3-year course of multiple intravenous antimicrobial drug combinations and subsequent sinus operations (*14*). Recently, a new member of the *M. abscessus-chelonae* complex, *M. franklinii*, was isolated from patients in the northeastern United States who have chronic sinusitis (*16*).

NTM are environmental opportunistic pathogens found in natural and human-engineered waters, including drinking water distribution systems (*17*) and household plumbing (*18*–*20*). NTM species can be classified into 2 groups on the basis of growth rates; rapidly growing mycobacteria (e.g., *M. chelonae* and *M. abscessus*) form colonies in <7 days at 37°C, and slowly growing mycobacteria (e.g., *M. avium* and *M. intracellulare*) take >7 days at 37°C to form colonies. Because NTM are resistant to commonly used antimicrobial drugs (*21*) and are found in drinking water, they might be responsible for antimicrobial drug–resistant, chronic rhinosinusitis. We report the isolation, identification, and fingerprinting of NTM isolates from patients with CRS and from their household plumbing.

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Methods

Patient Histories

We reviewed the charts of 33 adult outpatients in whom CRS was diagnosed in the medical practice of W.S. Tichenor, whose endoscopically directed sinus cultures yielded NTM. The 33 represent ≈1% of patient samples collected over a 10-year period. In all patients, CRS had been diagnosed on the basis of a combination of initial evaluation; appearance of the sinuses by endoscopic examination; results of computed tomographic scan; and endoscopically directed cultures for bacteria, fungi, and NTM. From all patients, bacterial isolates had been cultured at the time of endoscopy.

Initial symptoms, NTM identity, surgical history, HIV status, cystic fibrosis history and carrier status, diabetes and immune-deficiency status, current nasal irrigations, presence of polyps, treatment, repeat culture results, and symptom reduction were assessed (Table 1). Common patient conditions at the time of nasal endoscopy included headache, nasal blockage or congestion, thick postnasal drip, and decreased ability to taste or smell. Thirty (91%) of the 33 patients had previously undergone endoscopic sinus surgery; 10 (30%) had histories of primary immunodeficiency. Twelve (36%) of the 33 patients had evidence of polyps at the time of nasal endoscopy; however, no clear association was found between NTM species and the presence of polyps. Thirty-one (94%) of 33 patients were using some form of nasal irrigation at the time of endoscopy. Of those, 26 were known to have used tap water to irrigate the sinuses.

Table 1. Characteristics of patients whose sinuses yielded NTM in study of NTM in household plumbing, New York, New York, USA, 2001–2011*

Characteristics	Value
Total patients	33 (100)
Age range, y	25–74
Prior functional endoscopic sinus surgery	30 (91)
Nasal polyps	12 (36)
Primary immunodeficiency	10 (30)
HIV positive	0†
Cystic fibrosis carrier state	1 (20)‡
Diabetes	0
Repeat culture NTM negative	21 (64)
Repeat culture NTM positive	2 (6)
Repeat culture not performed or lost	10 (30)
Symptoms improved	14 (42)
Symptoms unchanged	6 (18)
Other persistent microorganism§	1 (3)
Refused treatment	3 (9)
Currently treated	9 (27)

*Values are no. (%) patients except as indicated. NTM, nontuberculous mycobacteria.
†No patients were known to be HIV positive; 16 were tested.
‡No patients were known to have cystic fibrosis; 5 were tested.
§Methicillin-resistant *Staphylococcus aureus*.

Patient Sample Collections

Endoscopically directed samples were taken directly from the sinuses, middle meatus, or ostiomeatal unit by using a flexible catheter with a self-contained Lukens trap as described (*22*). Samples (0.5–3 mL) were sent to the microbiology laboratories (Mayo Medical Laboratories, Rochester, MN, USA; Specialty Laboratories, Valencia, CA, USA; Quest Laboratories, Peterboro, NJ, USA) in sterile 5-mL containers.

Household Collections

Members of households with occupants who had NTM-associated CRS volunteered to participate in studies of their households' water systems. Informed consent was obtained from each collaborating patient, and the study was reviewed by the Virginia Tech Institutional Research Board and granted exempt status. NTM isolates from the patients were obtained through laboratories that cultured NTM from endoscopy samples. Containers, swabs, and tubes were sent to each collaborating patient's household. Directions were provided for the collaborating patient or family member to collect hot and cold water samples (500 mL) and biofilms/sediment from water taps and showerheads. Biofilm samples were collected by swabbing the inside of taps and showerheads, and swab specimens were placed in tubes containing 2 mL of tap water (from Blacksburg, VA, USA), sterilized by autoclaving. If in-line or point-of-use water filters were submitted by the patients, a 4-cm^2 area was swabbed, and the swab was placed in 2 mL of sterile tap water.

NTM Isolation, Identification, and Fingerprinting

Patient NTM isolates were identified by various methods, depending on the laboratory: DNA probe, high-performance liquid chromatography, gas-liquid chromatography, internal transcribed spacer region or 16S rDNA sequencing. NTM in water and swab (taps and filters) samples were enumerated and isolated as described (*19*). Household NTM isolates and those from patients were identified by nested PCR of 16S rRNA gene (*23*) and PCR amplification and analysis of restriction endonuclease digestion fragments of the *hsp-65* gene (*24*). When the *Mycobacterium* species of the patient and household water system isolates were identical, isolates were fingerprinted by *rep*-PCR (*25*) and pulsed-field gel electrophoresis (PFGE) of *Ase*I and *Xba*I restriction endonuclease digests of genomic DNA (*26*). To interpret PFGE in categories of "indistinguishable," "closely related," and "different," we used previously described criteria for the evaluation of *Mycobacterium avium* complex (MAC) isolates (*27*). With a minimum of 10 interpretable bands, strains were interpreted as indistinguishable (no band differences), closely related (1–3 band differences), possibly related (4–6 band differences), and different (>7 band differences). These isolates underwent species confirmation by sequencing

of the internal transcribed spacer 1. *M. intracellulare* and *M. chimaera* are indistinguishable without gene/region sequencing (*28*).

Results

Review of the charts of the 33 CRS patients showed that 39 NTM isolates belonging to 10 *Mycobacterium* species were recovered from samples from the ostiomeatal unit or paranasal sinuses (Table 2). The patients' mycobacterial isolates were identified by Mayo Clinic, Quest, and Specialty Laboratories. Two different *Mycobacterium* species were isolated from 6 patient samples (Table 2). Most isolates (25 [64%] of 39) were rapidly growing mycobacteria, primarily *M. abscessus* or *M. chelonae*. One laboratory that received patient samples did not distinguish *M. abscessus* from *M. chelonae*. The predominant slowly growing *Mycobacterium* species was MAC (6 [15%] of 39). *M. gordonae* was isolated from 4 (12%) of the 33 patients. Although the organism is normally considered a saprophyte, *M. gordonae* infection has been reported in immunodeficient persons (*29*–*31*), and thus its isolation should not be dismissed.

Table 2. NTM isolated from sinus cavity samples of 33 patients in study of NTM in household plumbing, New York, New York, USA, 2001–2011*

NTM species	No. (%) patients
Mycobacterium abscessus-chelonae	19 (58)
M. chelonae	4 (12)
M. abscessus	2 (6)
M. avium	4 (12)
M. avium complex	2 (6)
M. immunogenum	1 (3)
M. asiaticum	1 (3)
M. mucogenicum	1 (3)
M. mageritense	1 (3)
M. gordonae	4 (12)

*NTM, nontuberculous mycobacteria.

NTM Isolates from Households of Current CRS Patients

A total of 80 samples (i.e., 43 water, 31 biofilm, and 6 from filters) for NTM isolation were received from the 8 collaborating CRS patients. NTM were isolated from water, biofilm, or filter samples from at least 1 sample from 5 (63%) of the 8 households sampled and from 35 (40%) of the 80 samples (Table 3). The frequency of NTM recovery from water (44%), biofilm (42%), and filter (50%) samples was not significantly different ($p = 0.6065$, Kruskal-Wallace test). NTM colony counts varied widely in samples from the different households (Table 4). In 4 households, at least 1 of the samples yielded an NTM isolate that was of the same species and had the same *rep*-PCR fingerprint as that of the patient according to published criteria (*25*) (Figure 1). The band patterns illustrate the large number and wide range of *rep*-PCR bands and illustrate the discrimination provided by *rep*-PCR fingerprinting (*25*). To confirm the relatedness between isolates from patient and household plumbing, PFGE was performed (*26*) for the same isolates (Figure 2). The PFGE band pattern of the isolate from patient 2 and the pattern from the patient's household (lanes 10 and 11) appear almost identical ("closely related"). The PFGE patterns for 2 isolates from the household of patient 5 were "indistinguishable" and are "closely related" (clonal) to the respective patient isolates and thereby clonal (Figure 2, panel A). Isolates from patient 8 and the patient's household plumbing (not shown) gave faint signals by PFGE with repeat testing and both restriction enzymes. However, the patterns appeared "indistinguishable"(profile not shown). The lack of clear band patterns for the isolates from patient 8 and his or her household plumbing is likely because of the shared characteristic of resistance to lysis in the agar plugs (*26*). The absence of a match for patient 1 (not shown) might be because the person moved throughout the United States, and some places where the patient lived were not sampled. Samples of showerheads were collected from 6 of the 8 households, and although NTM isolates of the same species as that of the patient (i.e., *M. avium*) were recovered from 2 households, none of the showerhead isolates shared the same fingerprint with isolates from the patient. Notably, the samples from the household plumbing of the patients with *M. gordonae* and *M. immunogenum* isolates did not yield any NTM.

Table 3. Recovery of NTM from households in study of NTM in household plumbing, New York, New York, USA, 2001–2011*

Patient household no.	Patient isolate	No. samples collected	No. (%) samples yielding NTM	Species found in patient household	*rep*-PCR match	PFGE match
1	*M. abscessus*	9	5 (55)	None	NA	NA
2	*M. avium*	9	4 (44)	Yes	Yes	Yes
3	*M. immunogenum*	10	0	NA	NA	NA
4	*M. gordonae*	5	2 (40)	Yes	No	–
5	*M. avium*	10	9 (90)	Yes	Yes	Yes
6	MAC-X†	21	7 (33)	Yes	Yes	No
7	*M. gordonae*	10	0	NA	NA	–
8	*M. avium*	14	8 (57)	Yes	Yes	Yes

*NTM, nontuberculous mycobacteria; NA, not applicable; MAC-X, *Mycobacterium avium* complex "X" cluster; PFGE, pulsed-field gel electrophoresis.
†MAC-X is a mycobacterium that tests positive by DNA probe analysis for *M. avium* complex but is negative with specific *M. avium* and *M. Intracellulare* probes and PCR analysis.

Table 4. Numbers of NTM in household samples in study of NTM in household plumbing, New York, New York, USA, 2001–2011*

Patient household no.	Water		Biofilm	
	No.	Average CFU/mL	No.	Average CFU/cm^2
1	4	5,632 ± 3,372	2	36,000 ± 49,500
2	5	49 ± 18		6 ± 2
3	0		0	
4	1		0	
5	13	420 ± 1,000	8	23,310 ± 41,700
6	3	17,052 ± 11,200	11	21,100 ± 27,700
7	0		0	
8	7	27 ± 26	8	513 ± 632
Total	33	2,487	31	13,835

*NTM, nontuberculous mycobacteria.

Discussion

Our study confirms the possibility of the involvement of NTM in sinuses of patients with CRS (*3*,*11*,*16*). CRS patients who have not responded to medical treatment should undergo endoscopically directed sinus cultures for microorganisms, including fungi and NTM and other bacteria. Endoscopically directed sinus cultures have been shown to accurately replicate sinus puncture culture techniques (*22*). The American Thoracic Society and the Infectious Diseases Society of America discourage the use of swabs for sampling because swabs may decrease the likelihood of recovering NTM (*21*). Using a suction device to remove larger volumes of mucus helps increase the chances of obtaining representative sinus microflora (*22*). Spurious recovery of NTM, because of endoscope contamination, is possible (*32*), as is the possibility that glutaraldehyde may not adequately kill NTM (*33*). However, in the current study, endoscope contamination is an unlikely source of NTM because water samples from the physician's office did not reveal NTM. In addition, the patient and household samples were processed in different laboratories.

Besides establishing NTM as a potential agent of CRS, our results strongly suggest that in 3 of the 8 CRS patients studied here, the household plumbing was the source of infection, on the basis of identity of *rep*-PCR fingerprints of patient and household isolates and their clonal relatedness as determined by PFGE. Clonal variation in *Mycobacterium* species isolates is characteristic of isolates recovered from household plumbing, but because single *Mycobacterium* species isolates are typically recovered from patient samples, DNA fingerprint matches are not always obtained (*19*,*20*). A study of persons with NTM pulmonary disease found that in 7 (41%) of 17 households, patient and household plumbing isolates were identical as shown by *rep*-PCR fingerprints (*20*). Because NTM are found in household tap water (*19*,*20*,*34*), CRS patients should avoid sinus irrigation with unsterilized tap water.

A major question concerning isolation of NTM from the sinus cavities of patients with CRS is whether NTM were involved in pathogenesis. No guidance exists for the diagnosis and treatment of NTM sinus infection. For pulmonary NTM disease, it is recommended that multiple cultures be obtained over time (*21*) to rule out transient colonization and avoid sampling deficiencies. Our experience suggests that multiple cultures may be necessary to find NTM because endoscopy samples from many patients will be found NTM positive only by 1 of 2–5 endoscopies. For example, 1 patient had cultures that yielded *M. mageritense*, but cultures obtained 1 week later were negative, even in the absence of antibacterial drug treatment. In addition, smears from 2 patients showed acid-fast bacilli, but cultures failed to yield any *Mycobacterium* species isolate; yet upon subsequent endoscopy, NTM

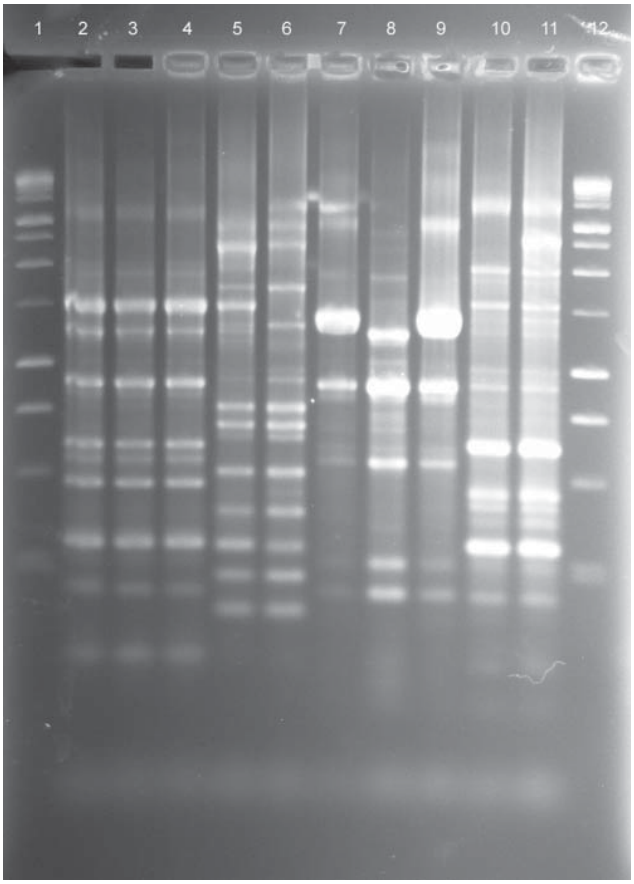

Figure 1. *rep*-PCR fingerprint patterns of patient and household isolates, New York, New York, USA, 2001-2011. Lane 1, 100-bp ladder; lane 2, patient no. 5 *Mycobacterium avium* isolate AG-P-1; lane 3, patient no. 5 household filter *M. avium* isolate AG-F-2–0-2; lane 4, patient no. 5 household filter *M. avium* isolate AG-F-2-I-1; lane 5, patient no. 6 *M. avium* complex "X" cluster isolate GG-P-1; lane 6, patient no. 6 household swab *M. chimaera* isolate GG-Sw-9–1; lane 7, patient no. 8 *M. avium* isolate GW-P-1; lane 8, patient no. 8 household water *M. avium* isolate GW-W-1–1; lane 9, patient no. 8 household swab *M avium* isolate GW-Sw-7–2; lane 10, patient no. 2 *M. avium* isolate BB-P-1; lane 11, patient no. 2 household water *M. avium* isolate BB-W-4–5; lane 12, 100-bp ladder.

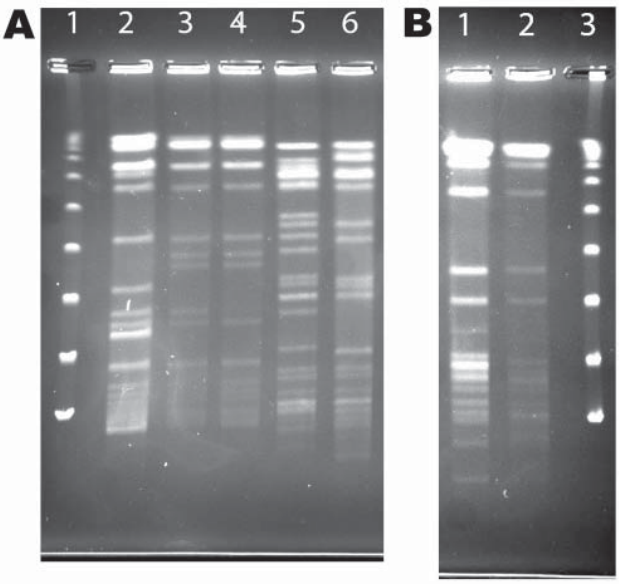

Figure 2. Pulsed-field gel electrophoresis (PFGE) of *Ase*I digest patterns of patient and household isolates, New York, New York, USA, 2001–2011. A) Patient and household isolates. Lane 1, λ ladder; lane 2, patient no. 5 *Mycobacterium avium* isolate AG-P-1; lane 3, patient no. 5 household filter *M. avium* isolate AG-F-2-0-2; lane 4, patient no. 5 household filter *M. avium* isolate AG-F-2-I-1 (environmental isolates in lanes 3 and 4 are indistinguishable; patient isolate in lane 2 considered clonal with 2 environmental isolates [6 bands difference]); with digestion with *Xba*I, the 3 were considered closely related.); lane 5, patient no. 6 *M. avium* complex "X" cluster isolate GG-P-1; lane 6, patient no. 6 household swab *M. chimaera* isolate GG-Sw-9–1 (despite overall similarity, isolates in lanes 5 and 6 belong to different species and differ by 10 bands and are therefore unrelated). B) Additional patient and isolate from the person's household. Lane 1, patient no. 2 *M. avium* isolate BB-P-1; lane 2, patient no. 2 household water *M. avium* isolate BB-W-4–5; lane 3, λ ladder.

were cultured. Several possible reasons could account for this low yield. First, hydrophobic NTM cells are likely adhering to the walls of the sinus cavity, and thereby a low number are in fluid removed during endoscopy. Second, the small volume of mucus removed at the time of culture also might reduce the likelihood of recovering NTM (*22*). Third, topical anesthetics, typically lidocaine, are used for anesthesia for endoscopy and might inhibit the growth of many microorganisms, including NTM (*35*). Although NTM could merely be colonizing the sinuses, several factors suggest otherwise. The samples that we collected were primarily mucus, and previously published reports on NTM in sinus samples from immunocompromised CRS patients were primarily based on biopsy specimens (*3*,*6*,*15*). In addition, our patients typically have persistent symptoms despite treatment with multiple different antimicrobial drug regimens over several months. Because the results of NTM culture and sensitivity testing take several months to obtain, patients are typically treated for other possible infecting microorganisms until the results of the NTM cultures are reported. Resolution typically occurred only after an extended course of multiple antimycobacterial agents given simultaneously. Unfortunately, the combination of insufficient experience and the absence of an established treatment protocol for CRS caused by NTM (*21*), prevent any meaningful review of treatment regimens for CRS caused by NTM. Such patients are treated with 2 oral antimycobacterial drugs and urged to irrigate sinuses with sterile or boiled water or saline, followed by irrigation with a topical antimycobacterial agent for 3–18 months, depending on clinical response and, in some cases, on subsequent positive cultures for NTM

The role of NTM in infectious disease processes is only starting to be recognized. This work documents that a proportion of patients with CRS could be infected with NTM and that sinus samples should be cultured for NTM. In addition, CRS patients should avoid sinus irrigation with tap water because tap water may contain NTM, and it may not be possible to remove it. Sterile saline should be used instead.

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Autochthonous and Dormant *Cryptococcus gattii* Infections in Europe

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Until recently, *Cryptococcus gattii* infections occurred mainly in tropical and subtropical climate zones. However, during the past decade, *C. gattii* infections in humans and animals in Europe have increased. To determine whether the infections in Europe were acquired from an autochthonous source or associated with travel, we used multilocus sequence typing to compare 100 isolates from Europe (57 from 40 human patients, 22 from the environment, and 21 from animals) with 191 isolates from around the world. Of the 57 human patient isolates, 47 (83%) were obtained since 1995. Among the 40 patients, 24 (60%) probably acquired the *C. gattii* infection outside Europe; the remaining 16 (40%) probably acquired the infection within Europe. Human patient isolates from Mediterranean Europe clustered into a distinct genotype with animal and environmental isolates. These results indicate that reactivation of dormant *C. gattii* infections can occur many years after the infectious agent was acquired elsewhere.

During the past decade, the basidiomycetous yeast *Cryptococcus gattii* has stepped out of the shadows of its sibling *C. neoformans*. The latter species mainly infects immunocompromised persons, and *C. gattii* mainly infects apparently immunocompetent persons. *C. neoformans* is found globally, and *C. gattii* has been mostly limited to tropical and subtropical areas in Central Africa, northern Australia, and Central and South America (*1*). However, this distribution pattern changed after an unprecedented outbreak of *C. gattii* emerged in the temperate climate of British Columbia, Canada, and expanded to the Pacific Northwest region of Canada and the United States (*1*,*2*). Epidemiologic studies have shown that *C. gattii* occurs in areas other than tropical or subtropical zones, such as in Mediterranean Europe, northern Europe, and western Australia (*3*–*5*).

For the purpose of studying the epidemiology of *C. gattii*, a broad variety of molecular biological techniques have been developed, including PCR fingerprinting, restriction fragment length polymorphism analysis of the *PLB1* and *URA5* loci, amplified fragment length polymorphism (AFLP) fingerprint analysis, and several multilocus sequence typing (MLST) approaches (*6*–*9*). These laboratory investigations have shown that *C. gattii* can be divided into 5 distinct genotypes: AFLP4/VGI, AFLP5/VGIII, AFLP6/VGII, AFLP7/VGIV, and AFLP10/VGIV (*8*,*9*). Serotype B strains occur in genotypes AFLP4/VGI, AFLP6/VGII, and AFLP10/VGIV; serotype C strains are restricted to genotypes AFLP5/VGIII and AFLP7/VGIV (*8*).

Recently, a consensus MLST scheme was proposed for epidemiologic investigations of *C. gattii* and *C. neoformans*, specifically, the nuclear loci *CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5* (*9*). So far, this consensus MLST scheme has been used to study the population structure of *C. neoformans* strains from Thailand and *C. gattii* strains from Australia (*3*,*10*).

We investigated the occurrence of *C. gattii* in Europe, focusing on whether this pathogen is emerging and, if so, how to explain this emergence pattern. Furthermore, we

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explored whether the infections originated from Europe or were introduced from other continents. To achieve these goals, members of the European Confederation of Medical Mycology were asked to send recently obtained human patient isolates of the species for detailed AFLP and MLST analyses. Thus, the genetic diversity of the yeast was used to trace its geographic origin to identify where the infections were acquired. AFLP genotyping results were combined with published *C. gattii* MLST results from Byrnes et al. (*11*) and Fraser et al. (*12*), which were extended to match the *Cryptococcus* consensus MLST scheme (*9*). Our study produced the following 5 conclusions: all hitherto known genotypes of *C. gattii* are emerging in Europe; genotype AFLP4/VGI isolates predominate; a *C. gattii* cluster, which is endemic to Mediterranean Europe and genetically distinct from the other populations, exists; several human infections are caused by travel-related acquisition of *C. gattii* outside Europe; and autochthonous cases occur in Europe.

Materials and Methods

Strains and Media

We compared a collection of 107 isolates collected from Europe with 194 isolates collected globally (Table 1; online Technical Appendix Table 1, wwwnc.cdc.gov/EID/pdfs/12-0068-Techapp.pdf). All isolates were checked for purity and cultivated on malt extract agar medium (Oxoid, Basingstoke, UK). Cultures were incubated for 2 days at 30°C. A working collection was made by growing *C. gattii* strains on malt extract agar slants for 2 days at 30°C, after which the strains were stored at 4°C. Strains were stored long term at −80°C by using the Microbank system (Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada).

Amplification and Sequencing of MLST Loci

Genomic DNA extraction, AFLP genotyping, and mating-type determination by amplification of either the *STE12***a** or *STE12*α locus were performed as described (*8*). The 7 nuclear consensus MLST loci (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*) were amplified by using the preferred primer combinations (*9*). To compare the current set of *C. gattii* strains with those from a published *C. gattii* population biology study, we included the 3 nuclear loci that are not part of the consensus MLST scheme (*CAP10*, *MPD1*, and *TEF1*α) (*12*). We also included isolates from a global study by Byrnes et al. (*11*) and Fraser et al. (*12*) and subjected them to amplification and sequencing of the *CAP59*, *SOD1*, and *URA5* loci.

Amplifications were conducted in a 25-μL PCR mixture containing 37.5 mmol/L $MgCl_2$ (Bioline, London, UK), 1× PCR buffer (Bioline), 1.9 mmol/L dNTPs (Bioline), 0.5 U Taq DNA polymerase (Bioline), 5 pmol of both primers (Biolegio, Nijmegen, the Netherlands) (online Technical Appendix Table 2), and ≈100 ng of genomic DNA. PCRs were conducted with an initial denaturation step at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30s, annealing for 30s (see Technical Appendix Table 2 for optimal annealing temperatures), extension at 72°C for 1 min, followed by 72°C for 5 min and a final dwell at 21°C.

Sequencing reactions were conducted with the BigDye version 3.1 chemistry kit (Applied Biosystems, Foster City, CA, USA) as described (*13*). For all amplification products except *CAP59*, the initial amplification primers were used for sequencing reactions. For *CAP59*, the newly designed forward primer CAP59L-Fwd and the original reverse primer JOHE15438 (*12*) were used.

Sequence Alignment and Phylogenetic and Recombination Analyses

Consensus sequences were assembled and checked for ambiguities by using SeqMan version 8.0.2 (DNASTAR, Madison, WI, USA). Sequence alignments were generated with MEGA version 5 (*14*) by using the standard settings and manual correction. The genome sequence databases of reference strains H99 (culture collection no. CBS8710; *C. neoformans* variety *grubii*; AFLP1/VNI; Broad Institute [www.broadinstitute.org/annotation/genome/cryptococcus_neoformans/]) and JEC21 (culture collection no. CBS10513; *C. neoformans* variety *neoformans*; AFLP2/VNIV; Stanford Genome Technology Center [www-sequence.stanford.edu/group/C.neoformans/]) were used to extract the corresponding sequences for all 10 investigated nuclear loci to serve as an outgroup. The best fitting nucleotide substitution model was determined by using MrModeltest version 2 (*15*) and was conducted for the complete *C. gattii* 10-loci MLST, for the accepted consensus MLST scheme (*CAP59*, *GPD1*, IGS1, *LAC1*, *PLB1*, *SOD1*, and *URA5*) (*9*), and a previously launched *C. gattii* MLST scheme (*CAP10*, *GPD1*, IGS1, *LAC1*, *MPD1*, *PLB1*, and *TEF1*) (*12*). As a result, the HKY G+I model (Hasegawa-Kishino-Yano plus gamma distributed with invariant sites) was the best model to use for analyzing the phylogeny of the *C. gattii* isolates for all 3 datasets. The evolutionary history was inferred by using the maximum-likelihood method in MEGA version 5 (*14*). A bootstrap consensus tree was inferred from 1,000 replicates to show the relevant lineages obtained in this analysis.

We calculated the haplotype diversity (H_R), equal to the Simpson diversity index (*D*), by using the Microsoft Excel (Microsoft, Redmond, WA, USA) add-in called Haplotype Analysis (*16*). For this purpose, sequences were collapsed into sequence type numbers (online Technical Appendix Table 1).

Results

AFLP Genotypes and Geographic Distribution

The 301 *C. gattii* isolates collected from Europe and other areas around the world could be divided into the following genotypes: 146 AFLP4/VGI (50.2%; 72 from Europe), 22 AFLP5/VGIII (7.6%; 1 from Europe), 108 AFLP6/VGII (37.1%; 23 from Europe), 13 AFLP7/VGIV (4.5%; 2 from Europe), and 2 AFLP10/VGIV (0.7%; both from Europe). From 10 isolates (7 AFLP8 [5 from Europe] and 3 AFLP9 [2 from Europe]), genotypes represented interspecies *C. neoformans* × *C. gattii* hybrids. These 10 isolates were excluded from further analysis because amplified fragments of hybrid isolates will result in mixtures of different alleles. The 57 human patient isolates from Europe were obtained from 40 patients (online Technical Appendix Table 1). The genotypic diversity of the remaining 291 *C. gattii* isolates (100 from Europe, 191 from other areas) with a haploid genotype is shown in the Table.

Phylogeographic Origin

Sequence type diversity was calculated for each of the MLST loci, all 10 loci, and the combined datasets according to Fraser et al. (*12*) and Meyer et al. (*9*) (online Technical Appendix Table 3). The *CAP10* locus showed the lowest overall diversity (n_{ST} = 19; D_{ST} = 0.765) and the IGS1 locus showed the highest diversity (n_{ST} = 52; D_{ST} = 0.930). The 10-loci MLST dataset showed the highest diversity (n_{ST} = 150; D_{ST} = 0.975), followed by the MLST scheme of Meyer et al. (D_{ST} = 0.971) (*9*) and Fraser et al. (D_{ST} of 0.959) (*12*). The latter MLST scheme differentiated more sequence types than the consensus MLST scheme (n_{ST} = 136 vs. 127).

Maximum-likelihood analysis of the MLST data showed that the *C. gattii* isolates clustered in 5 monophyletic clusters, which were highly supported and agreed with the AFLP genotypes (Figure; online Technical Appendix Figure). When each genotypic cluster was separately analyzed, support values of the branches were low, <75

Table. Distribution of *Cryptococcus gattii* strains*

Source of isolation	Genotype					Total†	Total (%)
	AFLP4/VGI	AFLP5/VGIII	AFLP6/VGII	AFLP7/VGIV	AFLP10/VGIV		
All *C. gattii* isolates	146	22	108	13	2	0	291 (100)
Human	84	16	68	12	2	0	182 (62.5)‡
Environment	37	5	17	0	0	0	59 (20.3)‡
Animal	24	0	23	1	0	0	48 (16.5)‡
Unknown	1	1	0	0	0	0	2 (0.7)‡
Africa							
Human	18	0	3	8	0	29	36 (12.7)§
Environment	6	0	0	0	0	6	
Animal	0	0	0	1	0	1	
Asia (clinical)	19	0	5	2	0	26	26 (8.9)§
Australia							
Human	2	1	8	0	0	11	18 (6.2)§
Environment	5	0	1	0	0	6	
Animal	0	0	1	0	0	1	
Europe¶							
Human	29#	1**	23††	2‡‡	2§§	57	100 (34.4)§
Environment	22	0	0	0	0	22	
Animal	21	0	0	0	0	21	
North America¶							
Human	3	8	19	0	0	30	69 (23.7)§
Environment	4	3	10	0	0	17	
Animal	2	0	20	0	0	22	
South America¶							
Human	12	3	10	0	0	25	36 (12.4)§
Environment	0	2	6	0	0	8	
Animal	1	0	2	0	0	3	
Unknown							
Human	1	3	0	0	0	4	6 (2.1)§
Unknown	1	1	0	0	0	2	

*The number of *C. gattii* isolates is provided for each geographic area and further subdivided per genotype and source of isolation. AFLP, amplified fragment length polymorphism; human, human clinical patient.
†Total number of isolates per geographic area.
‡The percentage of isolates is given for the source of isolation subset.
§The percentage of isolates is given as a percentage of all isolates.
¶Ten interspecies hybrid *C. gattii* × *C. neoformans* isolates were excluded from the set of isolates from Europe (n = 7), North America (n = 1), and South America (n = 2).
#Twelve patients with an autochthonous acquired infection (16 isolates) and 11 patients with an infection acquired outside Europe (13 isolates).
**Infection acquired outside Europe.
††Four patients with an autochthonous infection (10 isolates) and 10 patients with an infection acquired outside Europe (13 isolates).
‡‡Two phenotypically different isolates from an emigrant from Zambia.
§§Two phenotypically different isolates from an emigrant from Mexico.

(online Technical Appendix Figure). For genotype AFLP4/VGI isolates, bootstrap support values for nearly all branches were low; for the second largest group formed by AFLP6/VGII isolates, branches were better supported.

Phylogenetic analysis demonstrated that several human patient genotype AFLP4/VGI isolates from Europe had an autochthonous origin because they formed a separate cluster with environmental and animal isolates from the same area. A set of isolates from human patients on the Iberian Peninsula (CCA232, CCA242L, CCA242T, CCA311, CCA312, CL6148) were found to be genetically indistinguishable from isolates obtained from animals or the environment in the same area (indicated in online Technical Appendix Figure as the European Mediterranean cluster). The human patient isolates from Europe with genotype AFLP4/VGI, which were probably acquired within Europe because patients did not have histories of travel outside Europe, are IP2005/215 and IP2006/958 (France), RKI85/888 (Germany), 5UM and 75UM (Italy), RKI97/482 (Portugal), and CBS2502 (the Netherlands). Some of these human patient isolates were closely related (e.g., IP2005/215 and RKI97/482) or even genetically indistinguishable (e.g., CBS2502 and RKI85/888).

A large proportion of the human patient and environmental isolates of *C. gattii* AFLP4/VGI from Italy and Spain formed a novel autochthonous Mediterranean MLST cluster that was genetically homogeneous, irrespective of their origin or mating type (Figure, online Technical Appendix Figure). *C. gattii* AFLP4/VGI was involved with numerous small outbreaks among goats in Spain, and the isolates were genetically indistinguishable from recently obtained human patient, animal, and environmental isolates from different provinces in Spain as well as from AFLP4/VGI mating-type **a** isolates from Italy (online Technical Appendix Figure).

Several *C. gattii* genotype AFLP6/VGII infections were found to have originated in Europe (e.g., the IP1998/1037–1 and –2, IP2003/125, and CCA242 isolates), and all fell within a cluster that could not be linked to an environmental source. The same holds true for the human patient isolates from Greece (AV54S, –W, and IUM01–4731), which came from the same patient who had no history of travel outside Greece.

In the phylogenetic analysis, isolates from human patients in Europe were also observed next to those originating from other geographic areas. The most striking example was a set of 4 human patient isolates from citizens of Denmark, the Netherlands, Germany, and Switzerland, in whom cryptococcosis developed after they had visited Vancouver Island, British Columbia, Canada. The isolates obtained from these tourists (CBS10485, RKI06/496, RKI01/774, and CBS10866, respectively) had MLST profiles identical to that of *C. gattii* AFLP6A/VGIIa, the genotype that caused the Vancouver Island outbreak (Figure 1; online Technical Appendix Figure) (*17*–*19*). The outbreak-related sets of AFLP6A/VGIIa and AFLP6C/VGIIc isolates from the Vancouver Island and Pacific Northwest outbreaks, respectively, are within outbreak-specific clusters (Figure, online Technical Appendix Figure). A similar finding was observed for a set of 5 human patient *C. gattii* AFLP6B/VGIIb isolates (IP1996/1120–1 and –2, IP1999/901–1 and –2, and IP2000/87) in France,

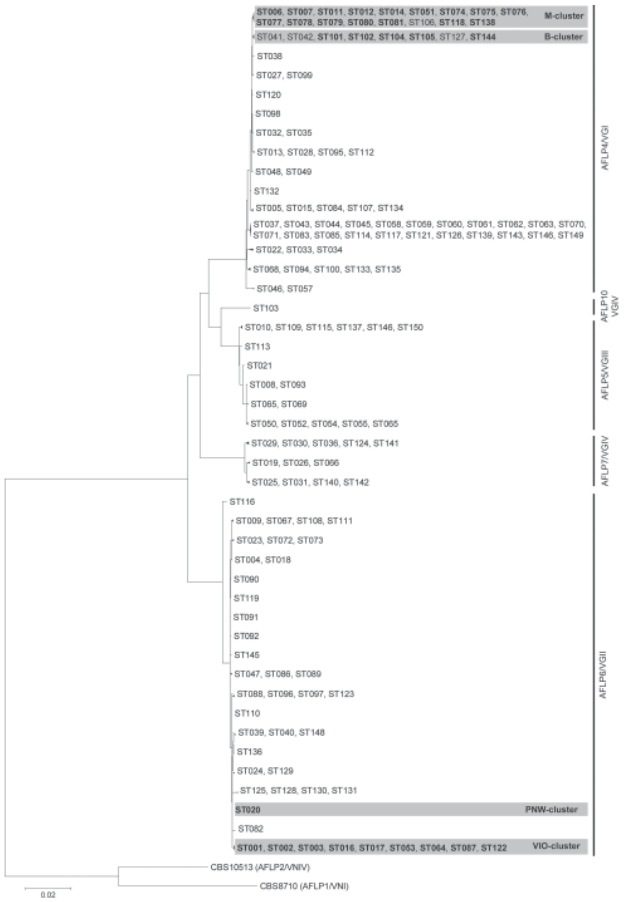

Figure. Maximum-likelihood phylogenetic analysis based on 10-loci multilocus sequence type data of *Cryptococcus gattii* isolates (condensed). Phylogenetic relatedness of 150 STs representing the 291 *C. gattii* isolates, calculated by using the maximum-likelihood algorithm and rooted by using the 2 *C. neoformans* reference strains CBS8710 (genotype AFLP1/VNI) and CBS10513 (genotype AFLP2/VNIV). Closely related sequence types were collapsed into 1 branch shown by multiple sequence type numbers. **Boldface** indicates sequence types that are within a shaded area belong to a specified *C. gattii* cluster; B, M, PNW, and VIO represent clusters from Brazil; Mediterranean Europe; the US Pacific Northwest outbreak; and the Vancouver Island, British Columbia, Canada, outbreak, respectively. AFLP, amplified fragment length polymorphism; ST, sequence type. Scale bar indicates number of substitutions per site. See online Technical Appendix Figure (wwwnc.cdc.gov/EID/pdfs/12-0068-Techapp.pdf) for a detailed phylogenetic analysis.

which had been obtained from patients who had emigrated from Africa to France and which were genetically indistinguishable from an isolate (IP2001/935–1) from a resident of Senegal (online Technical Appendix Figure). This set of 5 isolates from France and 1 from Senegal were closely related to isolates from the Vancouver Island and Pacific Northwest outbreaks; however, it seems unlikely that the patient from Senegal had traveled to these outbreak areas, and none of the patients in France reported having traveled to Canada or the United States.

A set of *C. gattii* AFLP4/VGI mating-type α isolates from Portugal (IP1997/18) and Belgium (IHEM19725B and –S) was found to be indistinguishable from human patient isolates from the Democratic Republic of the Congo and Rwanda (B3939 and CBS6289, which were both mating-type **a**, and IHEM10602S, IHEM10769S, and IHEM10769W, which were mating-type α) (Figure; online Technical Appendix Figure). Both phenotypically different isolates of IHEM19725 were obtained from an HIV-infected patient from Rwanda who had emigrated to Belgium. The CBS1622 isolate from Europe, obtained from a patient with the oldest documented case of a *C. gattii* infection (*20*), was found to be genetically indistinguishable from a set of isolates from North America.

The single *C. gattii* AFLP5/VGIII isolate from a 24-year-old immunocompetent patient from Germany (isolate RKI97/310) (*21*) clustered with human patient and environmental isolates from Mexico (online Technical Appendix Figure). The 2 *C. gattii* AFLP7/VGIV isolates CBS7952D and CBS7952S, obtained from an HIV-infected patient in Sweden, were genetically indistinguishable from each other and had a unique MLST profile that clustered with human patient isolates from Africa. According to the patient's history, years before the onset of cryptococcal infection, she had emigrated from Zambia to Sweden (online Technical Appendix Figure). Another example of a reactivated dormant *C. gattii* infection is that of the human patient isolate from Italy, IUM92–6682 (AFLP4/VGI), obtained from an immunocompetent immigrant from Brazil, which had an identical MLST profile to 6 human patient mating-type α isolates (IHEM14934, IHEM14956, IHEM14965, IHEM14968, IHEM14976, and IHEM14984) from Brazil (Figure, online Technical Appendix Figure).

Infection was acquired outside Europe for 24 of these patients (31 isolates) and within Europe for 16 patients (26 isolates) (Table; online Technical Appendix Figure). Among these 57 human patient isolates, most (47 [82.5%]) were obtained since 1995, the remaining 10 (17.5%) were isolated during 1895–1994. One of these isolates originated from 1985, another 2 isolates (CBS1622 and CBS2502), from 1895 and 1957, were retrospectively found to represent *C. gattii* isolates from Europe (*20*,*22*).

Discussion

In Europe, *C. gattii* has been reported as a rare cause of apparently autochthonous cryptococcal infections (*1*). The earliest documented case that turned out to be caused by *C. gattii* in Europe was made by Curtis in 1896 (*20*). Until the 1980s, cryptococcosis was rarely observed in Europe and *C. gattii* infections were especially rare; only 2 cases have retrospectively been found (*20*,*22*). In the 1980s, infections were reported for 2 immunocompetent citizens of Germany, who had never traveled abroad (*23*,*24*). Subsequent case reports described *C. gattii* infections that were imported or acquired in Europe (*25*,*26*). Since 1995, the number of reports of *C. gattii* infections increased and describe *C. gattii* infections in humans and animals from Greece (*27*), Italy (*28*,*29*), and Spain (*5*,*30*–*33*). In the current study, 8 (20%) cases were observed until 1995, and 32 (80%) cases in humans have been observed since 1995. We excluded cases for which an isolate was not available for confirmation. This exclusion is especially relevant in that we re-identified a reported *C. gattii* infection as actually being caused by *C. neoformans* (*34*). These data suggest that during the past 2 decades, *C. gattii* has been emerging in Europe.

In the current study, 16 (40%) of the human patient isolates from Europe were found to have an autochthonous origin in Europe that either could be linked to environmental isolates from the Mediterranean area or that came from patients who had never traveled outside their resident country. A total of 24 (60%) isolates could be linked to *C. gattii*–endemic regions in Brazil, the United States, Africa, and the Vancouver Island outbreak region. These observations demonstrate that *C. gattii* infections can be imported subclinically and can cause infections after being dormant for many years.

Nearly all isolates from Mediterranean Europe belonged to genotype AFLP4/VGI, and MLST analysis showed that these isolates form a separate cluster within this genotype (Figure; online Technical Appendix Figure) (*5*). *C. gattii* genotype AFLP4/VGI has also recently been reported from the environment in the Netherlands, but these isolates were not similar to any of the AFLP4/VGI isolates from Mediterranean Europe or to isolate CBS2502, which was isolated in 1957 from a pregnant citizen of the Netherlands, who had never traveled abroad but who died of cryptococcosis (*4*). Isolate CBS2502 is genotypically identical to isolate RKI85/888, which was isolated from a previously healthy citizen of Germany, who also had never traveled outside Germany. These 2 cases strongly suggest that different genotypes of *C. gattii* AFLP4/VGI occur in the environment of northwestern Europe because isolates from patients were genetically different from the recently reported isolates from the environment of the Netherlands (this study; *4*).

In conclusion, *C. gattii* is emerging in Europe, and the isolates from Europe can be divided into 5 genotypic clusters. Most *C. gattii* infections in Europe are probably autochthonous, and several infections are proven to have been acquired outside the European continent, e.g., during visits to *C. gattii*–endemic regions, such as the Vancouver Island outbreak area or before migration to Europe from *C. gattii*–endemic regions in Africa and South America. Reactivation of dormant *C. gattii* infections and of infections acquired outside Europe after immune suppression occurs more often than previously assumed. This finding might suggest that *C. gattii* infections caused by certain genotypes are associated with altered immune status of the human host. Thus, *C. gattii* is probably a more opportunistic pathogen, as has recently been hypothesized, than a strictly primary pathogen (*18*,*35*). *C. gattii* isolates with genotypes AFLP5/VGIII and AFLP7/VGIV were rarely found in Europe and were all acquired outside the European continent. However, these genotypes have frequently been isolated from HIV-infected persons and other immunocompromised patients in Africa and the American continents (*1*,*7*,*36*). A connection seems to exist between these *C. gattii* genotypes and the host's immune status. Further epidemiologic and immunologic research is needed to unravel this apparent correlation.

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Echinococcus multilocularis in Urban Coyotes, Alberta, Canada

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Echinococcus multilocularis is a zoonotic parasite in wild canids. We determined its frequency in urban coyotes (*Canis latrans*) in Alberta, Canada. We detected *E. multilocularis* in 23 of 91 coyotes in this region. This parasite is a public health concern throughout the Northern Hemisphere, partly because of increased urbanization of wild canids.

Echinococcus multilocularis is the causative agent of alveolar echinococcosis in humans. This disease is a serious problem because it requires costly long-term therapy, has high case-fatality rate, and is increasing in incidence in Europe (*1*). This parasitic cestode has a predominantly wild animal cycle involving foxes (*Vulpes* spp.) and other wild canids, including coyotes (*Canis latrans*), as definitive hosts. However, it can also establish an anthropogenic life cycle in which dogs and cats are the final hosts. Rodents are the primary intermediate hosts in which the alveolar/multivesicular hydatid cysts grow and are often fatal. Humans are aberrant intermediate hosts for *E. multilocularis* (*2*).

In North America, *E. multilocularis* was believed to be restricted to the northern tundra zone of Alaska, USA, and Canada until it was reported in red foxes (*Vulpes vulpes*) from North Dakota, USA (*3*). This parasite has now been reported in the southern half of 3 provinces in Canada (Manitoba, Saskatchewan, and Alberta) and in 13 contiguous states in the United States (*1*).

Foxes are the traditional definitive hosts for *E. multilocularis* worldwide. However, in North America, coyotes may be prominent hosts, particularly when they are more abundant than foxes. *E. multilocularis* was reported in 7 (4.1%) of 171 coyotes in the northcentral United States in the late 1960s (*3*), and subsequently prevalences ranging from 19.0% to 35.0% have been reported in coyotes in the central United States (*4*).

In Canada, *E. multilocularis* was detected in 10 (23.0%) of 43 coyotes in Riding Mountain National Park, Manitoba (*5*). In Alberta, 1 case was recorded from the aspen parkland in 1973 (*5*) but it was not found in coyotes from forested regions and southern prairies (*6*,*7*). Nonetheless, *E. multilocularis* is generally considered enzootic to central and southern Alberta on the basis of its prevalence in rodent intermediate hosts. During the 1970s, sixty-three (22.3%) of 283 deer mice (*Peromyscus maniculatus*) trapped in periurban areas of Edmonton were positive for alveolar hydatid cysts (*8*), and *E. multilocularis* was also detected in 2 deer mice collected <1.8 km from Lethbridge in southern Alberta (*9*).

Because mice and voles (family Cricetidae, including *Peromyscus* spp.) have been reported as main prey (70.1%) of coyotes in Calgary (*10*), and coyotes are common in urban areas of Calgary and Edmonton, we suspected a role for this carnivore in the maintenance of the wild animal cycle of *E. multilocularis* in such urban settings. Thus, we aimed to ascertain the frequency of *E. multilocularis* in coyotes from metropolitan areas in Alberta, Canada.

The Study

Ninety-one hunted or road-killed coyotes were collected during October 2009–July 2011. Most (n = 83) of the carcasses were from the Calgary census metropolitan area (CMA) (Figure 1). The remainder (n = 8) were opportunistically collected from the Edmonton CMA. Of those from the Calgary CMA, the exact location of collection was known for 60 animals: 27 were from Calgary and 33 were from the rural fringe, including 2 near Strathmore. Of the carcasses from the Edmonton CMA, 7 were from Edmonton and 1 was from a periurban site. Sex and age of 90 of the coyotes were recorded.

Before necropsy, all carcasses were stored at –20°C. Gastrointestinal tracts collected at necropsy were refrozen at –80°C for 3–5 days to inactivate *Echinococcus* spp. eggs. Once thawed and dissected, intestinal contents were washed, cleared of debris, and passed through a sieve (500-μm pores), and the material in the sieve was examined for *Echinococcus* spp.

Adult tapeworms were counted and identified as *E. multilocularis* on the basis of morphologic features (Figure 2). To confirm morphologic identification, PCR was performed by using species-specific primers (*11*). Briefly, a representative adult worm from each positive animal was

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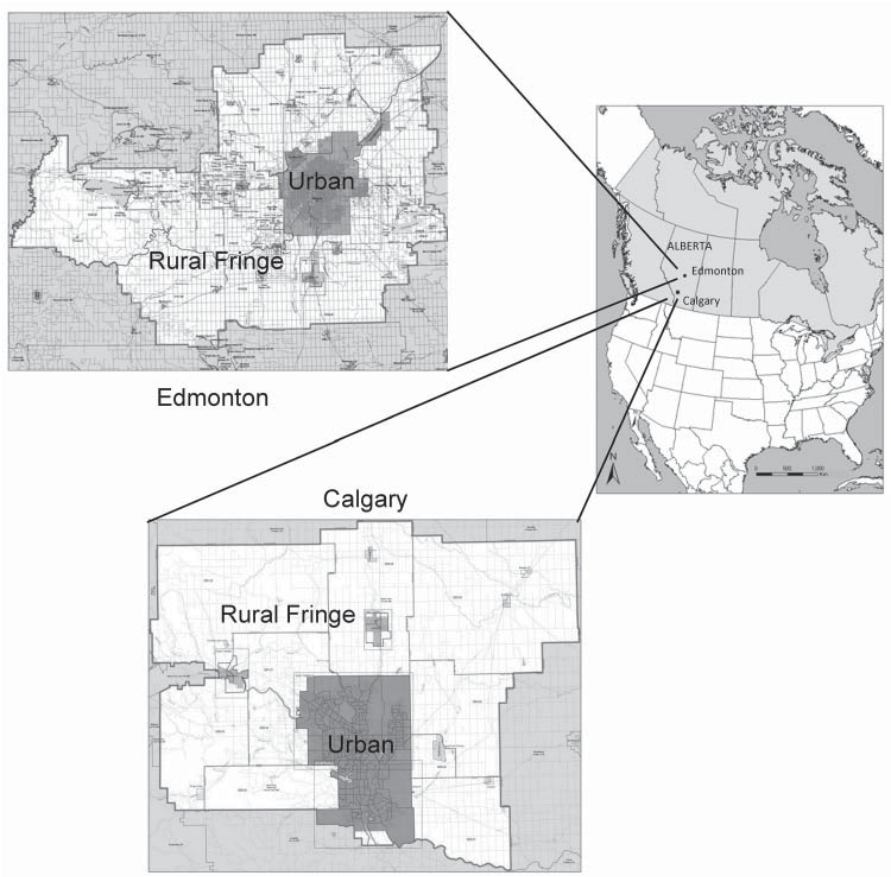

Figure 1. Calgary and Edmonton, Alberta, Canada, census metropolitan areas in which 91 coyote carcasses were collected during 2009–2011 and tested for *Echinococcus multilocularis*. Reference maps (2006) were obtained from the Geography Division, Statistics Canada (www12.statcan.gc.ca/census-recensement/2006/geo/index-eng.cfm). Urban core areas and surrounding rural fringes are indicated. For Edmonton, 5 (62.5%) of 8 carcasses were positive. For Calgary, 18 (20.5%) of 83 carcasses were positive: 9 (27.3%) of 33 from the rural fringe, 4 (14.8%) of 27 from the urban area, and 5 (21.7%) of 23 whose locations of collection were not accurate enough to be classified as urban or from the rural fringe.

lysed in 50 μL of DNA extraction buffer (500 mmol/L KCl, 100 mmol/L Tris-HCl, pH 8.3, 15 mmol/L $MgCl_2$, 10 mol/L dithiothreitol, and 4.5% Tween 20) containing 2 μL of proteinase K. This lysate was further diluted (1:20 in double-distilled water), and 2 μL was used for PCR. Amplicons of an expected 395 bp confirmed infection with *E. multilocularis*.

E. multilocularis was identified in 23 (25.3%) of 91 coyotes by using morphologic and molecular identification. Among positive animals, 18 (20.5%) of 83 were from the Calgary CMA and 5 (62.5%) of 8 were from the Edmonton CMA. In the Calgary CMA, 4 (14.8%) of 27 positive animals were found in the city and 9 (27.3%) of 33 were found in the rural fringe (Figure 1). Five (21.73%) of 23 coyotes for which the location was not recorded were also positive.

E. multilocularis intensity (number of cestodes per host) ranged from 1 to 1,400 (median 20.5). The frequency of infection was significantly higher in male coyotes (n = 44, 34.19%) than in female coyotes (n = 46, 15.2%; χ^2 4.337, df 1, P_{exact} = 0.05) (Table). No difference was detected between 43 juvenile coyotes and 47 adult coyotes (Table).

Conclusions

We demonstrated that *E. multilocularis* is common in coyotes of metropolitan areas of Calgary and Edmonton, Alberta, Canada, including their urban cores. This finding might indicate an emerging phenomenon similar to that observed in Europe with infiltration of urban centers by *E. multilocularis* caused by an increase in red foxes in cities such as Copenhagen, Geneva, and Zurich (*2*). In Alberta,

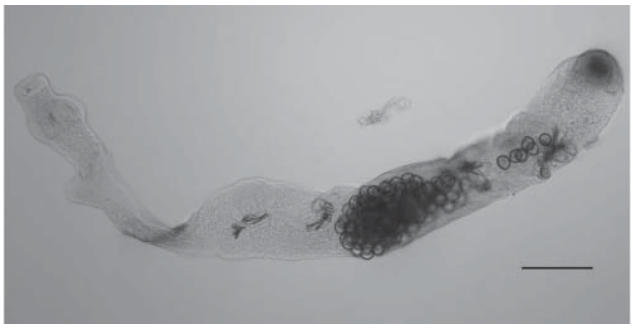

Figure 2. Differential interference contrast micrograph of a representative *Echinococcus multilocularis* isolate from a coyote carcass in Alberta, Canada, October 2009–July 2011. The parasite was 2,059.72 μm long, as measured by using an Olympus BX53 microscope and software (http://microscope.olympus-global.com/en/ga/product/bx53/sf04.cfm). Scale bar = 200 μm.

Table. *Echinococcus multilocularis* in coyotes carcasses collected in Calgary (n = 83) and Edmonton (n = 8) census metropolitan areas, Alberta, Canada, October 2009–July 2011*

Characteristic	Total	No. (%) positive or median (range)	No. negative	IQ distance	χ^2 (z)	df	p_{exact} value†
Sex‡							
M	44	15 (34.1)	29	NA			
F	46	7 (15.2)	39	NA	**4.337**	1	**0.05**
Parasite intensity							
M	NA	9 (1–1,400)	NA	83			
F	NA	59 (9–822)	NA	137	(–1.406)		0.19
Age‡							
Juvenile	43	14 (33.3)	29	NA			
Adult	47	8 (17.0)	39	NA	1.661	1	0.226
Parasite intensity							
Juvenile	NA	9 (1–151)	NA	71			
Adult	NA	32 (1–1,400)	NA	520	(–0.737)		0.518

*Values in **boldface** indicate a significant difference. Higher prevalence in male coyotes suggests a role for sex in parasite dispersion. Frequencies of cestodes in males vs. females and juveniles vs. adults were analyzed by using χ^2 test. Parasite intensity (no. parasites per host) among sex and age classes was compared by using Mann-Whitney test for independent samples. IQ, interquartile distance; NA, not applicable.
†Probability of distribution was estimated by using the permutation approach (p_{exact}).
‡Sex and age of 1 coyote were not recorded.

this phenomenon may be caused by coyotes occupying the urban landscape or by city sprawl invading the natural habitats of coyotes.

Our data suggest that *E. multilocularis* is enzootic in coyotes in Alberta and that perpetuation of the wild animal cycle of *E. multilocularis* within cities and surroundings and potential infection of domestic dogs may pose a zoonotic risk, as documented on Saint Lawrence Island, Alaska, and in China (*2*,*12*). With a considerable increase in domestic dog population of Calgary (32.1% increase since 2001, a total of 122,325 dogs in 2010; Animal and Bylaw Services Survey 2010, www.calgary.ca/CSPS/ABS/Pages/home.aspx) and substantial human population growth (32.9% increase in Calgary since 1999; Statistics Canada, 2009, www.statcan.gc.ca/start-debut-eng.html), awareness is needed of potential transmission risks associated with changing city landscapes and *E. multilocularis* in the urban environment.

In Canada, only 1 autochthonous human case of alveolar echinococcosis has been reported in Manitoba (*13*). However, imported cases have been described. In Alberta, there are no known reports of alveolar echinococcosis. This finding may be caused by the long incubation time required for clinical manifestation in humans (*12*) or a strain of *E. multilocularis* with a low zoonotic potential. Although there is little evidence of human risk from the strain of *E. multilocularis* in central North America (*14*), a human case caused by this strain has been confirmed (*15*).

Our finding of *E. multilocularis* in coyotes in urban regions in Alberta suggests that surveillance for this parasite should be increased in North America. Although removal of this parasite from domestic dogs and cats is effective, eradication from free-ranging definitive hosts may be unfeasible (*2*,*12*). Interventions other than improving public awareness about prevention and transmission risk are probably unnecessary, and public health messages should target veterinarians and dog owners because domestic dogs probably represent the main infection route for humans in North America (*2*,*12*). Genetic characterization of *E. multilocularis* and spatially explicit transmission models should also be developed to better assess risks of this emerging zoonosis in North America and worldwide.

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Orthobunyavirus Antibodies in Humans, Yucatan Peninsula, Mexico

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We performed a serologic investigation to determine whether orthobunyaviruses commonly infect humans in the Yucatan Peninsula of Mexico. Orthobunyavirus-specific antibodies were detected by plaque reduction neutralization test in 146 (18%) of 823 persons tested. Further studies are needed to determine health risks for humans from this potentially deadly group of viruses.

We previously reported the isolation of Cache Valley virus (CVV), Kairi virus (KRIV), Cholul virus (CHLV), and South River virus (SOURV) from mosquitoes in the Yucatan Peninsula of Mexico (*1*–*3*). Antibodies to CVV, CHLV, and SOURV were also detected in livestock in this region (*4*). These viruses belong to the genus *Orthobunyavirus* (*5*). All viruses in this genus possess a tripartite, single-stranded, negative-sense RNA genome.

CVV is a recognized human pathogen (*5*) that has been linked to severe encephalitis and multiorgan failure. KRIV has not been implicated as a cause of human disease, although antibodies to this virus have been detected in humans in Argentina (*6*). Recent data suggest that CHLV is a reassortant that acquired its small RNA segment from CVV and medium and large RNA segments from Potosi virus (POTV) (*1*). No clear evidence exists for human susceptibility to infection with CHLV or SOURV. However, diagnostic laboratories rarely test for orthobunyavirus infection; therefore, the true disease incidence and seroprevalence of these viruses remains to be determined. Because orthobunyaviruses comprise a neglected but potentially deadly group of viruses and recent studies have provided evidence of orthobunyavirus activity in the Yucatan Peninsula (*1*–*4*), we investigated whether orthobunyaviruses commonly infect humans in this region.

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The Study

Serum samples were obtained from 823 febrile patients at the Secretaria de Salud de Yucatán and other health institutions in Merida during January–October 2007. The patients resided in all 3 states of the Yucatan Peninsula of Mexico: Yucatan (n = 809), Quintana Roo (n = 8) and Campeche (n = 6). The study was approved by the Institutional Biosafety Committees at Iowa State University (Ames, IA, USA) and the Universidad Autónoma de Yucatán (Mérida, Mexico).

All serum samples were examined at a dilution of 1:20 by plaque reduction neutralization test (PRNT) by using CVV (strain CVV-478), and PRNTs were performed as described (*7*). A subset of serum samples with antibodies that neutralized CVV were titrated and further analyzed by PRNT by using CVV, CHLV (strain CHLV-Mex07), KRIV (strain KRIV-Mex07), SOURV (strain NJO-94f), Maguari virus (strain BeAr7272), and Wyeomyia virus (strain prototype). All of these viruses belong to the Bunyamwera (BUN) serogroup except SOURV, which belongs to the California (CAL) serogroup.

Titers were expressed as the reciprocal of highest serum dilutions yielding >90% reduction in the number of plaques ($PRNT_{90}$). For etiologic diagnosis, the $PRNT_{90}$ antibody titer for each virus was required to be ≥4-fold greater than that to the other viruses tested.

Antibodies that neutralized CVV were detected in 146 (18%) of 823 study participants. The mean ages of patients with and without antibodies that neutralized CVV were 32.0 and 22.3 years, respectively. Logistic regression analysis showed that the risk for infection increased significantly with age ($p = 0.0001$).

Serum samples from 50 seropositive patients were titrated and analyzed by comparative PRNT to identify the orthobunyaviruses responsible for these infections. Six persons were seropositive for CVV, 5 for CHLV, and 1 for SOURV or a SOURV-like virus; 38 had antibodies to an undetermined orthobunyavirus (Table). Because SOURV was the only CAL serogroup virus used in this study, and another CAL serogroup virus may have been responsible for the infection, the person who had a SOURV $PRNT_{90}$ titer ≥4-fold than that to the other viruses tested received a conservative PRNT diagnosis of seropositive for SOURV or a SOURV-like virus. Because interserogroup crossreactivity of neutralizing antibodies to viruses in the BUN and CAL serogroups has not been seen, the 17 persons with antibodies that neutralized SOURV and ≥1 of the BUN serogroup viruses might have been exposed to ≥1 viruses from each serogroup.

CHLV and POTV share the same medium RNA segment, so antibodies for these viruses cannot be differentiated by PRNT. Furthermore, antibodies to CHLV and POTV cannot be differentiated by complement fixation test (*4*). Thus, we cannot dismiss POTV as a possible cause of infection in some or all of the study participants who were seropositive for CHLV. However, it appears more likely that these persons had been infected with CHLV because this virus has been isolated in the Yucatan Peninsula, whereas no direct evidence has been found for POTV in this region.

As already noted, serum samples from 38 (76%) of the study participants analyzed by comparative PRNT had antibodies to an undetermined orthobunyavirus. Most of these persons had low $PRNT_{90}$ titers; the highest $PRNT_{90}$ titer for 29 of these persons did not exceed 40. Because neutralizing antibody levels decline over time, these findings may indicate that many of these infections

Table. Endpoint titers of serum samples collected from persons in Mexico and analyzed by using comparative PRNT*

Patient	Demographic characteristics		$PRNT_{90}$ titers						
ID no.	Residence	Age, y/sex	CVV	CHLV	KRIV	SOURV	MAGV	WYOV	Diagnosis
28	Yucatan	18/M	80	20	–	–	40	–	UND
34	Yucatan	39/F	80	20	–	–	20	–	CVV
52	Quintana Roo	39/F	160	–	–	40	80	–	UND
54	Yucatan	32/M	40	20	–	–	40	–	UND
62	Yucatan	44/M	40	–	–	20	20	–	UND
72	Yucatan	23/M	80	–	–	–	40	–	UND
81	Yucatan	60/F	20	20	–	20	–	–	UND
92	Yucatan	13/M	160	20	–	20	80	–	UND
93	Yucatan	42/F	40	20	–	–	20	–	UND
113	Yucatan	24/F	20	–	–	–	–	–	UND
114	Yucatan	29/F	40	–	–	–	20	–	UND
120	Yucatan	60/F	20	–	–	320	–	–	SOURV or SOURV-like virus
159	Yucatan	54/M	20	–	–	–	20	–	UND
161	Yucatan	53/M	20	20	–	–	–	–	UND
163	Yucatan	27/F	160	80	40	–	40	–	UND
167	Yucatan	16/M	160	–	–	–	40	–	CVV
183	Yucatan	69/F	20	–	–	–	20	–	UND
184	Yucatan	34/M	160	40	–	–	80	–	UND
185	Yucatan	25/F	20	–	–	–	–	–	UND
192	Campeche	54/F	80	40	–	–	20	–	UND
193	Yucatan	16/F	80	–	–	–	20	–	CVV
194	Yucatan	69/F	20	–	–	20	–	–	UND
200	Yucatan	3/F	40	–	–	40	40	–	UND
205	Yucatan	53/M	40	–	–	20	20	–	UND
208	Yucatan	57/F	20	160	–	–	–	–	CHLV
210	Yucatan	42/M	20	–	–	–	20	–	UND
224	Yucatan	34/M	20	20	–	20	–	–	UND
234	Yucatan	39/F	20	80	–	–	20	–	CHLV
236	Yucatan	74/F	20	–	–	20	–	–	UND
386	Yucatan	14/F	20	–	–	20	–	–	UND
388	Yucatan	60/M	160	1,280	160	–	40	–	CHLV
389	Yucatan	5/M	20	–	–	20	–	–	UND
390	Yucatan	33/M	40	20	40	20	40	–	UND
392	Yucatan	22/M	20	–	–	–	–	–	UND
393	Yucatan	29/M	40	20	–	–	40	–	UND
396	Yucatan	34/F	80	–	–	–	20	–	CVV
397	Yucatan	32/M	80	–	–	–	40	–	UND
399	Yucatan	27/M	320	1,280	160	–	320	–	CHLV
400	Yucatan	37/F	20	–	–	–	–	–	UND
401	Yucatan	30/F	160	–	–	–	40	–	CVV
402	Yucatan	18/M	20	–	–	–	20	–	UND
403	Yucatan	50/F	20	20	–	–	–	–	UND
407	Yucatan	27/M	80	20	–	–	40	–	UND
408	Yucatan	40/F	20	–	–	–	–	–	UND
412	Yucatan	60/M	20	320	80	40	40	–	CHLV
415	Yucatan	17/F	20	–	–	20	–	–	UND
420	Yucatan	16/F	160	–	–	–	20	–	CVV
429	Yucatan	37/F	20	–	–	–	–	–	UND
442	Yucatan	10/F	20	40	–	20	–	–	UND
455	Yucatan	30/F	20	–	–	20	20	–	UND

*PRNT, plaque reduction neutralization test; CVV, Cache Valley virus; CHLV, Cholul virus; KRIV, Kairi virus; SOURV, South River virus; MAGV, Maguari virus, WYOV, Wyeomyia virus; –, titer <20; UND, undetermined orthobunyavirus.

occurred years ago, and the trace amounts of neutralizing antibodies that remained were insufficient to yield a ≥4-fold difference between the titers of the virus responsible for the infection and the other viruses used in the PRNTs. Another explanation is that some of these persons had been infected with an orthobunyavirus not included in the PRNTs, although all orthobunyaviruses known to occur in the Yucatan Peninsula were represented.

Conclusions

We found 18% of the 823 Yucatan residents participating in our study had evidence of orthobunyavirus exposure. This number is presumably an underestimate; additional seropositive persons might have been identified if the initial PRNTs had not been restricted to CVV. In particular, additional seropositive persons likely would have been detected if SOURV was used in the initial PRNTs, because a screening algorithm that includes only a BUN serogroup virus would likely miss many CAL serogroup virus infections. Nevertheless, we provide evidence that orthobunyaviruses commonly infect humans in the Yucatan Peninsula.

Previous serosurveys have provided information on the seroprevalence of orthobunyaviruses in humans in the United States. For example, antibodies that neutralized CVV were detected in 42/356 (12%) residents in Maryland and Virginia in 1961–1963 (*8*). Antibodies that neutralized Maguari virus or Tensaw virus were detected in 71/≈300 humans in Florida in the 1980s (*9*); as observed in our study, the highest PRNT titers for most of the seropositive persons in that study did not exceed 40.

All persons in our study cohort initially sought care for unspecified fever; however, we could not determine whether any of these febrile illnesses were a direct consequence of orthobunyavirus infection. The detection of acute orthobunyavirus infections is limited because no IgM-capture ELISA for orthobunyavirus diagnosis exists. PRNTs can be used to identify recent orthobunyavirus infections when paired acute and convalescent serum samples are available, but for our study, only single serum samples were available from each participant. Orthobunyavirus viremias in humans are transient and of low magnitude, which makes reverse transcription PCR ineffective for the detection of orthobunyavirus RNA in serum samples. However, a duplex reverse transcription PCR was recently developed for the detection of CVV RNA in human cerebrospinal fluid (*10*).

In conclusion, we provide evidence that orthobunyaviruses commonly infect humans in the Yucatan Peninsula. These viruses are also a common cause of infection of livestock in this region (*4*). Our findings underscore the need to determine whether orthobunyaviruses represent an unrecognized cause of illness in humans and vertebrate animals in Mexico.

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Tetanus as Cause of Mass Die-off of Captive Japanese Macaques, Japan, 2008

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In 2008 in Japan, 15/60 captive Japanese macaques died. *Clostridium tetani* was isolated from 1 monkey, and 11 had tetanus-specific symptoms. We conclude the outbreak resulted from severe environmental *C. tetani* contamination. Similar outbreaks could be prevented by vaccinating all monkeys, disinfecting housing areas/play equipment, replacing highly *C. tetani*–contaminated soil, and conducting epidemiologic surveys.

Tetanus is a wound infection caused by a potent neurotoxin produced by *Clostridium tetani*. The bacterium is difficult to isolate, and no pathologically characteristic lesion is present during infection; thus, tetanus diagnosis is based on tetanus-specific clinical symptoms (*1*–*4*). Tetanus is a highly lethal zoonosis, and cases usually occur sporadically. Outbreaks among humans have occurred only after earthquakes and tsunamis (*4*). We report on an outbreak of tetanus in 2008 among a captive colony of Japanese macaques (*Macaca fuscata*) in Japan.

The Study

In 2008, deaths suddenly increased among Japanese macaques housed in a facility in the Kantou area of Japan. At that time, the facility, which had been in service for >40 years, housed ≈60 macaques, 15 (25%) of which died. This mortality rate was much higher than that during 2006 (10.9%, 7/64 monkeys), 2007 (7.1%, 4/56), 2009 (13.8%, 9/65), 2010 (5.2%, 3/58), and 2011 (5.7%, 4/70) (Figure 1). A total of 42 monkeys died during 2006–2011, and investigations at the time of death showed that 14 of the monkeys had tetanus-specific symptoms: 1 of 4 that died in 2007, 11 of 15 that died in 2008, and 2 of 9 that died in 2009). Nine of the 11 monkeys that died with characteristic symptoms of tetanus in 2008 died during the breeding season (November and December). Thus, the observed number of presumed tetanus cases during the 2008 breeding season (9/60) was 8.4× greater than the number during the 2007 breeding season (1/56).

The soil in the monkeys' enclosure was clay-like and without vegetation. In 2008 before the increase in deaths, there were no changes in maintenance procedures, such as feeding, at the facility and no evident pathogenic contamination of the monkeys' food or environment.

We performed necropsies on 3 monkeys (animal nos. 1, 2, and 3) that died 5, 2, and 3 days, respectively, after the onset of symptoms. At death, all showed a specific posture: the jaw was elevated, the back straightened, and the tail tightly stretched; the forelimbs were crossed in front of the body with the wrists bent; and the hind limbs were extended backward (Figure 2). Rigidity was abnormally severe and did not remit after death; at necropsy, the mouth was difficult to open. Congestion of the visceral organs and pulmonary edema were noted, but there were no findings to suggest poisoning, such as foreign bodies in the stomach or erosive changes in the gastrointestinal tract. No wound that might have led to infection was found in monkeys 1 or 2, but a lesion with purulent incrustation was present on a toe tip on the right hind limb of monkey 3. *C. tetani* was isolated from this lesion, and the tetanus toxin gene was detected by PCR. A mouse toxicity test confirmed tetanus toxin activity.

We obtained samples from the soil in monkey enclosures, from wooden playground equipment, and from the soil surrounding the enclosures and tested them for *C. tetani*; 67%, 75%, and 53% of the samples, respectively, were positive for *C. tetani*, indicating marked

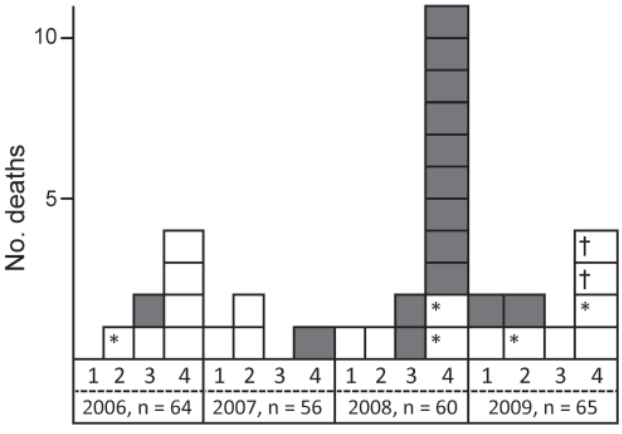

Figure 1. Number of deaths during 2006–2009 among macaques (*Macaca fuscata*) housed in an animal facility in the Kantou area of Japan. Grey boxes, monkeys with tetanus-specific clinical symptoms; white boxes, monkeys without tetanus-specific clinical symptoms. 1, January–March; 2, April–June; 3, July–September; 4, October–December; n, total number of monkeys. *Juvenile animal; †Accident at time monkeys captured for vaccination (death due to hyperthermia).

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contamination. *C. tetani* was not isolated from the monkeys' food or from soil sampled >1 km from the facility. We performed pulsed-field gel electrophoresis on isolates from the soil at the facility and from monkey number 3, and the results were identical, showing >90% homology.

On 3 occasions (October 27 and December 17, 2009, and December 8, 2010), macaques housed in the facility (total 65) were intramuscularly administered 0.5 mL of tetanus toxoid (Nisseiken Co., Ltd., Tokyo, Japan). In 1 monkey, the prevaccination serum level of tetanus toxoid antibody was higher than the level for tetanus prevention (0.1 IU/mL). At 51 days after the first vaccination, 83.3% (51/61) of the animals were antibody-positive, and 1 year after the second vaccination, 100% were antibody-positive. Since then, no tetanus symptoms have occurred in any of the monkeys. Caretakers for monkeys at the facility were examined at a community medical office and inoculated with tetanus toxoid.

Conclusions

On the basis of these findings, we diagnosed the disease as tetanus, and we concluded that it was an unprecedented, large-scale outbreak. Many animal exhibition facilities in Japan maintain Japanese macaques, and tetanus has been reported in captive macaques in other countries (*5*–*7*). Results of a 5-year study (July 1, 1976–June 30, 1981) among the free-ranging rhesus monkey (*Macaca mulatta*) colony on the island of Cayo Santiago, Puerto Rico, showed a high incidence of tetanus among the monkeys during the breeding seasons, but the report did not clarify the cause (*6*).

In facilities maintaining animals, the soil is often contaminated with *C. tetani* at a relatively high rate (*1*,*2*). In the facility in Japan, *C. tetani* was isolated at a high rate from soil and from play structures. The genotype of these isolates was consistent with that for an isolate obtained from a monkey housed at the facility, suggesting that the soil was the source of the infection.

The facility has >40 years' experience raising monkeys, and the cause of the sudden outbreak in 2008 is unclear. The outbreak was concentrated during the breeding season, suggesting that injuries sustained through fighting during the mating season in an environment with severe *C. tetani* contamination may have led to the outbreak. *C. tetani* is present in the intestinal contents of various animal species (*1*,*3*). Thus, bacteria in the feces of infected monkeys may have added to the level of indigenous *C. tetani* contamination in the soil.

In Japan, tetanus is still reported in >100 persons each year: 115 cases were reported in 2005, 117 in 2006, 89 in 2007, 124 in 2008, 113 in 2009,and 106 in 2010) (*8*). It is a highly lethal zoonosis and a disease of concern with regard to public and animal health. After tetanus was diagnosed in the monkeys, we immediately administered tetanus vaccine to monkey caretakers at the facility and thoroughly enforced hygiene practices. To prevent tetanus infection in animals and animal caretakers in such facilities and in visitors, we recommend that newborn monkeys be vaccinated, housing areas and play equipment be disinfected, soil highly contaminated with *C. tetani* be replaced, and epidemiologic surveys be conducted.

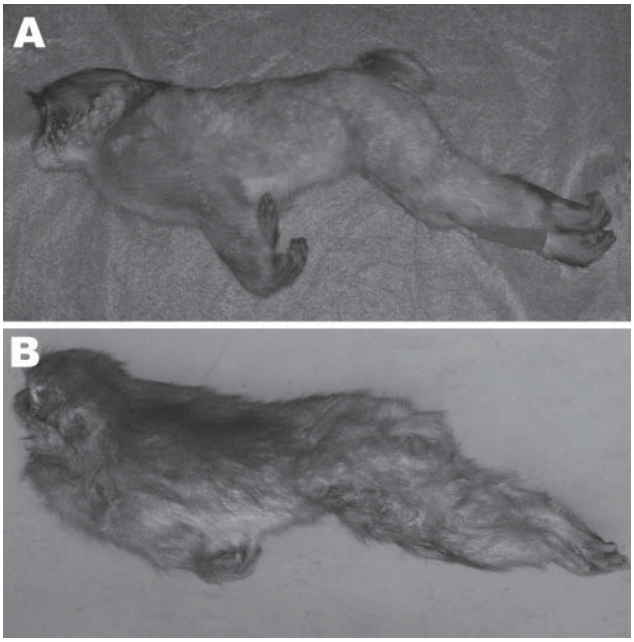

Figure 2. A) Opisthotonos as a tetanus-specific clinical symptom in a 1-year-old male Japanese macaque (*Macaca fuscata*). B) Opisthotonos with severe rigid posture in an adult male Japanese macaque.

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Miss Nakano, a graduate of Azabu University, Kanagawa, Japan, works as a clinical veterinarian; this paper is her graduation thesis. Her research interest is in infectious diseases of monkeys.

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etymologia

Tetanus

[tet′ə-nəs]

From the Greek *tetanos* (“tension,” from *teinein*, “to stretch”), an often fatal infectious disease caused by the anaerobic bacillus *Clostridium tetani*. Tetanus was well known to the ancients; Greek physician Aretaeus wrote in the first century CE, “Tetanus in all its varieties, is a spasm of an exceedingly painful nature, very swift to prove fatal, but neither easy to be removed.” Active immunization with tetanus toxoid was described in 1890, but cases continue to be reported (275 in the United States from 2001 through 2010), almost exclusively in persons who were never vaccinated or had not received a booster immunization in the previous 10 years. In developing countries, neonatal tetanus—when infants are infected through nonsterile delivery—is a major contributor to infant mortality. Worldwide, an estimated 59,000 infants died of neonatal tetanus in 2008.

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Human Infection with *Candidatus* Neoehrlichia mikurensis, China

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To identify *Candidatus* Neoehrlichia mikurensis infection in northeastern China, we tested blood samples from 622 febrile patients. We identified in 7 infected patients and natural foci for this bacterium. Field surveys showed that 1.6% of ticks and 3.8% of rodents collected from residences of patients were also infected.

Candidatus Neoehrlichia mikurensis was detected in 1999 in *Ixodes ricinus* ticks in the Netherlands and referred to as an *Ehrlichia* spp.–like agent (*1*). It was then classified as a new member of family *Anaplasmataceae* on the basis of ultrastructure and phylogenetic analysis (*2*). The agent was detected in ticks and small wild mammals in Europe and Asia (*1*–*6*) and has recently been reported to infect humans, especially immunocompromised patients in Europe (*7*–*10*). However, no cases of infection have been identified outside Europe. Moreover, the agent has not yet been isolated in pure culture, and its antigens are not available.

To investigate human infections with tick-borne agents in China, we initiated a surveillance study at Mudanjiang Forestry Central Hospital (Mudanjiang, China). This hospital is one of the largest hospitals treating patients with tick-borne infectious diseases in northeastern China, where various tick-borne agents have been detected in ticks and animal hosts (*11*–*15*).

The Study

During May 2–July 30, 2010, a total of 622 febrile patients, who had histories of recent tick bites and sought treatment at Mudanjiang Forestry Central Hospital (Figure 1) were screened for the infections of tick-borne agents. When patients were admitted, peripheral blood samples were collected and treated with EDTA. DNA as extracted by using the QIAmp DNA Blood Mini Kit (QIAGEN, Germantown, MD, USA).

Figure 1. Location of Mudanjiang, Heilongjiang Pro-vince, China, where *Candidatus* Neoehrlichia mikurensis was detected.

For a broad-range assay, a nested PCR specific for the 16S rRNA gene (*rrs*) was used to detect organisms in the family *Anaplasmataceae*. For positive samples, 2 heminested PCRs were used to amplify the entire *rrs* gene. For further confirmation, a nested PCR specific for the 60-kDa heat shock protein gene (*groEL*) was performed. Detailed cycling conditions for all amplifications are described in the Technical Appendix (wwwnc.cdc.gov/EID/pdfs/12-0594-Techapp.pdf).

Seven patients were found to be infected with *Candidatus* N. mikurensis by amplifications of the *rrs* and *groEL* genes. Amplified *rrs* gene (1,501 bp) and partial *groEL* gene (1,230 bp) sequences from these patients were identical. These sequences were also identical to genes of *Candidatus* N. mikurensis detected in ticks and rodents in the Asian region of Russia (*5*).

Serum samples were collected from patients during the acute (2–12 days after onset of illness) or convalescent (34–42 days after onset of illness) phases of illness. All samples were negative for IgG against *Anaplasma phagocytophilum*, *Ehrlichia chaffeensis*, *Borrelia burgdorferi*, *Rickettsia heilongjiangensis*, and tick-borne encephalitis virus when tested by indirect immunofluorescence assay.

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All 7 patients were farmers residing in the villages in Mudanjiang. Their median age was 41 years (range 29–67 years) and 5 were men. None had been vaccinated against tick-borne encephalitis. The patients had onset of illness during May 20–July 13, 2010. The median time from the tick bite to the onset of illness and from the onset of illness to the physician visit was 8 days (range 2–35 days) and 7 days (range 1–12 days), respectively.

All patients were otherwise healthy, and none had a history of underlying immunocompromised conditions. Fever, headache, and malaise were reported for all 7 patients. Other major manifestations included nausea (5/7), vomiting (5/7), myalgia (4/7), and stiff neck (4/7). Less common symptoms were arthralgias (2/7), cough (2/7), diarrhea (1/7), confusion (1/7), and erythema (1/7). Skin erythema (multiple and oval) was seen on the neck of 1 patient.

Laboratory test results showed leukopenia in 1 patient, leukocytosis in 1 patient, thrombocytopenia in 2 patients, and anemia in 2 patients. Serum levels of alanine aminotransferase and aspartate aminotransferase were within reference ranges for all patients. Wright–Giemsa stained peripheral blood smears did not show morulae or other blood parasites.

To identify local natural foci, we performed a field investigation on infections of *Candidatus* N. mikurensis in ticks and rodents from areas of residences of the patients. During May–July 2010, a total of 516 host-seeking ticks, including 316 *I. persulcatus*, 187 *Haemaphysalis concinna*, and 13 *Dermacentor silvarum*, were collected on vegetation and individually examined. *Candidatus* N. mikurensis DNA was detected in 6 (1.9%) *I. persulcatus* and 2 (0.8%) *H. concinna* ticks, but no DNA was detected in *D. silvarum* ticks (Table).

A total of 211 rodents of various species were captured by using snap traps. After rodent species was identified, spleen specimens were collected for DNA extraction and PCR. Eight rodents of 3 species, 5 (4.6%) *Clethrionomys rufocanus*, 2 (5.7%) *Rattus norvegicus*, and 1 (33.3%) *Tamias sibiricus*, were positive for *Candidatus* N. mikurensis (Table).

Nucleotide sequences of *rrs* and *groEL* genes of 8 ticks and 8 rodents were identical to each other and to sequences obtained from the 7 patients. Phylogenetic analysis of *rrs* genes showed that nucleotide sequences identified were identical to those of *Candidatus* N. mikurensis from Japan and the Asian region of Russia but different from sequences from Europe (99.6%–99.8% similarity) (Figure 2, panel A). Similar phylogenetic relationships were observed in a neighbor-joining tree based on *groEL* gene nucleotide sequences. In comparison with sequences from humans and ticks in Europe, the *groEL* gene sequences identified in the study showed 97.6%–98.4% similarity (Figure 2, panel B).

Table. Prevalence of *Candidatus* Neoehrlichia mikurensis in ticks and rodents, Mudanjiang, China

Species	No. positive/no. tested (%)
Tick	
Ixodes persulcatus	6/316 (1.9)
Haemaphysalis concinna	2/187 (0.8)
Dermacentor silvarum	0/13 (0)
Total	8/516 (1.6)
Rodent	
Clethrionomys rufocanus	5/109 (4.6)
Rattus norvegicus	2/35 (5.7)
Apodemus peninsulae	0/30 (0)
Apodemus agrarius	0/25 (0)
Mus musculus	0/9 (0)
Tamias sibiricus	1/3 (33.3)
Total	8/211 (3.8)

Conclusions

We have detected *Candidatus* N. mikurensis DNA in blood samples from 7 patients collected during the period of acute illness, which suggests that this bacterium was the etiologic agent of the infections. Our findings demonstrated human infections with *Candidatus* N. mikurensis in China. The *rrs* and *groEL* gene nucleotide sequences of this *Candidatus* N. mikurensis variant were identical to those obtained from ticks and rodents in the Asian region of Russia, which have not been reported to cause human infection.

Unlike reported cases in elderly or immunocompromised patients in whom disease developed (*7–10*), all 7 patients in our study had relatively mild disease. Major clinical manifestations and laboratory findings of the cases in our report, such as leukocytosis, were not similar to those of previously reported cases. It is noteworthy that the patients reported in this study were previously healthy. Thus, their clinical manifestations might be typical of *Candidatus* N. mikurensis infection in an otherwise healthy population. However, the number of cases in our study was limited, and clinical data were not inclusive. Clinical characteristics of *Candidatus* N. mikurensis infection should include detailed descriptions of additional cases.

Our finding of a *Candidatus* N. mikurensis variant in 1.6% of ticks and 3.8% of rodents tested suggested natural foci of the bacterium in Mudanjiang. Therefore, clinical diagnosis of *Candidatus* N. mikurensis infection should be considered in patients who have been exposed to areas with high rates of tick activity. It is noteworthy that *Candidatus* N. mikurensis was originally detected in *R. norvegicus* from Guangzhou Province in southeastern China (*4*), thereby indicating the potential threat to humans in areas other than northeastern China.

In summary, we identified *Candidatus* N. mikurensis as an emerging human pathogen in China. Further studies should be conducted to isolate this bacterium and investigate its epidemiologic, genetic, and pathogenic features. To

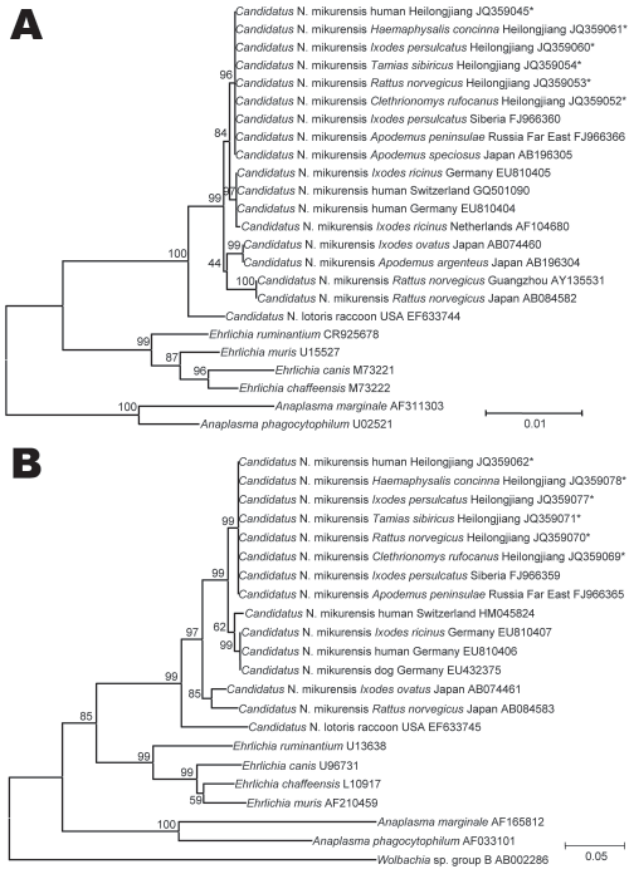

Figure 2. A) Neighbor-joining trees based on the 16S rRNA gene (*rrs*) and B) the 60-kDa heat shock protein gene (*groEL*) of *Candidatus* Neoehrlichia mikurensis, China, generated by using Molecular Evolutionary Genetics Analysis software version 4.0, (www.megasoftware.net/) the maximum composite-likelihood method, and bootstrap analysis of 1,000 replicates. Asterisks indicate nucleotide sequences of *Candidatus* N. mikurensis determined in this study. Numbers on branches indicate percentage of replicates that reproduced the topology for each clade. Scale bars indicate estimated evolutionary distance. A total of 1,303 positions for *rrs* and 953 positions for *groEL* were analyzed. Sources of *Candidatus* N. mikurensis sequences are shown between species names and GenBank accession numbers.

guide diagnostic testing and treatment, physicians should be aware that human *Candidatus* N. mikurensis infections are in Heilongjiang Province and that PCR can be used as a diagnostic technique for identifying suspected infections.

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Anthroponotic Enteric Parasites in Monkeys in Public Park, China

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Cryptosporidium spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* were detected in 45, 35, and 116 of 411 free-range rhesus monkeys, respectively, in a popular public park in the People's Republic of China. Most genotypes and subtypes detected were anthroponotic, indicating these animals might be reservoirs for human cryptosporidiosis, giardiasis, and microsporidiosis.

Cryptosporidiosis, giardiasis, and microsporidiosis are enteric diseases in humans and are mainly caused by *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi*, respectively (*1*–*3*). These protozoan parasites are also commonly found in animals and are considered zoonotic. However, the role of nonhuman primates in the transmission of the diseases remains unclear because few studies have been conducted on the genetic characteristics of the parasites in these animals. In a recent study in Kenya, 5 (2.0%) and 13 (5.5%) of 235 captive baboons had human-pathogenic *C. hominis* subtypes and *E. bieneusi* genotypes, respectively. This finding implies that nonhuman primates might be reservoirs for human cryptosporidiosis and microsporidiosis (*4*). We determined the genotypes and subtypes of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* in free-range rhesus monkeys in a popular public park to assess the potential for transmission of these parasites from rhesus monkeys to humans.

The Study

In November 2010, we collected 411 fecal specimens from rhesus monkeys (*Macaca mulatta*) in Qianling Park, Guiyang, People's Republic of China (www.qlpark.cn). The park, a major tourist attraction of the city, is visited by 10,000–70,000 persons each day. It has the highest number (≈700) of domesticated free-range monkeys in China, which originated from a troop of 20 animals in 1985. Visitors are allowed to bring or buy food to feed the animals, watch them from a short distance, or play with them (Figure, panel A).

We collected fecal droppings at 3 locations with different animal densities. A total of 187 specimens were collected from the Macaque Garden, where animal density was the highest, at ≈400 animals in a small open area between 2 mountains. Another 74 specimens were collected from the Tanquan Spring area, where animal density was the lowest. The remaining 150 specimens were collected from the Hongfu Temple, which had moderate animal density. Twenty-three 100-mL grab samples of high-turbidity water were collected at various points of a small lake near the Macaque Garden and Tanquan Spring, where the rhesus monkeys frequently bathed (Figure).

We detected *Cryptosporidium* spp., *G. duodenalis* subtypes, and *E. bieneusi* genotypes in the fecal specimens and differentiated them by using PCR and sequence analysis of the small subunit rRNA gene (*5*), triosephosphate isomerase

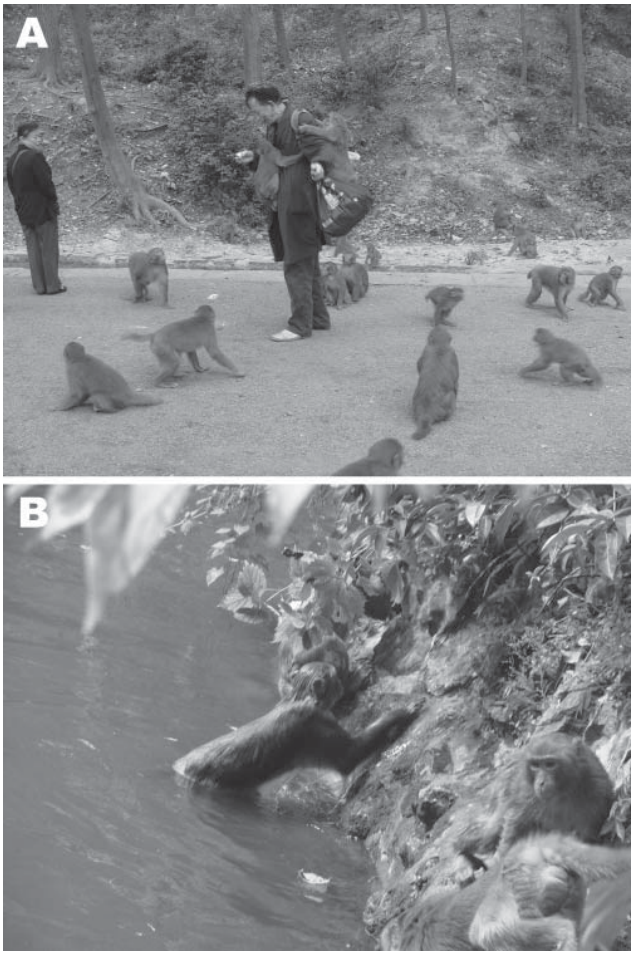

Figure. Potential zoonotic and waterborne pathways of parasites in Qianling Park, Guiyang, China. A) Close contact of rhesus monkeys with humans. B) Potential contamination of recreational water with pathogens from rhesus monkeys.

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gene (*6*), and ribosomal internal transcribed spacer (*4*), respectively. We similarly analyzed water samples after concentrating pathogens by centrifugation at 3,000 × *g* for 15 min. We subtyped *C. hominis* and *C. parvum* by using sequence analysis of the 60-kDa glycoprotein gene (*5*). We analyzed each specimen at least 2× by using PCR, with the inclusion of positive and negative controls in each run. We used the χ^2 test to compare differences in rates of each parasite.

We detected *Cryptosporidium* spp. in 45 (10.9%) of the 411 fecal specimens, belonging to 3 species: *C. hominis* (39 specimens), *C. parvum* (5), and *C. felis* (1). The rate at Macaque Garden (16.6%) was significantly higher than at Hongfu Temple (6.0%; p = 0.003) and Tanquan Spring (6.8%; p = 0.038) (Table). Among 44 specimens successfully subtyped, 7 subtypes in 5 subtype families were identified: 4 families (Ia, Id, Ie, and If) of *C. hominis* and 1 family (IIc) of *C. parvum* (Table). The most common subtypes were IaA13R8 (8 specimens), IdA20 (13), IeA11G3T3 (13), and IIcA5G3a (5).

G. duodenalis was identified in 35 (8.5%) of the 411 fecal specimens. The rates at Macaque Garden (10.2%; p = 0.016) and Hongfu Temple (10.0%; p = 0.018) were significantly higher than at Tanquan Spring (1.4%). All positive specimens except for 1 were successfully genotyped and subtyped and belonged to assemblages A (10) and B (24). All assemblage A isolates belonged to subtype A2 (10 specimens). In assemblage B, 7 subtypes were identified: 1 known subtype in 11 specimens and 6 new subtypes at low frequencies (Table).

Table. Anthroponotic enteric parasites in free-range rhesus monkeys (*Macaca mulatta*) and water samples in Qianling Park, Guiyang, China*

Species, genotype, or subtype	GenBank accession no.	Fecal specimens positive for organism			Water samples positive for organism, n = 23
		Macaque Garden, n = 187	Hongfu Temple, n = 150	Tanquan Spring, n = 74	
Cryptosporidium					
C. hominis					
IaA13R7	EU095261	2	0	0	0
IaA13R8	JX000568†	6	2	0	2
IaA14R7	JX000569†	1	0	1	0
IdA20	EU095265	10	3	0	0
IeA11G3T3	DQ665689	8	3	2	7
IfA16G2	JX000570†	1	0	0	0
Unknown‡	NA	0	0	0	2
C. parvum					
IIcA5G3a	AY738195	2	1	2	0
C. felis		1	0	0	0
Subtotal (mean %; 95% CI)§		31/187 (16.6; 10.7–22.4)	9/150 (6.0; 2.1–9.9)	5/74 (6.8; 0.8–12.7)	11/23 (47.8; 19.6–76.1)
Giardia duodenalis					
Assemblage A2	U57897	10	0	0	0
Assemblage B					
B1	AY368164	3	7	1	8
B2d	JX000562†	0	2	0	0
B3d	JX000563†	0	1	0	0
B4d	JX000564†	2	0	0	0
B5d	JX000565†	2	3	0	3
B6d	JX000566†	2	0	0	0
B7d	JX000567†	0	1	0	0
Unknown‡	NA	0	1	0	1
Subtotal (mean %; 95% CI)§		19/187 (10.2; 5.6–14.7)	15/150 (10.0; 4.9–15.1)	1/74 (1.4; 0–4.0)	12/23 (52.2; 22.7–81.7)
Enterocytozoon bieneusi					
Peru11	AY371286	46	21	2	4
WL15	AY237223	15	9	4	5
EbpC	AY371279	4	0	0	1
Type IV	AY371277	6	0	0	2
LW1d	JX000571†	0	0	0	1
Macaque1¶	JX000572†	0	0	1	0
Macaque2¶	JX00057†	1	0	0	0
Unknown‡	NA	4	2	1	0
Subtotal (mean %; 95% CI)§		76/187 (40.6; 31.5–49.8)	32/150 (21.3; 13.9–28.7)	8/74 (10.8; 3.3–18.3)	13/23 (56.5; 25.8–87.2)

*Values are number of positive samples unless otherwise indicated. NA, not applicable.
†From this study.
‡PCR positive but sequence unavailable.
§No. positive /no. tested (% positive; 95% CI).
¶New subtypes or genotypes identified during this study.

E. bieneusi was identified in 116 (28.2%) of the 411 fecal specimens. The occurrence rate at Macaque Garden (40.6%) was significantly higher than at Hongfu Temple (21.3%; p = 0.00016) and Tanquan Spring (10.8%; p = 0.000033). Among the 109 specimens successfully sequenced, 6 genotypes were identified: 4 known genotypes (Peru11 [69 specimens], WL15 [28], EbpC [4], and Type IV [6]) and 2 new genotypes at low frequencies (Table).

Cryptosporidium spp., *G. duodenalis*, and *E. bieneusi* were detected in 11 (47.8%), 12 (52.2%), and 13 (56.5%), respectively, of the 23 water samples collected from the lake where the animals bathed. Fewer *C. hominis* and *G. duodenalis* subtypes and *E. bieneusi* genotypes were detected in water samples than in fecal specimens. Most of the common *C. hominis* (IaA13R8 and IeA11G3T3) and *G. duodenalis* (B1 and B5) subtypes and all common *E. bieneusi* genotypes (Peru11, W15, EbpC, and Type IV) in animals were found in water samples (Table). We deposited unique nucleotide sequences obtained in GenBank under accession nos. JX000562–JX000573.

Conclusions

All *C. hominis* and *C. parvum* subtypes found in this study are well-known parasites of humans and have rarely been found in animals. The C. *hominis* subtype families Ia, Id, Ie, and If had been reported in humans and urban wastewater in China (*5*,*7*–*9*). Although it has not been found in humans in China, the *C. parvum* IIc subtype family identified in rhesus monkeys in this study is a well-known anthroponotic parasite in developing countries (*1*).

The *G. duodenalis* subtypes found in Qianling Park are also major pathogens in humans. The subtype A2 of assemblage A is a common pathogen in humans in most areas studied and is less frequently found in animals than the A1 subtype (*2*). The dominant B1 subtype found in Qianling Park is also identical to an assemblage B subtype (GenBank accession no. GU564280) previously identified in humans in China (*9*).

Most *E. bieneusi* genotypes identified in this study had also been reported in humans. Among the dominant *E. bieneusi* genotypes, Peru11 had been seen only in humans and baboons (*3*,*4*). Although genotypes IV, EbpC, and WL15 have been reported in animals, they are common parasites of humans in many areas (*3*).

The origin of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* parasites in the rhesus monkey population is not clear. Because these parasites are common human pathogens, they could have been introduced by humans. However, rhesus monkeys can be natural hosts of these organisms, as supported by recent identification of some of these organisms in newly captive baboons from rural and forested areas (*4*). Regardless of the initial origin of the parasites, they can be transmitted efficiently among rhesus monkeys, as supported by the higher occurrence of *Cryptosporidium* spp., *G. duodenalis*, and *E. bieneusi* at places with higher animal density.

Our results indicate that rhesus monkeys in close contact with humans are commonly infected with human-pathogenic *C. hominis*, *C. parvum*, and *G. duodenalis* subtypes and *E. bieneusi* genotypes. Therefore, they can serve as reservoirs of human cryptosporidiosis, giardiasis, and microsporidiosis. Zoonotic transmission of infection from these monkeys can occur directly by close contact of monkeys and humans (Figure, panel A), or indirectly through contamination of drinking water or recreational water (Figure, panel B). Efforts should be made to educate the public about the potential risk for zoonotic transmission of enteric pathogens from rhesus monkeys and to minimize contamination of drinking and recreational water by parasites of rhesus monkey origin.

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Schmallenberg Virus as Possible Ancestor of Shamonda Virus

Katja V. Goller,[1] Dirk Höper,[1] Horst Schirrmeier, Thomas C. Mettenleiter, and Martin Beer

Schmallenberg virus (SBV), an orthobunyavirus of the Simbu serogroup, recently emerged in Europe and has been suggested to be a Shamonda/Sathuperi virus reassortant. Results of full-genome and serologic investigations indicate that SBV belongs to the species *Sathuperi virus* and is a possible ancestor of the reassortant Shamonda virus.

A novel virus, Schmallenberg virus (SBV), was discovered in Europe in October 2011, and since then, cases of SBV infection have been reported in sheep, cattle, and goats in several European countries (*1*–*4*). Preliminary phylogenetic analyses revealed that SBV is a member of the genus *Orthobunyavirus* within the family *Bunyaviridae* and is related to Simbu serogroup viruses (*1*). Similar to Akabane virus (AKAV), another Simbu serogroup virus, SBV can cause fatal congenital defects by infection of fetuses during a susceptible stage in pregnancy (*2*). Vaccines for SBV are not available. Thus, SBV poses a serious threat to naive populations of ruminant livestock in Europe.

Orthobunyaviruses are arthropod-borne viruses with a negative-stranded tripartite RNA genome comprising large (L), medium (M), and small (S) segments. Genetic reassortment occurs naturally among these viruses, which results in the emergence of new virus strains that have altered biologic properties (*5*). The L segment encodes the RNA-dependent RNA polymerase; antigenic determinants are the M-encoded viral surface glycoproteins Gn and Gc, which are responsible for viral attachment, cell fusion, hemagglutination, and the induction of neutralizing antibodies, and the S-encoded nucleocapsid protein N, which plays a role in complement fixation (*6*). In the pregenomics era, orthobunyavirus relationships were determined solely by serologic cross-reactivity analyses (*7*), but since DNA sequencing became available, phylogenetic relationships have additionally been assessed by comparison of partial genome sequences (*8*,*9*). However, published full-length genome sequence information is sparse, which makes in-depth phylogenetic analysis difficult. Therefore, a detailed taxonomic classification of SBV could not be made initially when the virus emerged.

The first report of SBV showed highest similarities of M- and L-segment sequences to partial Aino virus and AKAV sequences, whereas the N gene was most closely related to Shamonda virus (SHAV) (*1*). Additionally, results of recent investigations on complete N and M genes and partial L genes of SHAV, Douglas virus (DOUV), and Sathuperi virus (SATV) suggested that SBV is a reassortant consisting of the M segment from SATV and the S and L segments from SHAV (*9*). Conversely, in 2001, SHAV was described as a reassortant virus comprising the S and L segments of SATV and the M segment from the unclassified Yaba-7 virus (*8*). To clarify the phylogenetic relationships and classification of SBV within the Simbu serogroup, we conducted genetic and serologic investigations of its relationship to 9 other Simbu serogroup viruses.

The Study

To enable comparative sequence analysis and phylogenetic investigations, we determined almost full-length S-, M-, and L-segment sequences for 9 Simbu serogroup viruses belonging to 5 species (Table 1): SHAV, Peaton virus, and Sango virus, species *Shamonda virus*; DOUV and SATV, species *Sathuperi virus*; Aino virus and Shuni virus, species *Shuni virus*; Sabo virus, species *Akabane virus*; and Simbu virus, species *Simbu virus*. Sample preparation and sequencing were done by using the Genome Sequencer FLX (Roche, Mannheim, Germany) as described (*10*). Sequence data obtained in this study are archived in the International Nucleotide Sequence Database Collaboration databases (www.insdc.org; accession nos. HE795087–HE795110 and HE800141–HE800143). In addition to the newly determined sequences, published full-genome sequences of AKAV and Oropouche virus (OROV) from the National Center for Biotechnology Information reference genome database (www.ncbi.nlm.nih.gov/sites/genome) were used for sequence comparisons and the reconstruction of phylogenetic relationships. Coding sequences of each genome segment were aligned by using ClustalW (www.clustal.org) for codons, and phylogenetic analyses were performed by using maximum-likelihood methods in MEGA5 (*11*). For the N and L gene analysis Tamura-Nei parameter, the M gene analysis Tamura 3-parameter was used. The robustness of the trees was tested by bootstrap analysis by using 1,000 replications. Sequence identities were calculated by using BioEdit version 7.0.9.0 (*12*).

SBV N gene nucleotide sequence identities to other viruses ranged from 69.8% (OROV; 69.9% aa identity)

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Table 1. Viruses, isolates, and sequence lengths used in classification of Schmallenberg virus within the Simbu serogroup

Virus	Isolate	Sequence length, nt		
		S	M	L
Aino	38K	834	4,335	6,966
Douglas	93–6	813	4,365	6,857
Peaton	CSIRO 110	851	4,324	6,829
Sabo	IB AN 9398	894	4,307	6,857
Sango	An 5077	838	4,314	6,828
Sathuperi	NA	843	4,330	6,861
Schmallenberg	BH80/11–4	830	4,415	6,864
Shamonda	Ib An 5550	927	4,314	6,863
Shuni	Ib An 10107	850	4,326	6,880
Simbu	SA Ar 53	860	4,417	6,895

*S, small segment; M, medium segment; L, large segment; NA, not available.

to 97.7% (SHAV; 100% aa) (Table 2, Appendix, www.cdcnc.gov/EID/article/18/9/12-0835-T2.htm). The L gene sequence of SBV had the lowest identity to OROV (60.4% nt; 57.5% aa) and highest identity to SHAV (92.9% nt; 98.4% aa); the SBV M gene showed the highest sequence identity to SATV (82.1% nt; 90.1% aa), whereas identity of SBV and SHAV M gene was low (48.2% nt; 36.5% aa). In general, identity of the SHAV M gene to the other Simbu serogroup viruses was low, from 45.6% nt (33.4% aa; OROV) to 55.0% nt (47.9% aa; Sango virus), which indicates that its M segment belongs to another virus, as previously suggested (*8*). On the other hand, the high sequence identity of all SBV genes to SATV and DOUV indicates that SBV belongs to the species *Sathuperi virus*.

A phylogenetic tree derived from the M gene (Figure, panel A) demonstrates that SBV and DOUV cluster closely with SATV, whereas SHAV was placed distantly from all other viruses. In contrast, phylogenetic trees of the L (Figure, panel B) and N (Figure, panel C) genes showed a close relationship between SBV and SHAV. Results for all genome segments show that SBV should be classified within the species *Sathuperi virus* and is likely to be the ancestor of SHAV, which is in contrast a reassortant virus comprising the S and L segments from SBV and the M segment from another virus, as proposed previously (*8*).

To further clarify the placement of SBV within the Simbu serogroup, we determined the ability of SBV antibodies to neutralize other Simbu serogroup viruses. We performed titer reduction assays of the 9 other viruses with anti-SBV serum; the viruses were propagated in BHK-21 cells clone CT (L164, Collection of Cell Lines in Veterinary Medicine, Friedrich-Loeffler-Institute, Riems, Germany). Neutralizing potencies of SBV antibodies against Simbu serogroup virus strains were tested by titrating virus in the presence of anti-SBV serum (neutralizing titer 32); results were expressed as percentage titer reduction in relation to a parallel test without antiserum. Although DOUV and SATV were well neutralized, SHAV titers were not reduced at all (Figure, panel A), which supports the M gene phylogeny. Thus, phylogeny and cross-reactivity identified SHAV, but not SBV, as a reassortant within the Simbu serogroup.

Conclusions

Although our results do not support the suggestions of Yanase et al. (*9*), they are fully consistent with the conclusions of Saeed et al. (*8*). On the basis of our results and those of Saeed et al. (*8*), we suggest that SHAV should be reclassified into the species *Sathuperi virus* and that the species *Shamonda virus* should be renamed *Peaton virus* or *Sango virus*.

In addition to showing that SBV belongs to the species *Sathuperi virus*, our results show that the virus is most likely not a reassortant and is likely to be one of the ancestors of SHAV, whereas SHAV is a reassortant comprising the SBV S and L genomic segments and the M segment from an unclassified virus. These detailed insights into the phylogeny of SBV could be the basis for

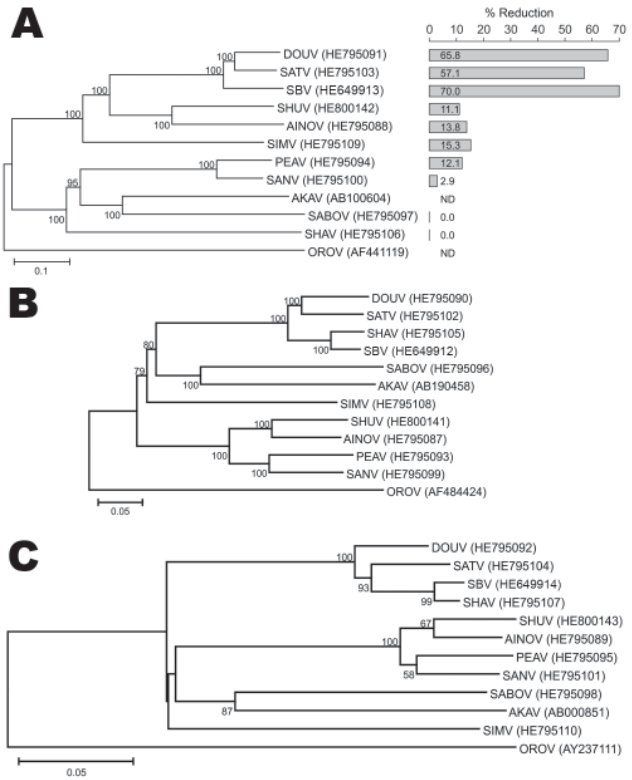

Figure. Maximum-likelihood trees showing phylogenetic relationships of Simbu serogroup viruses for the M (A), L (B), and S coding regions (C). The bar plot in panel A indicates the percentage of titer reduction of each virus by anti–Schmallenberg virus serum. GenBank accession numbers are shown. Numbers at nodes indicate percentage of 1,000 bootstrap replicates (values <50 are not shown). Scale bars indicate nucleotide substitutions per site. DOUV, Douglas virus; SATV, Sathuperi virus; SBV, Schmallenberg virus; SHUV, Shuni virus; AINOV, Aino virus; SIMV, Simbu virus; PEAV, Peaton virus; SANV, Sango virus; AKAV, Akabane virus; SABOV, Sabo virus; SHAV, Shamonda virus; OROV, Oropouche virus. ND, not determined.

the development of efficient, cross-protective vaccines. Our results also highlight the importance of full-genome analyses to identify potential genetic reassortments and to investigate the evolutionary history of viruses with segmented genomes.

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Monkey Bites among US Military Members, Afghanistan, 2011

Luke E. Mease[1] and Katheryn A. Baker[2]

Bites from *Macaca mulatta* monkeys, native to Afghanistan, can cause serious infections. To determine risk for US military members in Afghanistan, we reviewed records for September–December 2011. Among 126 animal bites and exposures, 10 were monkey bites. Command emphasis is vital for preventing monkey bites; provider training and bite reporting promote postexposure treatment.

Military members deployed to Afghanistan face many risks; among these are bites from *Macaca mulatta* monkeys and possible subsequent infections. In August 2011, a 24-year-old US Army soldier died of a rabies infection contracted while in eastern Afghanistan. This tragedy highlights the threat that animal bites pose to deployed military members.

During 2001–2010, a total of 643 animal bites among deployed US military members were reported (*1*). Dogs were implicated in 50% of these bites, but several other animals pose risk as well. Prominent among these is the nonhuman primate *M. mulatta* (rhesus macaque), native to and commonly kept as a pet in Afghanistan (*2*) (Figure). Risks from *M. mulatta* monkey bites include physical trauma and/or infection with B-virus (Macacine herpesvirus 1), oral bacteria (including *Clostridium tetani*), and rabies virus. Although not well characterized in Afghanistan, the risk for exposure to *M. mulatta* monkeys has been described (*3*) for researchers (*4*), tourism workers (*5*), and US pet owners (*6*). We examined this risk for US military members deployed to eastern Afghanistan. The work presented herein was reviewed and deemed exempt from internal review board oversight by the Joint Combat Casualty Research Team, the human subjects review board responsible for oversight of human subjects research affecting US military members in Afghanistan.

The Study

Information about all reported animal bites and exposures affecting US military and coalition personnel is collected by preventive medicine officers assigned to Combined Joint Task Force–1 in eastern Afghanistan. We evaluated these records to identify and describe monkey bites and high-risk exposures among US military members serving in eastern Afghanistan during September–December 2011. For this study, eastern Afghanistan refers to North Atlantic Treaty Organization Regional Command East, which covers ≈43,000 square miles (110,000 km^2). The US military population in eastern Afghanistan during the study period was ≈23,500 persons. Case information obtained included patient age, sex, rank, branch of military service, animal exposures, and treatment details.

We evaluated the cases for the 5 parameters that comprise appropriate initial treatment according to the literature. The parameters are wound care (appropriate cleansing of the wound) (*7*), antiviral medications for B-virus (valacyclovir) (*8*), antimicrobial drugs for oral bacteria (amoxicillin/clavulanic acid or clindamycin plus sulfamethoxazole/trimethoprim) (*3*), verification of up-to-date tetanus vaccination status or vaccine administration in accordance with Advisory Committee on Immunization Practices guidelines (*9*), and rabies postexposure prophylaxis (PEP). US military policy advised that rabies PEP should adhere to World Health Organization guidelines (*10*), which recommend giving human rabies immunoglobulin plus 5 doses of rabies vaccine. In accordance with the same policy, adherence to Advisory Committee on Immunization Practices guidelines for rabies PEP with human rabies immunoglobulin plus 4 doses of rabies vaccine was also acceptable (*11*).

When appropriate initial treatment was not administered, subsequent follow-up was conducted to ensure that patients received required treatment. Appropriate treatment was accomplished by contacting and coordinating with the responsible provider, the patients, and their commanders.

During the study period, we identified 126 cases of animal bites or serious exposures (involving animal neural tissue or saliva affecting the mucosal surfaces or open wounds of the patient). Among these cases, 10 were cases of monkey bites.

Among the 10 military members who had been bitten by monkeys, age range was 22–44 years (Table); most (7) were <30 years of age, and 8 were male. All were junior enlisted or noncommissioned officers; 8 were members of the Army, and 2 were members of the Air Force (Table).

In terms of treatment, 6 received appropriate wound care and washing, 5 received appropriate B-virus prophylaxis, and 8 received appropriate antimicrobial drugs (Table). In terms of prophylaxis, only 4 were evaluated for

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Figure. Pet monkey (*Macaca mulatta*), Afghanistan, 2011. Photograph courtesy of Ronald Havard.

tetanus status, and 8 received appropriate rabies PEP. Beyond the initial trauma and follow-up visits for rabies PEP, no visits for any illness possibly associated with the bite or exposure were recorded.

All cases involved different monkeys, 8 of which were kept as pets. Of these 8 pet monkeys, 4 belonged to Afghan National Security Forces (ANSF), 3 belonged to Afghan civilians, and 1 belonged to US military members. For the other 2, no ownership data were available; they could have been wild or pets. One monkey was euthanized and sent to US Army Veterinary Laboratory Europe for testing; brain samples were negative for rabies and B-virus.

Conclusions

Our identification of 126 reported bites or exposures over just 4 months suggests that the 643 animal bites reported for all deployed US military members for the past decade greatly underestimate the true number of animal bites in this population. The number of bites and exposures identified in this study might represent more accurate reporting because of increased attention to animal bites after the US soldier died in August 2011. It is possible that before that time, only more severe bites and exposures were reported but that after that time, more lower-risk exposures might have been reported.

The risk for monkey bites in other populations has been described. The 10 monkey bites reported in this study demonstrate that US and coalition military members in Afghanistan are also at risk for the trauma and the B-virus, bacterial, tetanus, and rabies infections that can result from monkey bites and exposures. The demographics of the population bitten (Army, age <30 years, and male) is representative of the underlying population at risk.

Most monkey-bite patients received appropriate care. This care is laudable, considering the recognized difficulties in treating monkey bites (*12*). Some patients, however, did not receive appropriate medical treatment initially. Because treatment of monkey bites is not a standard part of US medical education, inadequate treatment could reflect insufficient training and lack of familiarity among US-trained health care providers. It is imperative that before providers are deployed to Afghanistan, they receive proper instruction on the care of animal bites and exposures. Appropriate reporting of any animal bite to military preventive medicine personnel is crucial because it permits oversight of care and timely correction of deficiencies.

Table. Characteristics of US military members bitten by monkeys, eastern Afghanistan, September–December, 2011*

Patient no.	Age, y/sex	Military branch	Treatment received					Monkey ownership
			Wound care	Valacyclovir	Antimicrobial drug	Tetanus vaccine	Rabies vaccine, HRIG	
1	39/M	Army	–	+	+	+	+	ANSF
2	27/M	Army	+	+	+	+	+	CIV†
3	22/M	Army	–	+	+	–	+	CIV
4	44/F	Army	+	–	+	–	–	CIV
5	31/M	Army	+	–	+	+	+	ANSF
6	26/M	Air Force	+	–	–	–	–	US military
7	26/M	Army	–	+	–	–	+	ANSF
8	27/M	Army	+	–	+	+	+	ANSF
9	22/M	Army	–	–	+	–	+	Unknown
10	25/F	Air Force	+	+	+	–	+	Unknown

*HRIG, human rabies immunoglobulin; –, not administered; +, administered; ANSF, Afghan National Security Forces; CIV, Afghan civilian.
†Monkey euthanized. Brain, tested at US Army Veterinary Laboratory Europe, was negative for rabies and B-virus.

Most (7/10) monkeys involved were pets owned by ANSF or Afghan civilians. As the mission in Afghanistan shifts from combat to ANSF mentoring and reconstruction, US and coalition troops will come into increasingly close contact with ANSF and Afghan civilians. Accordingly, the likelihood of deployed US military members being exposed to monkeys in Afghanistan will probably increase. However, although risk for contact with monkeys might increase, an increase in bites is not inevitable. Explicit orders prohibit deployed US military members from adopting local mascots and from interacting with animals or pets owned by ANSF or Afghan civilians. To mitigate the risk for animal bites, it is crucial that commanders enforce these regulations (*13*).

The risk of being bitten by a monkey could increase as US forces work more closely with ANSF and Afghan civilians. Bites could be prevented by appropriate emphasis from command and enforcement of existing policies prohibiting pet adoption and animal contact. Treatment of patients who are bitten could be improved by further training of military health care providers on appropriate treatment for animal bites, including monkey bites.

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Human Parvovirus 4 in Nasal and Fecal Specimens from Children, Ghana

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Nonparenteral transmission might contribute to human parvovirus 4 (PARV4) infections in sub-Saharan Africa. PARV4 DNA was detected in 8 (0.83%) of 961 nasal samples and 5 (0.53%) of 943 fecal samples from 1,904 children in Ghana. Virus concentrations ≈6–7 $\log_{10}$ copies/mL suggest respiratory or fecal–oral modes of PARV4 transmission.

Human parvovirus 4 (PARV4; human partetravirus) is a single-stranded DNA virus discovered in 2005 (*1*). PARV4 has been detected in persons at risk for parenteral infections, suggesting blood-borne transmission (*2*,*3*) although other transmission routes have not been ruled out. Studies in northern Europe demonstrated a high prevalence of antibodies against PARV4 in injection drug users, persons co-infected with HIV and hepatitis C virus, and persons with hemophilia who were exposed to nonvirally inactivated clotting factors; however, antibodies were not detected in the general population (*4*,*5*).

In contrast, PARV4 seroprevalence was 25%–37% in adults in the Democratic Republic of Congo, Cameroon, and Burkina Faso who were not infected with HIV and hepatitis C virus. (*6*). PARV4 DNA was detected in blood of 8.6% of children 15 or 24 months of age in Ghana (*7*). There was no history of exposure to multiple-use needles or blood transfusion in any of these children. These data suggested alternative modes of PARV4 transmission in countries in Africa. Nonparenteral modes of transmission of PARV4 have also been suggested in South Africa (*6*), Taiwan (*8*), India, (*9*), China (*10*), and Thailand (*11*).

PARV4 has been classified into 3 genotypes. Genotypes 1 and 2 are found in North America, Europe, and Asia (*1–3*,*9–11*), and genotype 3 is found in in sub-Saharan Africa (*7*,*12*). To investigate whether PARV4 is found in the respiratory or intestinal tract, we analyzed previously collected specimens from 1,904 children in Ghana.

The Study

Ethical approval for this study was provided by the Committee on Human Research Publication and Ethics, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana. Informed consent was obtained from parents or guardians of all children.

A total of 1,904 anonymous nasal and fecal specimens were obtained during a study on molecular diagnostics for respiratory and enteric tract infections in symptomatic children <15 years of age at the Presbyterian Hospital in Agogo, Ghana. Nasal swab specimens were obtained from children with upper or lower respiratory tract symptoms. Fecal samples were obtained from 504 children with gastrointestinal symptoms (53.4% of sampled children; 294 [58.3%] of symptomatic children with vomiting, 190 [37.7%] with diarrhea, and 144 [28.6%] with acute malnutrition; 9 [1.8%] with incomplete clinical data) and 439 (46.6%) children without gastrointestinal symptoms.

A total of 961 nasal swabs were obtained during February–November 2008 from 520 boys and 441 girls (median age 19 months, range 0–162 months, interquartile range 8–38 months). Nasal swabs were placed in 1.5 mL of RNAlater (QIAGEN, Hilden, Germany). A total of 943 fecal samples were obtained during May–October 2009 from 500 boys and 443 girls (median age 36 months, range 0–165 months, interquartile range 17–78 months). Fecal samples were prepared as 10% suspensions in phosphate-buffered saline. No paired nasal and fecal specimens were available from individual patients.

Viral DNA was purified from 140 μL of nasal swab suspension or 200 μL of fecal suspension by using QIAamp Viral RNA and DNA Stool Mini Kits (QIAGEN), respectively. Two real-time PCRs were performed. One primer/probe set was designed to detect PARV4 genotypes 1 or 2 viruses (*13*), and a second primer set was designed to detect PARV4 genotype 3 viruses (*7*). The sensitivity of both protocols was 1–2 genome copies/reaction. Absolute quantification of PARV4 genome copy numbers relied on photometrically quantified genotype 3 plasmid standards, as described (*7*).

To exclude bias from DNA purification methods, PARV4-negative nasal and fecal specimens were spiked with quantified plasmid standards. Subsequent

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Table. Nucleotide sequence divergence of parvovirus 4 strains from nasal swab and fecal samples from children, Ghana, from genotype 1, 2, and 3 prototype strains*

Specimen type and no.	Nucleotide position according to GenBank accession no. EU874248	Nucleotide sequence divergence from parvovirus 4 reference strains, %		
		Genotype 1 (GenBank AY622943)	Genotype 2 BR10627–5 (GenBank DQ873390)	Genotype 3 NG-OR (GenBank EU874248)
Nasal swab				
N1	1700–4660	6.56	7.39	0.92
N2	299–4660	7.51	8.07	0.88
N3	50–4660	7.37	8.38†	0.83
N4	1962–2056‡	9.16	6.73	2.14
N4	2117–3413	4.97	5.31	0.93
N5	1962–2056	9.16	6.73	2.14
N5	2117–4183	5.50	6.34	0.98
N6	299–4660	7.51	8.10	0.90
N7	1962–2056	9.16	6.73	2.14
N7	2431–2914	6.24	7.01	1.25
N7	3068–3246	4.61	5.19	1.12
N8	624–3246	7.36	7.84	0.84
Feces				
F1	1700–4183	6.20	6.82	0.89
F2	1700–4460	6.56	7.39	0.92
F3	1700–3716	6.08	6.52	0.85
F4	1700–4183	6.02	6.78	0.89
F5	1700–4183	6.93	6.73	1.04

*Pairwise nucleotide divergence was calculated by using the DNA distance matrix in BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html).
†Because the homologs of the first 92 nt of strain N3 are not given in the prototype strain BR10627–5, calculation of divergence started at N3 nt position 93.
‡Nucleotide sequence of the PCR product (primer sequences trimmed) was amplified by using screening PCR designed for detection of PARV4 genotype 3 as described (*7*).

quantification was equivalent between techniques and specimens, and differences between specimen types in several experiments were <0.5 $\log_{10}$ copies/mL. Standard procedures were used to prevent PCR contamination. Determination of PARV4 genotypes was conducted by nucleotide sequencing of several genomic target regions (Table).

Eight (0.83%) of 961 nasal swabs and 5 (0.53%) of 943 fecal samples tested were positive for PARV4 DNA. Virus concentrations ranged from 1.3×10^3 to 1.8×10^7 copies/mL (median 1.0×10^4 copies/mL) in nasal swab suspensions and from 2.3×10^3 to 4.6×10^6 copies/mL (median 6.8×10^4 copies/mL) in fecal suspensions (Figure 1). The difference in virus concentrations between the 2 groups was not significant (p = 0.056, by Mann-Whitney U test).

Nucleotide sequencing of amplicons generated by screening PCRs and sequencing of additional genomic regions classified all viruses as PARV4 genotype 3 (Table) (GenBank accession numbers JN183920–JN183932). This result was confirmed by phylogenetic analysis of a 483-nt fragment of the capsid-encoding open reading frame 2 (Figure 2).

Ages of the 8 children with PARV4-positive nasal swab specimens ranged from 9 to 58 months (median 32 months). Ages of the 5 children with PARV4-positive fecal samples were 1, 36, 43, 57, and 124 months. Nasal swab specimens with the highest viral loads were from a 9-month-old boy and a 29-month-old girl. Fecal samples with the highest viral loads were from 2 boys 43 and 57 months of age.

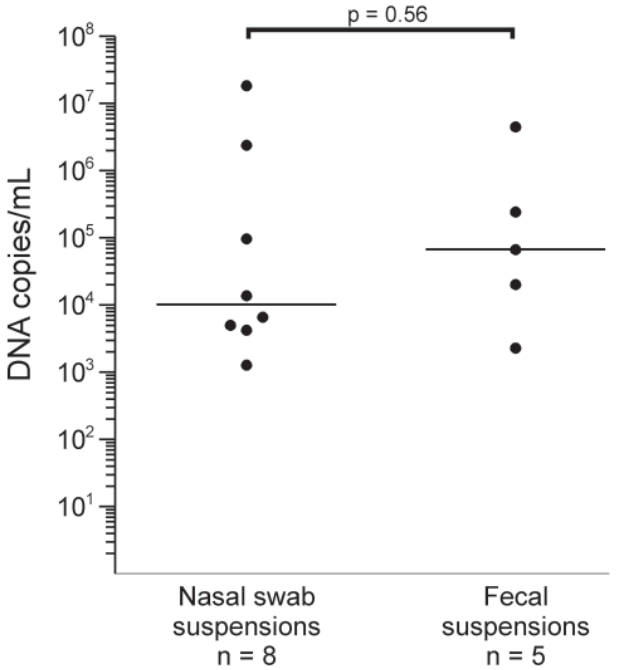

Figure 1. Parvovirus 4 DNA loads in virus-positive nasal and fecal specimens from children, Ghana. Virus concentrations are given on a log scale on the y-axis. Each dot represents 1 specimen. Horizontal lines represent median values for each sample type. For calculation of statistical significance of the difference in viral quantities between sample types, the Mann-Whitney U test was used. Virus quantities in nasal swabs and feces are given for sample suspensions (nasal swabs in 1.5 mL of stabilizing reagent and feces in a 10% suspension in phosphate-buffered saline).

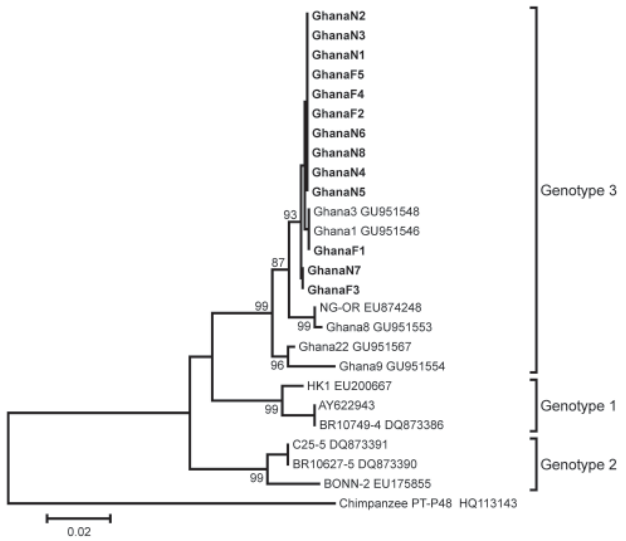

Figure 2. Phylogenetic analysis of a 483-nt fragment of the parvovirus 4 (PARV4) capsid-encoding open reading frame (ORF) 2 for PARV4 strains identified in children, Ghana. Neighbor-joining phylogeny was conducted in MEGA5.05 (www.megasoftware.net) by using a gap-free ORF2 fragment corresponding to positions 2,432–2,914 in the PARV4 genotype 3 prototype strain NG-OR (GenBank accession no. EU874248) with a nucleotide percentage distance substitution model and 1,000 bootstrap replicates. Scale bar indicates percentage uncorrected nucleotide distance. Previously published PARV4 sequences are given with strain names (if available) and GenBank accession numbers. Viruses newly identified are in **boldface**. The source of PARV4 strains identified in the study is indicated by capital letters (N, nasal specimen; F, fecal specimen). PARV4 genotypes are given to the right of taxa. A chimpanzee partetravirus was used as the outgroup.

Conclusions

We found PARV4 in 0.8% of nasal swab specimens and 0.5% of fecal specimens from 2 groups of children in Ghana symptomatic for respiratory illness and with or without diarrheal illness, respectively. Our results provide evidence to suggest that the higher prevalence of PARV4 reported among adults in countries in western Africa (*6*) might be caused by transmission by the respiratory or fecal–oral route.

However, demonstration of PARV4 in the respiratory tract and feces does not identify a transmission route. PARV4 in the respiratory tract could be caused by high viremia, which was recently reported in a child in India with a genotype 2 infection (*9*) and in 2 patients with hemophilia in the United Kingdom, 1 with a genotype 1 infection and 1 with a genotype 2 infection (*14*).

It is unclear to what extent the putative nonparenteral transmission routes of PARV4 genotype 3 in western Africa apply to other areas. Markedly lower PARV4 antibody prevalences observed in Europe (*4*,*5*) argue against PARV4 spread by nonparenteral routes, e.g., from infected injection drug users to the general population. Likewise, the higher prevalence of PARV4 antibodies in HIV-infected blood donors in South Africa compared with uninfected donors (*6*) appears incompatible with PARV4 transmission primarily by the respiratory route. Therefore, our results do not contradict those of a study conducted in Scotland, which showed no PARV4 in respiratory specimens (*15*).

Because of the small number of children with PARV4 DNA in nasal or fecal specimens, correlation of infection with age groups was not possible. A limitation of our study was the lack of blood specimens from children with current respiratory or fecal PARV4 shedding, and serologic studies are needed to evaluate susceptibility of different age groups to PARV4 infection. Furthermore, detection of PARV4 in patients with respiratory disease does not indicate that PARV4 was the cause of the disease. In 5 of 8 PARV4-positive nasal swabs, typical respiratory viruses (parainfluenza virus, influenza A virus, rhinovirus) were also detected and the pattern of symptoms in PARV4-positive children did not differ from symptoms in PARV4-negative children. Similarly, 3 of 5 children with PARV4-positive feces did not have gastrointestinal symptoms at the time of fecal sampling. One child had vomiting and another child had vomiting and diarrhea. Moreover, in 3 of these 5 children, in addition to PARV4, *Giardia lamblia*, a potential cause of diarrhea, was also detected.

Although data for exposure and risk factors and paired samples were not available, suggested transmission routes might explain the high infection rates in western Africa. Further studies are needed to assess the effect of PARV4 excretion on virus epidemiology and the chronology of PARV4 infection.

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Hepatitis E Virus Seroprevalence among Adults, Germany

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We assessed hepatitis E virus (HEV) antibody seroprevalence in a sample of the adult population in Germany. Overall HEV IgG prevalence was 16.8% (95% CI 15.6%–17.9%) and increased with age, leveling off at >60 years of age. HEV is endemic in Germany, and the lifetime risk for exposure is high.

In industrialized countries, hepatitis E virus (HEV) has long been regarded as a rare imported infection. However, sporadic cases without travel to disease-endemic areas and caused by genotype 3 are being increasingly reported (*1*,*2*). Epidemiologic and molecular studies have implicated undercooked pork and wild boar products as a source of HEV infection (*3*–*5*). An unexpectedly high prevalence of HEV-specific antibodies, e.g., among blood donors, has been shown by several studies in Europe and the United States (*6*–*11*).

In Germany, the number of notified hepatitis E cases rose from <50 annually in 2001–2003 to 238 in 2011 (incidence 0.3/100,000 population); the proportion of autochthonous cases increased from 30%–40% to 78%. We conducted a study to determine HEV seroprevalence in Germany's adult population and associations with sociodemographic characteristics by using an assay highly sensitive for HEV genotype 3.

The Study

We assessed HEV seroprevalence in a large subsample (n = 4,422) of the 2008–2011 German Health Examination Survey for Adults (Deutscher Erwachsenen Gesundheitssurvey; www.degs-studie.de), a 2-stage national probability sample that assessed the health status in the general population. The sampling frame comprised persons 18–79 years of age whose principal residence was in Germany and who were fluent in German. Overall response was 48.4% (7,116 respondents). Our subsample reflects the total adult population with respect to age, sex, and geographic region, but persons with migration background are underrepresented (non-German citizenship 4.6% in the sample vs. 8.7% in the total adult population).

Serum samples were screened for HEV IgG by using the *recom*Line HEV-IgG/IgM immunoassay (Mikrogen, Neuried, Germany). The assay is based on 7 recombinantly expressed antigens of genotypes 1 and 3 of open reading frames 2 and 3. According to the manufacturer's and our data (J.J. Wenzel et al., unpub. data), the test is 97%–100% sensitive for detecting acute or previous HEV infections. Test strips were scanned with the semiautomatic *recom*Scan software (Mikrogen). The intensity of 3 quality assurance and other bands was determined by densitometrical detection algorithms. Each antigen band with an intensity greater or equal to the cutoff was assigned a point value. The final results were classified into 3 categories: no antibodies detectable (negative), test inconclusive (borderline), and antibodies detectable (positive). Persons whose test results were borderline (n = 70) were excluded from further analysis.

We poststratified the remaining survey population (n = 4,352) by age group and location of residence (16 states) to account for per protocol oversampling in eastern Germany and to restore the distribution of age groups to match the distribution in the total population. Weighted seroprevalence estimates were calculated by using survey-weighted logistic regression. Associations between demographic characteristics and seropositivity were analyzed by using adjusted Wald test p values. We also estimated mean annual incidence of HEV seroconversion from the seroprevalence data by using a catalytic model with age-constant force of infection, similar to that of Faramawi et al. (*12*). Detailed methods and underlying assumptions are described in the online Technical Appendix (wwwnc.cdc.gov/EID/pdfs/11-1756-Techapp.pdf).

The 4,352 persons who were included in the analysis were from 108 communities of all federal states in Germany (Table 1). Weighted prevalence of HEV IgG was 16.8% (95% CI 15.6%–17.9%); prevalence ranged from 6.1% (95% CI 4.5%–7.8%) in the 18–34-year age group to >20% in the >50-year groups, with a maximum of 26.4% (95% CI 21.6%–31.1%) in the 60–64-year group (Figure). In the univariable analysis (Table 2), only age group was significantly associated with seropositivity (p<0.01); results were not significant for sex (p = 0.97), residence (northern/middle/southern Germany, p = 0.29; west/east, p = 0.43), or population of municipality (4 categories; p = 0.10). In separate multivariable models, each including age group and 1 other variable, age remained the only significant

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Table 1. Comparison of demographic characteristics of persons in study of hepatitis E virus seroprevalence and general adult population, Germany, 2008–2011

Characteristic	% Study population, n = 4,352	% Total population, n = 64,139,871*
Age group, y		
18–19	3.2	2.9
20–29	12.3	15.5
30–39	11.5	15.6
40–49	18.4	21.7
50–59	19.5	17.9
60–69	19.7	14.3
70–79	15.4	12.2
Female sex	51.3	50.3
State of residence		
Baden-Württemberg	11.4	13.0
Bavaria	10.7	15.2
Berlin	3.4	4.4
Brandenburg	6.2	3.2
Bremen	0.9	0.8
Hamburg	1.4	2.2
Hesse	6.4	7.4
Mecklenburg-Vorpommern	3.8	2.1
Lower Saxony	11.2	9.5
North Rhine-Westphalia	14.8	21.6
Rhineland-Palatinate	5.1	4.8
Saarland	2.2	1.3
Saxony	7.1	5.3
Saxony-Anhalt	6.2	3.0
Schleswig-Holstein	2.6	3.4
Thuringia	6.7	2.9

*Total no. adults 18–79 years of age as of December 31, 2009 (www.destatis.de).

variable. Mean annual incidence of HEV seroconversion estimated from the catalytic model was 3.9 (95% CI 3.6%–4.2%) per 1,000 population.

Conclusions

We found an overall HEV seroprevalence of 16.8% among adults in Germany; seroprevalence increased with age but was not dependent on sex or location of residence. Similarly high seroprevalence was found in blood donors in Denmark (20.6%), southwestern England (16%), and the United States (18%) (*6*,*7*,*9*). Estimates from Switzerland and the Netherlands, on the other hand, are considerably lower (*10*,*11*). Reasons for these differences could be effects of sample selection, different lifetime exposures (e.g., to foods that may serve as transmission vehicles), or use of different test systems with varying sensitivity. Mansuy et al. recently reported 53% prevalence of HEV antibodies in blood donors in southwestern France (*13*), a figure considerably higher than the 17% prevalence reported earlier for the same geographic region, when a different test system was used (*8*). The assay applied in our study was designed to also detect previous infections with HEV genotype 3; therefore, it is likely to be more sensitive than assays used in previous studies.

Our data show that HEV exposure is common among the general adult population in Germany, which is consistent with increasing evidence for pigs as a reservoir for foodborne transmission of HEV in industrialized countries. HEV seroprevalence is high in domestic pig herds in Germany and other countries; closely related HEV strains were found in pig livers on retail sale and in autochthonous cases of hepatitis E, and HEV seroprevalence was higher in persons with occupational exposure to pigs than in control groups (*4*,*10*,*14*,*15*).

Our data and other studies (*11*–*14*) have shown no significant difference in seroprevalence between sexes, despite an assumed higher frequency of alimentary or occupational exposure and the higher incidence of clinical cases among men. This finding may indicate sex-specific differences in disease development or application of laboratory testing or that foods frequently consumed by both sexes play a substantial role as vehicles for transmission. These results also may highlight the lack of evidence for 1 main risk factor or food vehicle (*14*).

The strong association between age and HEV seroprevalence in our study most likely reflects cumulative lifetime exposure to the virus. However, HEV seroprevalence remains relatively stable from 18 to 34 years of age, which could indicate a birth cohort effect resulting from a decrease in the overall risk over the past few decades, as was reported for Denmark (*6*). The leveling

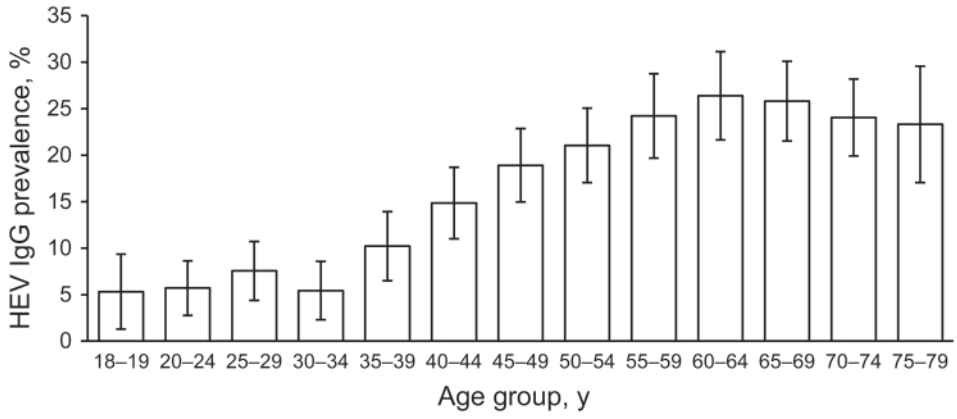

Figure. Estimated prevalence of hepatitis E virus (HEV) IgG, by age group, Germany, 2008–2011. Error bars indicate 95% CIs.

Table 2. Univariable survey-weighted logistic regression estimates of seroprevalence of hepatitis E virus IgG, by age, sex, and location of residence, Germany, 2008–2011*

Characteristic	Seroprevalence, % (95% CI)
Total	16.8 (15.6–17.9)
Age group, y	
18–19	5.3 (2.5–11.1)
20–29	6.6 (4.8–9.2)
30–39	8.0 (5.8–10.8)
40–49	16.9 (14.3–19.9)
50–59	22.6 (19.7–25.7)
60–69	26.1 (23.0–29.4)
70–79	23.8 (20.4–27.5)
Sex	
M	16.8 (15.2–18.5)
F	16.7 (15.2–18.5)
Place of residence (north–south)	
Northern states	15.5 (13.5–17.6)
Middle states	16.6 (14.8–18.6)
Southern states	17.9 (15.8–20.1)
Place of residence (east-west)	
Western states	16.6 (15.2–18.0)
Eastern states	17.6 (15.6–19.7)
Population of municipality	
<5,000	17.9 (15.3–20.8)
5,000–<20,000	17.5 (15.2–20.1)
20,000–<100,000	14.4 (12.4–16.6)
>100,000	17.7 (15.7–20.0)

*n = 4,352.

off of the seroprevalence above age 60 years could be caused by a loss of antibodies in the elderly.

We found a striking difference between the estimated annual incidence of seroconversion and the relatively low incidence of notified disease in Germany. Besides underdiagnosis, a possible explanation is a high proportion of asymptomatic infections.

The survey response rate is similar to those achieved in other comprehensive national health examination surveys in Europe. In the statistical analysis, we corrected for differences in the distribution of age and place of residence between the sample and the general population. However, foreign-born persons are underrepresented in the survey because participation required fluency in German. A high proportion of foreign-born persons in Germany are Muslim, and their avoidance of raw pork products likely decreases their risk for HEV infection, which means we may have slightly overestimated the seroprevalence in the adult general population.

Autochthonous HEV infections are common in the industrialized world, and testing for HEV is indicated in patients with acute hepatitis, irrespective of history of travel to developing countries. Recommendations against eating undercooked pork products exist, but further studies are needed to specify implicated food products and to better target recommendations for safer food production, preparation, and consumption.

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Scarlet Fever Epidemic, Hong Kong, 2011

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More than 900 cases of scarlet fever were recorded in Hong Kong during January–July, 2011. Six cases were complicated by toxic shock syndrome, of which 2 were fatal. Pulsed-field gel electrophoresis patterns suggested a multiclonal epidemic; *emm*12 was the predominant circulating type. We recommend genetic testing of and antimicrobial resistance monitoring for this reportable disease.

Scarlet fever is caused by infection with *Streptococcus pyogenes* and mainly affects children. An upsurge of scarlet fever occurred in Hong Kong, People's Republic of China, in 2011, exceeding baseline annual incidence rates for the previous 2 decades. We investigated possible changes in clinical severity, transmissibility, and characteristics of the causative pathogen for this outbreak.

The Study

Scarlet fever is a statutory notifiable disease in Hong Kong. A clinical case is defined as illness in a person who has clinical features of scarlet fever (fever and fine, sandpaper rash of characteristic distribution that blanches on pressure, with or without strawberry tongue, desquamation, or sore throat). A confirmed case is defined as a clinical case with positive throat or wound culture for *S. pyogenes* or antistreptolysin titer >200.

Epidemiologic, clinical, and laboratory data were collected by standard questionnaire for every reported case. A cluster was defined as >2 cases in persons sharing the same residential or school address within the incubation period. We compared epidemiologic, clinical, and microbiological features of the scarlet fever cases from January–July 2011 (outbreak period) with features of those reported during 2008–2010 (baseline period). We used SPSS version 14.0 (SPSS Inc., Chicago, IL, USA) for analyses; $p<0.05$ was considered significant.

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For comparison, we performed a retrospective review of hospital discharge records kept by public hospitals. We extracted records of patients hospitalized during January 2008–July 2011 who had diagnoses that are known complications of scarlet fever, including toxic shock syndrome, acute rheumatic fever, and acute glomerulonephritis. These cases were reviewed to determine whether the complications were related to scarlet fever.

Bacterial culture of *S. pyogenes* was performed on diagnostic specimens in hospital laboratories and the Public Health Laboratory Centre of the Department of Health; the latter serves as the diagnostic and public health reference laboratory in Hong Kong. Antimicrobial drug susceptibility testing, *emm* typing, and detection of various virulence genes were performed at the Public Health Laboratory Centre on *S. pyogenes* isolates received during 2011 and archived during 2008–2010 (*1*). Pulsed-field gel electrophoresis (PFGE) was performed on the basis of the gram-positive protocol, and PFGE profiles were analyzed by using BioNumerics 5.0 software (Applied Maths, Sint-Martens-Latem, Belgium).

In June 2011, the Department of Microbiology of the University of Hong Kong announced the discovery of a unique 48-kb insertion sequence in the genome of *S. pyogenes* isolated from a blood specimen from a 7-year-old girl who died of scarlet fever (*2*). We tested for this insert in a sample of strains collected during 2008–2011 using the method provided by the University of Hong Kong.

During January 1–July 31, 2011, a total of 996 cases of scarlet fever were reported, greatly exceeding the annual number of cases reported during 2008 (235), 2009 (187), and 2010 (128). Outbreak activity in 2011 peaked at week 26 (week ending June 25) (Figure 1). During the outbreak period (January–July 2011), the annualized incidence rate was 24.0/100,000 population, ≈9× higher than the average annualized incidence rate of 2.62/100,000 population during the baseline period of 2008–2010. During the previous 2 decades, baseline annual incidence rates ranged from 0.0351 to 3.37 cases/100,000 population.

Table 1 compares the epidemiologic features, clinical features, and laboratory results for scarlet fever cases reported during 2011 and 2008–2010. Highest incidence (547 cases/100,000 population) was reported for children 4–7 years of age (Table 1). Clinical features, complications, and case-fatality rate for cases reported in 2011 were largely comparable to those reported during the baseline period. The proportion of case-patients requiring hospitalization during 2011 was lower, and mean duration of hospital stay was ≈0.5 days shorter than for the baseline period. Details of the 9 complicated cases are shown in Table 2.

Among the 996 scarlet fever cases reported during January–July 2011, *S. pyogenes* isolates from samples

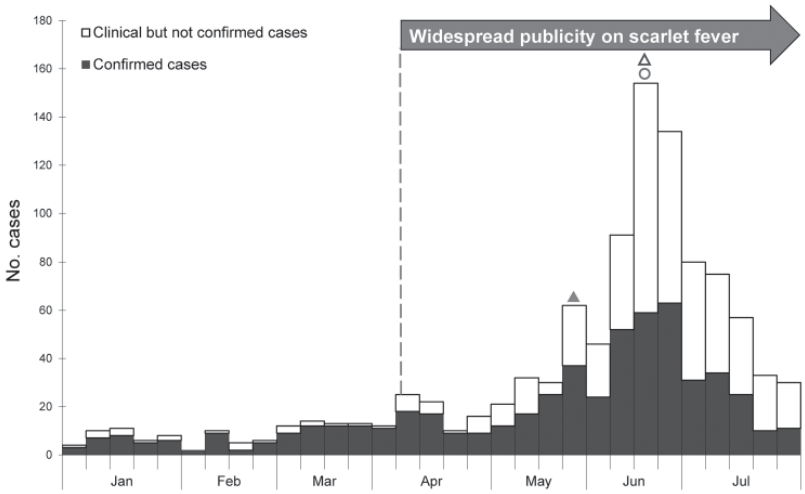

Figure 1. Weekly number of scarlet fever cases, by onset date, Hong Kong, January–July 2011. White bars indicate clinically diagnosed but not laboratory-confirmed cases; solid bars indicate laboratory-confirmed cases. Solid triangle indicates May 30 dissemination of press release about first fatal case (in a 7-year-old girl); open triangle indicates June 21 dissemination of press release about second fatal case (in a 5-year-old boy); circle indicates June 23 launch of health education campaign.

from 90 patients (mostly throat swab specimens) were characterized. Strains found belonged to the following *emm* types (number and percentage of strains): *emm*12 (70, 77.8%), *emm*1 (14, 15.6%), *emm*4 (2, 2.2%), *emm*22 (2, 2.2%), *emm*2 (1, 1.1%), and *emm*3 (1, 1.1%). All strains were susceptible to penicillin, but 77 (85.6%) strains were resistant to erythromycin. Of 59 strains tested for the 48-kb insert, 78.0% (46 strains) tested positive; 39 were *emm*12 strains and 7 *emm*1 strains. Antimicrobial drug susceptibility results were available for 39 strains

Table1. Epidemiologic characteristics, clinical features, and laboratory results for scarlet fever cases reported in Hong Kong during January–July 2011 compared with cases reported during 2008–2010

Characteristic*	2008–2010, n = 550	January 1–July 31, 2011, n = 996	p value
Epidemiology			
Sex ratio, M:F	1.6:1	1.5:1	0.50
Age range (median)	9 mo–40 y (5 y)	1 mo–51 y (6 y)	0.40
Local cases	98.0 (539/550)	97.4 (970/996)	0.56
Clustering			
Cases in a cluster	5.45 (30/550)	14.4 (143/996)	<0.0001†
Cases in home clusters	3.3 (18/550); 9 clusters	6.5 (65/996); 31 clusters	
Cases in each home cluster, range (median)	2 (2)	2–3 (2)	0.34
Cases in school clusters	2.2 (12/550); 4 clusters	7.8 (78/996); 28 clusters	
Persons affected in each school cluster, no. (median)	2–4 (3)	2–7 (2)	0.42
Clinical features			
Fever	95.6 (526/548)	93.2 (928/996)	0.065
Sandpaper rash	97.4 (534/548)	95.4 (950/996)	0.13
Strawberry tongue	45.1 (248/550)	51.4 (512/996)	0.020‡
Sore throat	74.4 (409/550)	78.5 (782/996)	0.073
Desquamation	27.8 (153/550)	23.7 (236/996)	0.084
Hospitalization	63.9 (351/549)	56.6 (561/991)	0.005§
Duration of hospitalization, d (mean)	1– 25 (3.8)	1–33 (3.3)	0.005¶
Concomitant chickenpox	5.5 (30/550)	1.9 (19/996)	0.0002#
Complications**	0.73 (4/550)	0.90 (9/996)	0.79
Toxic shock syndrome	0.18 (1/550)	0.60 (6/996)	0.43
Case-fatality rate	0	0.20 (2/996)	0.54
Laboratory results			
Laboratory confirmation	46.0 (253/550)	51.8 (533/996)	0.0055††
Positive throat or wound culture	95.3 (241/253)	97.2 (521/533)	0.094
Antistreptolysin O titer >200 IU/mL	4.74 (12/253)	4.37 (12/533)	0.094

*Values are % (no./total no.) unless otherwise indicated. Lower denominators indicate data missing or not applicable.
†χ^2 = 27.
‡χ^2 = 5.40.
§χ^2 = 7.85.
¶*t* = –2.8 (95% CI of difference 0.15–0.83 d).
#χ^2 = 14.
**Complications include toxic shock syndrome, septicaemia, parapharyngeal abscess, rheumatic fever, quinsy and hepatitis.
††χ^2 = 7.7.

Table 2. Clinical characteristics of patients with scarlet fever who had medical complications, Hong Kong, January–July 2011*

Characteristic	Case-patient no.								
	1	2	3	4	5	6	7	8	9
Patient age, y/sex	14/M	M/11	8/F	7/F	5/M	6/M	3/M	2/F	12/M
Month of illness onset	April	April	April	May	June	June	July	July	July
Days from symptom onset to hospital admission	1	4	7	7	4	10	1	1	0
Complications	TSS	Parapharyngeal abscess	TSS	TSS	TSS	Septicemia	TSS	TSS	Septicemia
Intensive care unit admission	No	No	Yes	Yes	Yes	Yes	No	Yes	No
Concomitant chickenpox infection	No	No	No	No	Yes	No	Yes	No	Yes
Recovered	Yes	Yes	Yes	No (died)	No (died)	Yes	Yes	Yes	Yes
S. pyogenes isolates	Throat	Throat	None	Blood, lower limb blister fluid	Blood and pus	Blood	Throat	Throat	Blood and pus
emm type	NA	NA	NA	*emm*12	*emm*1	*emm*12	NA	NA	*emm*12
48-kb insert	NA	NA	NA	+	+	+	NA	NA	–
Virulence geneprofile (*speA*¸*speB*, *speC, speF, speH, ssa*)	NA	NA	NA	–++–++	++++–+	–+++++	NA	NA	– + + + + –

*All case-patients were healthy before infection. TSS, toxic shock syndrome; NA, not available; +, positive; –, negative.

positive for the 48-kb insert; 3 (7.70%) were susceptible to erythromycin. Conversely, among all erythromycin-resistant *emm*12 strains in 2011 tested for the 48-kb insert, 6/42 (14.3%) yielded a negative result.

Forty-eight *emm*12 isolates during January–June 2011 that were subjected to virulence gene profiling showed 5 virulence gene profiles. No particular virulence gene profile was dominant among the 9 scarlet fever cases associated with medical complications (Table 2). Among 26 *emm*12 strains subjected to PFGE, 7 patterns were detected; the *emm*12 strain from 1 of the 2 fatal cases exhibited a unique PFGE pattern (Figure 2). For the other fatal case, an *emm*1 strain positive for *speA* was isolated.

Of the archived *S. pyogenes* strains collected during 2008–2010, few strains were from patients diagnosed with scarlet fever; therefore, we analyzed *S. pyogenes* strains isolated from throat and superficial wound specimens from outpatients <15 years of age. Among 28 such strains, *emm*28 was detected in 9 strains; *emm*4 in 4 strains; *emm*1 in 3 strains; *emm*12, 22, and 89 in 2 strains each; and 6 other *emm* types in 1 strain each. All strains were susceptible to penicillin; the erythromycin resistance rate was 10.7% (3/28). The 48-kb insert was found in 10 (35.7%) strains: 3 strains of *emm*28, and 1 strain each of *emm* types 1, 2, 4, 22, 44, 89, and stG485.

Conclusions

The 2011 *S. pyogenes* outbreak in Hong Kong attracted heightened media coverage, which might have increased reporting of cases; however, the higher proportion of laboratory-confirmed cases in 2011 than those during 2008–2010 suggests the upsurge was genuine. Overall clinical and epidemiologic profiles in 2011 did not differ from previous years. We found insufficient evidence that a particular *emm* type of virulence gene profile or presence of the 48-kb insert was associated with increased incidence or severity.

The reasons for the upsurge remain obscure. Laboratory findings showed diverse patterns of *S. pyogenes* strains, suggesting a multiclonal epidemic. The 48-kb insert identified in 2011 was found in *S. pyogenes* strains isolated in 2008–2010, albeit at lower rate (35.7% in 2008–2010 vs.78% in 2011). Thus, it is difficult to attribute the upsurge to the insert alone. A shift in prevailing *emm* type that occurred in 2011 might have contributed to fluctuations in the number of cases (*3*).

A higher rate of erythromycin resistance in *S. pyogenes* (>80%) was found in 2011 than in the reported previous

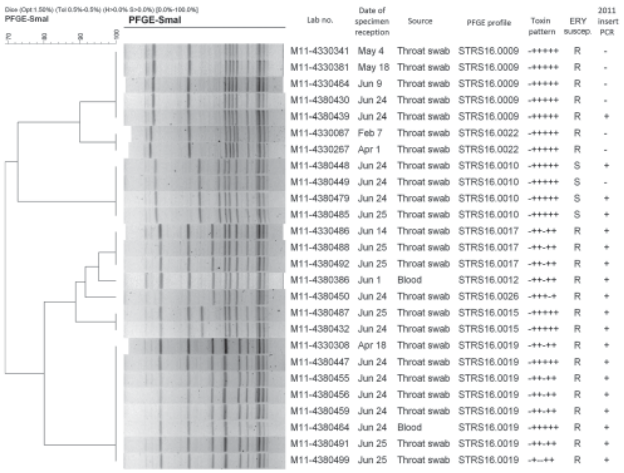

Figure 2. Pulsed-field gel electrophoresis patterns of 26 *emm* type 12.0 *Streptococcus pyogenes* strains, Hong Kong, 2011. Toxin profile results are shown as corresponding to the genes *speA*¸ *speB*, *speC*, *speF*, *speH*, and *ssa*. Strain M11–4380386 was from a fatal case. ERY suscep., erythromycin susceptibility result; R, resistant; S, susceptible. Scale bar indicates percent similarity.

years (20%–30%) (*4*). Because all erythromycin-resistant strains were also resistant to clindamycin (data not shown), we deduced the resistance mechanism to be resistance to macrolides, lincosamides, and streptogramins B system, as encoded by the *erm* genes (*5*).

The 48-kb insert provided a mechanism for macrolide resistance among *S. pyogenes* in Hong Kong, but our laboratory investigation found macrolide-resistant *S. pyogenes* strains and the macrolide-susceptible strains that bore them negative for this insert. Mutation of the PCR primer binding site might explain the former strains; further investigation is needed to explore this possibility.

The upsurge in scarlet fever cases in Hong Kong during 2011 likely reflects a regional phenomenon; a marked increase in cases was also observed in mainland China (*6*) and Macao (*7*) during this period. High resistance rates against macrolides were also observed for the outbreak in mainland China (*8*). We recommend close monitoring and surveillance of disease activity, genetic testing, antimicrobial susceptibility profiling, and maintaining scarlet fever's statutory notifiable status.

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Dr Luk is a medical and health officer at the Centre for Health Protection, Department of Health, Hong Kong, People's Republic of China. She was in the US Centers for Disease Control and Prevention's Field Epidemiology Training Program during this study. Her research interests include studying the epidemiology and transmission of communicable disease.

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Visceral Leishmaniasis in Rural Bihar, India

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To identify factors associated with incidence of visceral leishmaniasis (VL), we surveyed 13,416 households in Bihar State, India. VL was associated with socioeconomic status, type of housing, and belonging to the Musahar caste. Annual coverage of indoor residual insecticide spraying was 12%. Increasing such spraying can greatly contribute to VL control.

Visceral leishmaniasis (VL), a vector-borne parasitic disease caused by several *Leishmania* spp., is nearly always fatal if left untreated (*1*,*2*). The clinical syndrome is characterized by fever, weight loss, splenomegaly, hepatomegaly, and anemia. The disease is endemic in >60 countries, but 90% of all reported cases occur in just 5 countries: Bangladesh, Brazil, India, Nepal, and Sudan (*3*). On the Indian subcontinent, the disease is assumed to be an anthroponosis; the vector is a sand fly, *Phlebotomus argentipes*. Approximately 200 million persons on the Indian subcontinent are at risk for VL, and the annual incidence is ≈420,000 cases (*4*). The disease affects mainly poor rural communities; ≈80% of all cases in the region are reported from the state of Bihar in India (*4*).

Earlier studies on the Indian subcontinent have identified several risk factors for VL (*5*–*11*). At times, findings between studies have been conflicting, particularly in relation to the role of domestic animals (*7*). The use of bed nets was found to be protective in some studies (*2*,*5*), but this conclusion could not be confirmed in a recent cluster-randomized trial (*12*). Many of the earlier studies were conducted on fairly small populations, usually 1 or 2 villages (*5*,*6*,*8*,*9*); confounding by socioeconomic status was controlled to a varying extent. Most studies were conducted in high-incidence villages or in villages in which a recent outbreak had occurred. Because VL has a strongly clustered distribution, understanding the reasons behind widely varying incidence levels among villages and hamlets could also be useful. We therefore studied factors associated with VL in an area made up of villages with variable levels of VL incidence and constructed an asset index to control for confounding by socioeconomic status.

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The Study

The study area is a geographically continuous area comprising 50 villages in the Muzaffarpur District of Bihar State, India, a district where VL is highly endemic. The 50 villages can be further subdivided into 200 hamlets, also known as *tolla*. We conducted 3 annual surveys in September and October of 2008, 2009, and 2010. In each survey, we visited all households in the study area, collected demographic information, and asked whether the house had been covered by indoor residual insecticide spraying in the year preceding the survey. In each survey, we also collected information about VL in the household since the previous survey. For the first survey, we used a recall period of 1.5 years. A case of VL was defined as the combination of a clinical history typical for VL (fever of >2 weeks' duration, lack of response to antimalarial drug treatment); a positive result by the rK39 rapid diagnostic test; and a good response to specific VL treatment, with or without confirmation of parasites. Each case reported was verified from medical records by a study physician. At the time of the second survey in 2009, we also collected information about assets owned by each household, including domestic animals, and we recorded characteristics of the structure of the house and the surrounding vegetation. Information about assets owned (other than domestic animals) was used to subdivide the study population into 5 quintiles of socioeconomic status. To study potential associations with household environment, remote sensing (15-m resolution ASTER images), and ground data were combined to derive a map of the study area showing 7 types of land cover. Data were analyzed in a binomial multilevel model with *tolla* as a random effect.

We enrolled a study population of 81,210 persons, divided over 13,416 households (92% of all households in the study area). During the study period, we registered 207 VL cases, equivalent to an average annual incidence of 72.8/100,000 population. Cases were strongly clustered at the *tolla* level, with an intraclass correlation of 32%. None of the types of land cover was significantly associated with disease. With the villages spread out along roads and farming land split up into small plots, the environment experienced by persons from different villages or from different locations did not vary greatly.

VL was strongly associated with age; the odds of having VL was lowest for children <5 years of age and highest

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for children 5–14 years of age (odds ratio [OR] 2.5, 95% CI 1.5–4.0). Higher socioeconomic status was associated with reduced risk; comparing the wealthiest to the poorest quintile, we observed an OR of 0.5 (95% CI 0.3-1.0). Having at least 1 bed net per 3 household members was protective on univariate analysis, but the effect was weaker and no longer statistically significant when we controlled for confounding (OR 0.8, 95% CI 0.5–1.3). This finding can be explained by a strong association between socioeconomic status and ownership of bed nets.

Of ownership of all the domestic animals investigated, only ownership of goats was weakly, but significantly, associated with VL (OR 1.4, 95% CI 1.0–1.8). Other factors at household level that were statistically significant in multivariate analysis were the following: belonging to the Musahar caste (OR 2.9, 95% CI 1.3–6.8); presence of a bamboo tree (OR 1.5, 95% CI 1.2–2.0); and type of walls (OR 1.8, 95% CI 1.0–3.3 for unplastered brick walls and OR 2.5, 95% CI 1.3–4.6 for thatched walls, with plastered brick walls as reference for both). (Table) Thatched walls and presence of bamboo trees are likely to provide favorable breeding conditions for the sand fly vector (*13*).

Indoor residual insecticide spraying coverage was poor. In 2009 (the last year for which data were collected for the full year), only 12% of all households had reportedly been sprayed at least once.

Conclusions

In this large cohort study, controlled for potential confounding by socioeconomic status and other contextual factors, we identified several factors associated with VL. Ownership of goats and presence of bamboo trees near the house are risk factors, but are not strong enough to warrant specific interventions. Poor housing is a stronger risk factor; thus, housing plans launched by the Indian government may positively affect control of VL. Persons in the Musahar caste were at increased risk; they made up 2.4% of the study population but had >15% of VL cases. The Musahars are known to be among the poorest of the poor, but even after we controlled for confounding by socioeconomic status, the association remained statistically significant. Some residual confounding cannot be ruled out, but other factors probably play a role. One such factor could be long delays in seeking health care by Musahars, which was documented in another recent study (*14*). When devising improved VL control strategies, it would certainly be justified to pay special attention to the hamlets inhabited by the Musahar caste. Overall, however, the most benefit can be expected from strengthening vector control efforts. In the 1960s, as a byproduct of intensive indoor residual insecticide spraying for malaria eradication campaigns, VL was all but eliminated from the area, and biannual indoor residual insecticide spraying is one of the cornerstones of the regional VL elimination strategy (*15*). Thus, it defies imagination that in this highly VL-endemic area, the annual indoor residual insecticide spraying coverage can be as low as 12%.

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Dr Hasker is a scientific collaborator at the Institute of Tropical Medicine in Antwerp, Belgium. His major research interests

Table. Factors associated with visceral leishmaniasis, Bihar State, India, 2008–2010

Factor	No. (%) participants		Odds ratio*
	Total, N = 81,210	Case-patients, n = 207	
Demographic characteristic			
Mushahar caste	1,980 (2.4)	32 (15.5)	2.9 (1.3–6.8)
Age group, y			
0–4	12,787 (15.8)	20 (9.7)	Referent
5–14	21,020 (25.9)	79 (38.2)	2.5 (1.5–4.0)
15–24	14,282 (17.6)	33 (15.9)	1.7 (1.0–3.0)
25–34	10,993 (13.5)	31 (15.0)	2.0 (1.1–3.5)
35–44	8,462 (10.4)	23 (11.1)	1.9 (1.1–3.6)
>45	13,666 (16.8)	21 (10.1)	1.2 (0.6–2.2)
Socioeconomic status, by assets index level			
Level 1, poorest	16,515 (20.3)	70 (33.8)	Referent
Level 2	16,094 (19.8)	58 (28.0)	0.9 (0.6–1.3)
Level 3	16,124 (19.8)	31 (15.0)	0.7 (0.5–1.1)
Level 4	16,256 (20.0)	35 (16.9)	0.9 (0.6–1.4)
Level 5	16,221 (20.0)	13 (6.3)	0.5 (0.3–1.0)
Other			
Ownership of goats	25,703 (31.7)	86 (41.6)	1.4 (1.0–1.8)
Bamboo tree at <10 m	31,554 (38.9)	86 (41.6)	1.5 (1.1–2.0)
Type of walls			
Brick, plastered	19,169 (23.6)	15 (7.3)	Referent
Brick, unplastered	35,401 (43.6)	96 (46.4)	1.8 (1.0– 3.3)
Thatched	26,640 (32.8)	96 (46.4)	2.5 (1.3– 4.6)

*Based on multivariate model with *tolla* of residence as random effect.

are the epidemiology and control of visceral leishmaniasis, human African trypanosomiasis, and tuberculosis.

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Influenza A(H1N1) pdm09 Virus in Pigs, Réunion Island

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During 2009, pandemic influenza A(H1N1)pdm09 virus affected humans on Réunion Island. Since then, the virus has sustained circulation among local swine herds, raising concerns about the potential for genetic evolution of the virus and possible retransmission back to humans of variants with increased virulence. Continuous surveillance of A(H1N1)pdm09 infection in pigs is recommended.

Influenza A(H1N1)pdm09 virus, which caused the last influenza pandemic among humans, is a unique reassortant derived from swine influenza viruses of the triple reassortant swine North American lineage and the avian-like swine Eurasian lineage (*1*). Réunion Island, a tropical French overseas department in the southwestern Indian Ocean, was struck by the influenza pandemic during July–August 2009. The epidemic had a high attack rate in humans (estimated clinically at 12.5% and serologically at 40.0%) (2,*3*). A(H1N1)pdm09 virus was reported to cause a reverse zoonosis in pigs (*4*); thus, a long-term (2009–2011) serologic and virologic survey was designed to check for transmission of the virus to pigs on Réunion Island, where the pork industry is a key economic activity and no live pigs have been imported since 1978. At 6-month intervals, a local veterinary surveillance system conducts serologic surveillance for pathogenic swine influenza viruses (H1N1, H1N2, and H3N2) among local herds, and during the last 20 years, none have been detected.

The Study

During a first step (November 2009–February 2010), seroprevalence rates for A(H1N1)pdm09 virus were assessed in 120 breeding pigs (>4 years old) from 57 farms. Blood was obtained from randomly selected pigs at the only slaughterhouse on the island, where pigs are held for ≤3 hours. We screened the samples for antibodies to influenza A viruses by using the ID Screen Antibody Influenza A kit (ID.vet, Montpellier, France), and titers were determined by using hemagglutination-inhibition (HI) assays (*5*) against all classical swine influenza viruses and A(H1N1) pdm09 virus (Table 1). Ninety-eight (81.7%; 95% CI 74.7%–88.5%) of the 120 serum samples were positive for A(H1N1)pdm09 virus (HI titers ≥20); the range of positive titers was 40–640, and 54.2% of the samples expressed high HI titers (160–640). Of the 98 serum samples, 5 reacted at low titer and with only 1 European A (H1N1) swine virus (titer ≤20), i.e., ≥4 dilutions lower than for A(H1N1)pdm09 virus, indicating cross reactivity (*6*). Thus, pigs from 47 (82.4%) of 57 tested farms had been infected by A(H1N1) pdm09 virus; the seroprevalence rate was 81%–100% for pigs on 79.0% of the farms. Farms with affected pigs were located throughout the island (Figure).

In a second step (June 2010, when A(H1N1)pdm09 infection was no longer detected among humans), we tested whether the virus was still circulating among pigs born that year. To obtain nasal swab and blood samples for testing, we randomly selected 390 fattening pigs (25–27 weeks old) at the slaughterhouse; the pigs originated from 45 farms. At the time of sampling, the veterinary surveillance system did not report any clinical signs suggesting virus circulation among herds. However, ≈3.5% of the serum samples (9% of tested farms) contained antibodies to A(H1N1)pdm09 virus (HI titers 20–160). Nasal swab specimens from 6.7% (26/390) of pigs were positive for A(H1N1)pdm09 virus as determined by using a specific real-time reverse transcription PCR (rRT-PCR); the pigs originated from 13 (28.8%) farms (*7*). Two strains, A/Sw/La Reunion/0164/10 and A/Sw/LaReunion/110348/10, were isolated onto MDCK cell cultures (*5*).

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Table 1. Antigenic characterization of A(H1N1)pdm09-like influenza viruses isolated from pigs, Réunion Island, 2010*

	HI titers† obtained with reference swine hyperimmune serum samples directed against				
Virus strain	California/04/09‡	Sw/England/ 117316/86§	Sw/Cotes d'Armor/0388/09¶	Sw/Flandres/1/98#	Sw/Scotland/ 410440/94**
A/Sw/La Reunion/0164/10	1,280	160	40	<10	<10
A/Sw/La Reunion/110348/10	1,280	80	160	10	20
A/Sw/La Reunion/0167/10	640	80	40	<10	<10
A/Sw/La Reunion/110194/10	640	80	20	<10	10

*HI, hemagglutination inhibition.
†HI tests were performed according to standard procedure (*5*).Titers are expressed as the reciprocal of the highest dilution of serum that inhibits 4 hemagglutination units of virus.
‡A(H1N1)pdm09 lineage.
§Classical swine A(H1N1).
¶Avian-like swine A(H1N1).
#Human-like reassortant swine A(H3N2).
**Human-like reassortant swine A(H1N2).

During July–December 2010, 11 farms reported influenza-like clinical signs in pigs, and proof of A(H1N1)pdm09 virus infection was established on 3 farms (farms A–C). In June 2010, fattening pigs on farm A were seronegative for A(H1N1)pdm09 virus. In July, when acute respiratory disease was reported among pigs, 12 of 39 fattening pigs (18–21 weeks old) sampled on farm A were still seronegative for A(H1N1)pdm09 virus; however, rRT-PCR results were positive for A(H1N1)pdm09 virus. Four weeks later, when pigs had recovered from influenza, only 7.7% (3/39) of sampled pigs on farm A had rRT-PCR results positive for A(H1N1)pdm09 virus, and all 39 were seropositive for the virus. High rates of rRT-PCR positivity were also noted for pigs on farms B (17/30 pigs) and C (6/15 pigs). Two A(H1N1)pdm09 strains (A/Sw/LaReunion/0167/10 and A/Sw/La Reunion/110194/10) were isolated from pigs on farms A and B, respectively.

Four influenza virus strains were isolated from pigs, and all induced a cytopathic effect and displayed hemagglutinating activity on chicken erythrocytes; all 4 were confirmed as A(H1N1)pdm09 virus by specific rRT-PCRs. In addition cross-HI assays (*5*) revealed that these strains exhibit antigenic relationships with swine influenza A(H1N1) viruses from classical and avian-like lineages, although they reacted most strongly with A(H1N1)pdm09 virus (Table 1). Genome sequencing of these strains showed high (>98%) nucleotide sequence homology to the corresponding genes of A/California/04/09 and 2009 human strains from Réunion Island, suggesting human-to-swine transmission (H. Pascalis, unpub. data).

In a third step (March, July–August, and October 2011), 3 other sampling campaigns were conducted at the slaughterhouse, including 831 fattening pigs from 104 farms. Nasal swab samples for 7 (8.4%) pigs from 3 (2.9%) farms still had rRT-PCR–positive results. However, serologic analyses revealed that pigs on ≈40% of the farms (distributed throughout the island) were seropositive for A(H1N1)pdm09 virus, indicating continuing circulation of the virus in swine herds (Table 2).

Conclusions

Consistent with findings elsewhere (*8*), our results show that A(H1N1)pdm09 virus has substantially affected swine herds in Réunion Island. Results of our long-term (≈2 years) investigation show that A(H1N1)pdm09 virus has circulated in pigs beyond the 5-week epidemic among

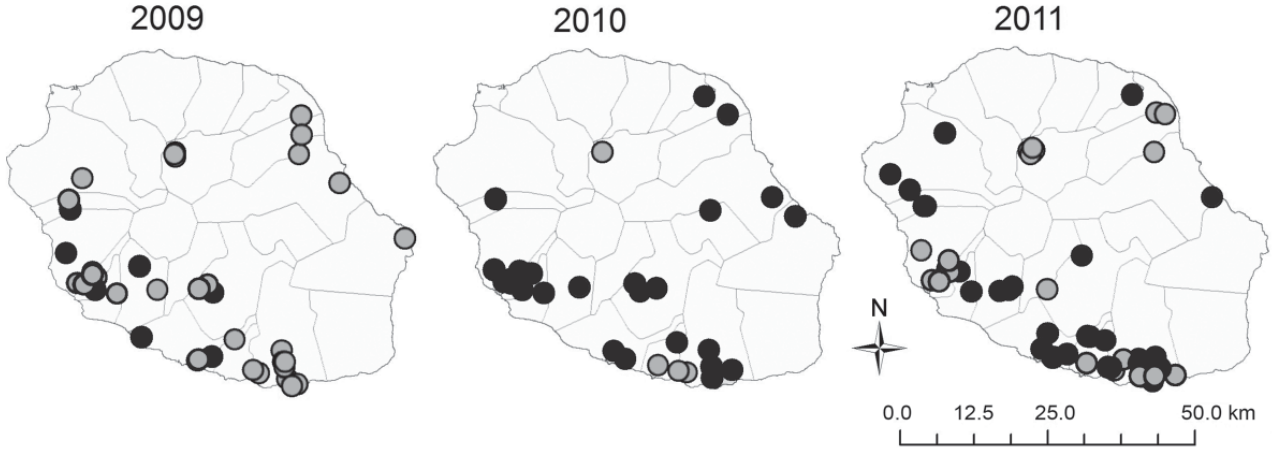

Figure. Location of farms tested for antibodies against influenza A(H1N1)pdm09 virus in serologic surveys, Réunion Island, 2009–2011. Black dots, seronegative farms; gray dots, seropositive farms.

Table 2. Seroprevalence rates for influenza A viruses among fattening pigs as determined by ELISA and HI testing, Reunion Island, 2010 and 2011*

Time of sampling campaign (total no. farms; total no. pigs)	Antinucleoprotein ELISA		A(H1N1)pdm09 HI test	
	Herd, %	Pigs, %	Herd†, %	Pigs, %
June 2010 (N = 45; n = 252)	13.33	5.55	8.89	3.57
March 2011 (N = 33; n = 256)	48.48	28.12	42.42	16.41
July–August 2011 (N = 38; n = 316)	47.37	34.49	39.47	27.21
October 2011 (N = 33; n = 259)	57.57	40.93	42.42	22.01

*Pigs were randomly sampled at the slaughterhouse during 4 campaigns. Seroprevalence rates are % farms positive at the herd level among all farms (N) and % pigs positive at the animal level among all animals (n). HI, hemagglutination inhibition.
†Within-herd prevalence of infected pigs ranged from 3% to 23%.

humans during the austral winter 2009 (*3*) and has become a novel enzootic pathogen in Réunion Island.

Several facts may account for the heavy human-to-swine transmission of A(H1N1)pdm09 virus. First, the reassortant pandemic virus contains genomic segments originating from swine influenza viruses established in pigs (*1*). Second, pigs are highly susceptible to experimental inoculations with A(H1N1)pdm09 virus and support high intraspecies transmissibility (*9*). Third, the pressure of infection caused by A(H1N1)pdm09 virus among humans in Réunion Island was high but most infections were mild or asymptomatic (*3*); therefore, people pursued their professional activities, acting as silent spreaders of the virus. Last, pigs on Réunion Island had no history of previous passages of swine influenza viruses; thus, the lack of specific immunity to influenza A viruses would have contributed to the high sensitivity of the pigs to infection, as described (*10*,*11*).

Despite serologic proof of large numbers of infected pigs during late 2009–early 2010 in Réunion Island, influenza-like signs were not exhibited and reported until July 2010; this finding was similar to that in New Caledonia (*8*). In July 2010, several herds showed symptomatic changes in infection that could indicate either a change in virulence of the circulating strain or the intervention of co-infecting pathogens or some other environmental factor(s). *Mycoplasma hyopneumoniae* and *Pasteurella multocida* were co-detected on farms with pigs with signs of infection (data not shown). Co-infection with swine influenza virus and these bacteria is known to contribute to severe respiratory disorders among pigs; thus, these bacteria may have enhanced pathogenicity of A(H1N1)pdm09 virus on affected farms (*12*).

Because specific immunity to A(H1N1)pdm09 virus will decline over time when the virus is no longer circulating among humans, persistence of the virus in an animal reservoir raises concerns about the risk for genetic evolution of the virus and retransmission back to humans of variants with potentially increased virulence. As an example, during the 2011 austral winter, only influenza A(H3N2) and B viruses were recorded (*13*). Novel reassortant viruses containing genomic segments from A(H1N1)pdm09 and enzootic swine influenza viruses have been isolated in pigs (*14*). Such a reassortant was responsible for several cases of influenza among humans in 2011 (*15*); these cases were mild, but other, more virulent pathogenic viruses could emerge. Hence continuous surveillance of A(H1N1)pdm09 infection in pigs is recommended.

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Powassan Virus Encephalitis, Minnesota, USA

Justin Birge and Steven Sonnesyn

Powassan virus (POWV) is a rare tick-borne agent of encephalitis in North America. Historically, confirmed cases occurred mainly in the northeastern United States. Since 2008, confirmed cases in Minnesota and Wisconsin have increased. We report a fatal case of POWV encephalitis in Minnesota. POWV infection should be suspected in tick-exposed patients with viral encephalitis,

Case Report

A 67-year-old woman from Aitkin, Minnesota, USA, sought treatment at a local hospital on May 30, 2011, with a 3-day history of dizziness, fever of up to 103°F (39.4°C), chills, malaise, nausea, and occasional confusion with slurred speech. She had no respiratory or gastrointestinal symptoms and no history of ill contacts, travel, environmental exposures, or other recent illness. She had not been exposed to animals or vectors, other than those endemic to her area of residence, which included mosquitoes and deer ticks. She had removed many deer ticks after gardening or hiking in the woods. The patient's past medical history was notable for colon cancer, which had been treated with a partial colectomy in October 2010 and chemotherapy through April 2011. She also had a history of hypertension, cutaneous lupus, and a remote cerebral aneurysm with surgical clipping. Medications she was taking were atenolol, hydroxychloroquine, and valsartan.

On admission, the woman was alert and reported mild neck tenderness. Her temperature was 100°F (37.8°C), blood pressure 138/77 mm Hg, pulse rate 83 beats/min, respiratory rate 22 breaths/min, and oxygen saturation 98% on room air. Results of neurologic, cardiac, and respiratory examinations were normal. Studies with normal test results included comprehensive metabolic panel, urinalysis, computed tomographic scan of the head, and chest radiograph. Results of serum screen for *Borrelia burgdorferi* antibodies (by ELISA) were negative. Leukocyte count was within reference range ($10.8 \times 10^3/mm^3$), with neutrophil (polymorphonuclear leukocytes) predominance (80%). Her cerebrospinal fluid (CSF) showed 80 leukocytes (89% polymorphonuclear leukocytes), 5 erythrocytes, and 64 mg/dL of protein. The patient was given piperacillin/tazobactam and doxycycline.

The next day, she was less responsive and was transferred to Abbott Northwestern Hospital in Minneapolis. Shortly thereafter, she became unresponsive and labored breathing developed. Her temperature reached 102°F (38.9°C), and the following laboratory values were outside the reference range: leukocyte count ($11.3 \times 10^3/mm^3$), sodium level (131 mmol/L), erythrocyte sedimentation rate (49 mm/h), and protein level (2.3 mg/dL). Neurology and infectious disease specialists suspected viral encephalitis. Magnetic resonance imaging (MRI) was deferred because of the unknown composition of the aneurysm clip, and the patient underwent a computed tomography angiogram of the head and neck. Infarction, vasculitis, meningeal enhancement, and structural abnormalities were not found. Twenty-four–hour electroencephalogram monitoring and administration of ceftriaxone (2 g intravenously [IV] every 24 h), acyclovir (500 mg IV every 8 h), and doxycycline (100 mg IV every 12 h) were initiated.

Overnight, the patient became apneic and required intubation. Examination revealed absent deep tendon reflexes, ocular deviation, positive Babinski response, and bilateral flaccid paralysis of the extremities. Pupillary light and corneal reflexes remained intact. No independent respirations were initiated. Complement levels were within reference range. No evidence of seizure was shown on electroencephalogram, although epileptiform discharges were seen. Given the severity of encephalopathy, prophylactic levetiracetam was initiated.

Results of repeat screen for *B. burgdorferi* antibodies, smear for *Ehrlichia* spp., and blood and urine cultures were unremarkable. Brain MRI revealed nonspecific inflammatory changes within the thalamus, midbrain, and cerebellum, with no evidence of meningeal irritation, temporal lobe abnormality, mass effect, acute infarct, or hydrocephalus (Figure 1, panel A, Appendix, wwwnc.cdc.gov/EID/article/18/10/12-0621-F1.htm). Acyclovir was dis-continued. Routine bacterial culture of CSF was negative. Ceftriaxone and doxycycline were continued because acute Lyme disease, which rarely manifests in this manner, could not be ruled out by serologic testing alone.

The patient remained unresponsive with flaccid paralysis and areflexia. Four days after the initial examination, repeat MRI showed substantial progression of signal abnormality in cerebral hemispheres, thalamus, and midbrain. Mass effect was evident with crowding of structures at the foramen magnum. Lateral and third ventricle dilation, consistent with acute hydrocephalus, was noted (Figure 1, panel B). A repeat lumbar puncture was not performed, given clinical interpretation of illness, known imaging findings, key pending results, and lack of indications for additional testing.

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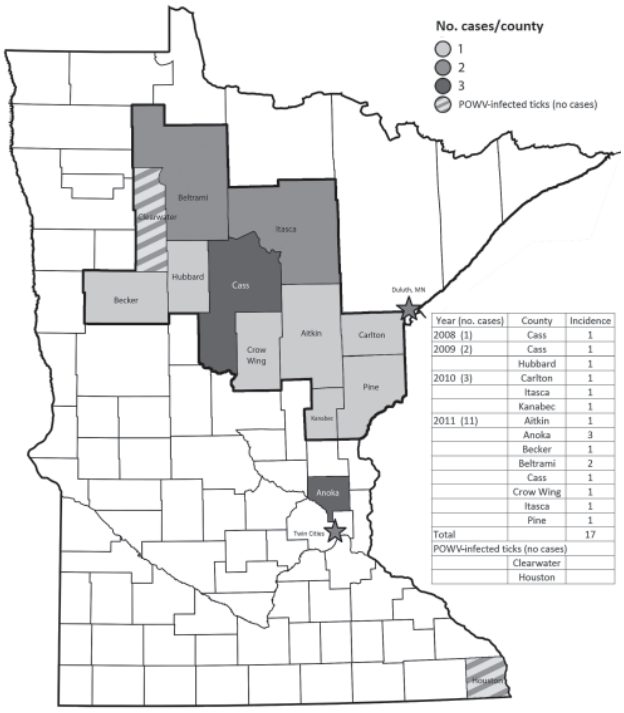
Thirteen days after illness onset, a serology panel was negative for the following viruses: West Nile, La Crosse, Eastern equine encephalitis, St. Louis encephalitis, and Western equine encephalitis. PCR of CSF was negative for enteroviruses and herpesviruses. The Minnesota Department of Health (MDH) reported positive IgM serologic results against Powassan virus (POWV). Ceftriaxone and doxycycline were discontinued. Given the patient's clinical deterioration and poor prognosis, she was electively extubated and then died. The Centers for Disease Control and Prevention confirmed POWV infection by IgM serology and reverse transcription PCR of CSF.

POWV is a tick-borne member of the family *Flaviviridae,* first reported in 1958 (*1*). It is the only tick-borne member of the genus *Flavivirus* with human pathogenicity in North America (*2*). Selection bias in identifying the infection may exist, diminishing the reported incidence to only patients with severe disease. Results of seroprevalence studies in Canada and the northeastern United States are variable but include seroprevalence estimates as high as 5.8% in Canada (*3*) and 0.7% in New York State (*4*). Small and medium-sized mammals are common reservoirs (notably, woodchucks [*Marmota monax*] and white-footed mice [*Peromyscus leucopus*]), and several species of tick (4 *Ixodes* spp., 2 *Dermacentor* spp.) act as vectors (*5*–*7*). Human infection has been documented in North America and Russia (*8*). Both prototypic (POWV) and deer tick virus (DTV) genotypes exist (*6*,*9*). In Canada and the northeastern United States, *I. cookei* ticks are the typical vectors for POWV. In Minnesota and Wisconsin, *I. scapularis* ticks are the typical vectors for DTV. Although *I. cookei* ticks are present in these states, they rarely attach to humans, and according to MDH data, all sequenced strains in Minnesota are of the DTV genotype. Transmission time from tick to host has been documented in mice as quickly as 15 minutes (*10*). The length of attachment time required for disease-causing viremia in humans is unknown. Literature estimates vary, but <40 cases were documented during 1958–2000 (*2*,*4*,*11*,*12*). According to the Centers for Disease Control and Prevention and the health departments of Minnesota and Wisconsin, 33 US cases were reported during 2001–2010: 12 in New York, 9 in Wisconsin, 8 in Minnesota, 2 in Maine, and 1 each in Michigan and Virginia. In 2011, of 16 confirmed cases in the United States, 11 occurred in Minnesota and 4 occurred in Wisconsin. Figure 2 illustrates the geographic distribution of human cases and infected ticks.

Patients with POWV infection typically exhibit encephalitis after an incubation period of 1–4 weeks. Fever and headache are common; the typical viral prodrome lasts 1–3 days. Mental status changes, cerebellar symptoms, and hemiplegia are also common and may be severe. Results of CSF testing and brain imaging are generally consistent with viral encephalitis. Reverse transcription PCR of CSF, serologic testing of CSF, and serologic testing of serum are the preferred diagnostic tests, but they are not widely available. Diagnostic testing for POWV should be referred to state and federal laboratories to ensure accuracy and standardization (*4*,*8*,*9*,*11*–*13*)



Year (no. cases)	County	Incidence
2008 (1)	Cass	1
2009 (2)	Cass	1
	Hubbard	1
2010 (3)	Carlton	1
	Itasca	1
	Kanabec	1
2011 (11)	Aitkin	1
	Anoka	3
	Becker	1
	Beltrami	2
	Cass	1
	Crow Wing	1
	Itasca	1
	Pine	1
Total		17
POWV-infected ticks (no cases)		
	Clearwater	
	Houston	

Figure 2. Geographic distribution of confirmed Powassan virus (POWV) infections (diagnosis made by serology, reverse transcription PCR) and counties with POWV–infected ticks in Minnesota. Data provided by the Minnesota Department of Health.

Pathogenesis is due to lymphocytic infiltration of perivascular neuronal tissue with a predilection for gray matter, including thalamus, midbrain, and cerebellum (*11*). Supportive care is the only therapy. A European vaccine against the related tick-borne encephalitis virus is available, but although the viruses are antigenically similar, its effectiveness against POWV is unknown (*12*). Approximately 10% of the reported infections have been fatal, and an additional 50% have produced long-term neurologic sequelae, including hemiplegia and headaches (*2*,*9*,*13*).

Conclusions

POWV is causing an emerging and potentially severe tick-borne infection in Minnesota and Wisconsin. POWV infection should be suspected when tick-exposed patients exhibit viral encephalitis, especially those with cerebellar

symptoms and/or thalamus/midbrain gray matter disease. Preventing tick attachment by using chemical prophylaxis and vigilance are essential in disease-endemic environments to prevent contraction of POWV infection.

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Influenza Virus Infection in Nonhuman Primates

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To determine whether nonhuman primates are infected with influenza viruses in nature, we conducted serologic and swab studies among macaques from several parts of the world. Our detection of influenza virus and antibodies to influenza virus raises questions about the role of nonhuman primates in the ecology of influenza.

Worldwide, infections with influenza A viruses are associated with substantial illness and death among mammals and birds. Public health and research have placed major focus on understanding the pathogenicity of different influenza virus strains and characterizing new influenza vaccines. Nonhuman primates (NHPs), including macaques, have become popular experimental models for studying the pathogenesis and immunology of seasonal and emerging influenza viruses. NHPs readily seroconvert after experimental inoculation with seasonal influenza virus and have been used to test candidate vaccines for strains of human and avian origin. Like humans, macaques infected with influenza virus exhibit fever, malaise, nasal discharge, and nonproductive cough; virus replication can be detected in the nasal passages and respiratory tract (*1*,*2*). However, whether NHPs are infected with influenza viruses in nature remains unknown.

Over the past decade, we have focused on the role of pet and performing monkeys in disease transmission throughout Asia. Commonly trapped in the wild, these monkeys might be sold at wet markets, the putative source of several zoonotic outbreaks (*3*), where they might be caged next to any number of animal species (Figure 1, panel A) (*4*). Pet and performing monkeys are likely conduits for cross-species transmission of respiratory pathogens like influenza viruses because of their close and long-term contact with their owners, audiences, domestic animals, wild animals, and birds (Figure 1, panel B) (*5*). However, the breadth and diversity of this interface presents a challenge for monitoring the emergence of infectious diseases. We have approached this challenge by conducting longitudinal studies at several sites and collecting biological samples and behavioral data representing various contexts of human–NHP contact (*4*–*7*). We used these historical and newly acquired samples, representing various countries and contexts of human–macaque contact, to determine whether NHPs are infected with influenza viruses in nature.

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The Study

As part of our decade-long longitudinal studies, ≈200 serum samples were collected from macaques. These included pet macaques (*Macaca nigra, M. nigrescens, M. hecki*) from Sulawesi, Indonesia; performing macaques from Java, Indonesia (*M. fascicularis*) and from Bangladesh (*M. mulatta*); *M. fascicularis* macaques from the Bukit Timah and Central Catchment Nature Reserves in Singapore, where they freely interact with wild avian fauna and visitors (occasionally entering residential areas) (*7*); *M. sylvanus* macaques from the Upper Rock Nature Reserve in Gibraltar, where international tourists frequently use food to entice the macaques to climb about their heads and shoulders (*6*); and free-ranging macaques (*M. fascicularis* and *M. nemestrina*) at temple shrines or *M. fascicularis* macaques that range throughout a wildlife rescue center and nearby villages in Cambodia (Figure 2). Serum was collected and stored as described (*8*). All samples were stored on cold packs in the field and transferred to dry ice for shipment to the United States, where they were then stored at −80°C.

For initial screening for antibodies against influenza virus, serum samples were treated with receptor-destroying enzyme as described (*9*) and tested by using a multispecies Influenza A Virus NP Antibody Inhibition Test (Virusys Corporation, Taneytown, MD, USA) according to manufacturer's instructions. ELISA results indicated nucleocapsid protein antibodies against influenza in samples from macaques from Cambodia (29.2%), Singapore (16.7%), Sulawesi (16.1%), Bangladesh (13.3%), and Java (6.0%) (Table 1). Antibodies were detected in animals 1–10 years of age at the time of sampling. No influenza virus–specific antibodies were detected from the 73 total samples from Gibraltar, perhaps because persons with influenza virus infection infrequently travel to the Upper Rock

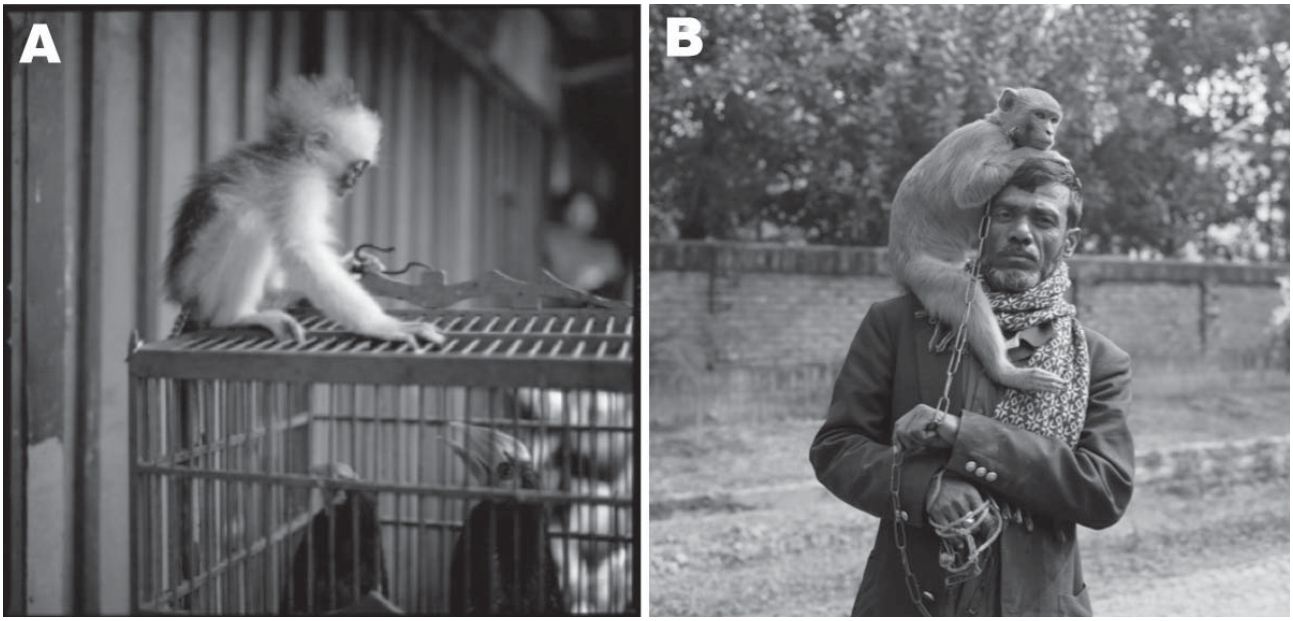

Figure 1. The interface between nonhuman primates, birds, and humans. A) A young, recently captured leaf monkey perched on a cage containing birds in a wet market in Java. B) A man and his performing monkey in Bangladesh. Reprinted with permission from Lynn Johnson, 2012.

Reserve (healthy-visitor effect) (*10*) or perhaps because monkeys from Gibraltar are less susceptible to infection. Seroprevalence of antibodies against influenza A virus, by site and collection year, human and NHP population densities, and prevalence of avian influenza viruses are shown in Figure 2.

Serum samples that were positive by ELISA were also screened by hemagglutination-inhibition assay as described (*9*). Based on the year and location of NHP sample collection, the estimated ages of the NHPs at the time of sample collection, and the presence of avian H5 and H9 influenza viruses in many of these countries during the sampling period (*11*–*13*), a panel of human vaccine strains and avian influenza virus strains was used in the hemagglutination-inhibition assay. Although not all ELISA-positive serum samples could be subtyped, antibodies against seasonal subtype H1N1 and H3N2 influenza A strains were detected from macaques in Bangladesh, Singapore, Java, and Sulawesi (Table 2). Of the performing macaques in Bangladesh, 2 had antibodies against A/chicken/Bangladesh/5473/2010, a strain of G1 clade subtype H9N2 avian influenza virus. Subtype H9N2 influenza viruses are prevalent in poultry in Bangladesh (*14*) and have been detected in humans (*12*). We did not detect antibodies against highly pathogenic avian influenza subtype H5 viruses, which might not be surprising given our relatively small sample size (Table 2). Also given the small sample size, we were unable to perform microneutralization studies, which would be useful to perform with future samples.

In 2011, to determine whether any macaques were actively infected with influenza virus, we collected oral swabs from 48 monkeys in Cambodia to test for influenza

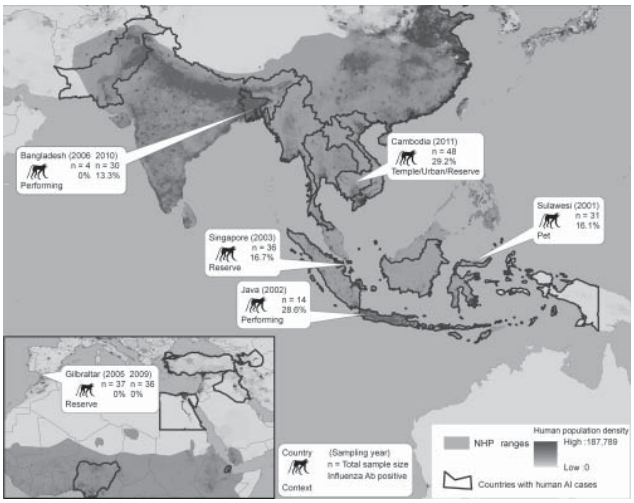

Figure 2. Nonhuman primate (NHP) habitat countries and approximate location of sampling sites, with sample size, year collected, context of human–macaque interaction, and seroprevalence of antibodies against influenza virus A. Countries that have reported human influenza infection of avian origin are outlined. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/10/12-0214-F2.htm).

Table 1. Anti-influenza nucleocapsid protein antibodies in nonhuman primate populations, 2001–2011*

Location, year	No.	*Macaca* species	Type(s)	No. (%) positive
Singapore, 2003	36	*fascicularis*	Reserve	6 (16.7)
Indonesia				
Java, 2002	14	*fascicularis*	Performing, pet	4 (28.6)
Sulawesi, 2001	31	*nigra*, *hecki*, *nigrescens*	Pet	5 (16.1)
Gibraltar				
2005	37	*sylvanus*	Reserve	0
2009	36	*sylvanus*	Reserve	0
Bangladesh				
2006	4	*mulatta*	Performing, pet	0
2010	30	*mulatta*	Performing, pet	4 (13.3)
Cambodia, 2011	48	*fascicularis, nemestrina*	Temple, urban, reserve	14 (29.2)

*Testing was conducted with a multispecies influenza A virus nucleocapsid protein antibody inhibition test for strain-specific antibodies.

virus by real-time reverse transcription PCR as described (*8*). In brief, the inside of the anesthetized and immobilized monkeys' mouths (cheeks, tongue, and gums) were swabbed. Swabs were immediately placed into viral transport media, stored, and shipped as previously described. RNA was isolated by using an Ambion MagMAX-96 AI/ND Viral RNA Isolation Kit (Life Technologies Corporation, Grand Island, NY, USA) on a Kingfisher Flex system (Thermo Fisher Scientific, Waltham, MA, USA). Viral RNA was analyzed in a Bio-Rad CFX96 Real-Time PCR Detection System and a C1000 Thermocycler (Bio-Rad, Hercules, CA, USA) with TaqMan Fast Virus 1-Step Master Mix (Applied Biosystems, Foster City, CA, USA) and the InfA primer/probe sets as described (*15*). Of the 48 respiratory samples, 1 (2.1%) was positive for influenza virus; cycle threshold value was 38 (limit of detection is 40). Attempts to amplify longer PCR fragments of matrix, hemagglutinin, or neuraminidase genes or to isolate the virus by blind passage in embryonated chicken eggs or MDCK cells were unsuccessful.

Conclusions

Our results indicate that NHPs that have contact with humans can be naturally infected with seasonal endemic human influenza viruses and with emerging pandemic-risk avian influenza viruses. We found serologic evidence of infection in several countries, contexts, and macaque species. Preliminary real-time reverse transcription PCR results also pointed to the presence of virus in a buccal swab from an adult macaque from Cambodia, indicating active infection at the time of sampling. On the basis of results from this study, it seems that pet, performing, and free-ranging macaques are susceptible to influenza virus infection. Given the close relationship between humans and NHPs in areas of the world where avian and human influenza viruses cocirculate, further surveillance of these populations is warranted. The ability to detect and eventually isolate strains of influenza virus currently infecting NHPs and humans at the animal–human interface is paramount to understanding how NHP–human interactions can affect the

Table 2. Seroprevalence of influenza A virus subtypes in monkeys with nucleocapsid protein–positive ELISAs, by location*

Virus strain	Virus subtype (H5 clade)	Years used in vaccine	No. tested/no. positive				
				Indonesia			
			Singapore	Java	Sulawesi	Bangladesh	Cambodia
A/Beijing/262/1995	H1N1	1999–2000	0/6	2/4	1/6†	NSA	NSA
A/Sydney/5/1997	H3N2	1999–2000	2/6‡	0/4	1/6§	NSA	NSA
A/New Caledonia/20/1999	H1N1	2000–2007	0/6	0/4	1/6†	0/4	0/14
A/Panama/2007/1999	H3N2	2000–2004	2/6‡	0/4	1/6§	0/4	NSA
A/California/07/2004	H3N2	2005–2006	NSA	NSA	NSA	0/4	0/14
A/Wisconsin/67/2005	H3N2	2006–2008	NSA	NSA	NSA	0/4	0/14
A/Brisbane/59/2007	H1N1	2008–2010	NSA	NSA	NSA	1/4	0/14
A/Brisbane/10/2007	H3N2	2008–2010	NSA	NSA	NSA	0/4	0/14
A/California/04/2009	H1N1	2010–present	NSA	NSA	NSA	0/4	0/14
A/Perth/16/2009	H3N2	2010–present	NSA	NSA	NSA	0/4	0/14
A/chicken/Bangladesh/5473/2010	H9 G1	NA	NSA	NSA	NSA	2/4	NSA
A/Vietnam/1203/2004	H5 (1)	NA	NSA	NSA	NSA	NSA	0/14
A/Cambodia/R0H05050/2007	H5 (1)	NA	NSA	NSA	NSA	NSA	0/14
A/duck/Hunan/795/2002	H5 (2.1)	NA	NSA	NSA	NSA	0/4	NSA
A/BHG/Qinghai/01/2005	H5 (2.2.2)	NA	NSA	NSA	NSA	0/4	NSA
A/JWE/Hong Kong/1038/2006	H5 (2.3.4.2)	NA	NSA	NSA	NSA	0/4	NSA
A/duck/Laos/3295/2006	H5 (2.3.4.2)	NA	NSA	NSA	NSA	0/4	NSA

*Samples were only tested for relevant strains based on the collection location, year of collection, and estimated age of the monkey. Monkeys from Indonesia were not tested for influenza subtype H5N1 viruses because samples were collected in 2001 and 2002 and subtype H5N1 viruses were first reported in poultry from Indonesia in February 2004. Samples with a hemagglutination inhibition value ≥1:10 were considered positive. NSA, no samples available for testing; NA, not applicable for use in vaccine; BHG, bar-headed goose; JWE, Japanese white-eye.
†Individual monkey gave positive results for both strains.
‡Individual monkeys gave positive results for both strains.
§Individual monkey gave positive results for both strains.

genetics, transmission, and public health risk for infection with influenza A viruses.

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Human Polyomaviruses in Children Undergoing Transplantation, United States, 2008–2010

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Immunocompromised patients are at risk for disease caused by infection by some polyomaviruses. To define the prevalence of polyomaviruses in children undergoing transplantation, we collected samples from a longitudinal cohort and tested for the 9 known human polyomaviruses. All were detected; several were present in previously unreported specimen types.

BK and JC polyomaviruses (BKPyV, JCPyV) cause disease in immunocompromised persons. Both are double-stranded DNA viruses in the family *Polyomaviridae*. Seven additional human polyomaviruses were discovered during 2007–2011: KI polyomavirus (KIPyV) (*1*), WU polyomavirus (WUPyV) (*2*), Merkel cell polyomavirus (MCPyV) (*3*), human polyomavirus 6 (HPyV6) (*4*), human polyomavirus 7 (HPyV7) (*4*), trichodysplasia spinulosa-associated polyomavirus (TSPyV) (*5*), and human polyomavirus 9 (HPyV9) (*6*).

The 7 novel polyomaviruses have been detected in various specimen types; detection has been extensively reviewed for KIPyV, WUPyV, and MCPyV (*7*). Polyomaviruses HPyV6, HPyV7, TSPyV, and HPyV9 have been detected in skin (*4*,*5*,*8*); TSPyV and HPyV9 have also been detected in urine, and HPyV9 was detected in serum (*6*). However, only 2 of these recently identified viruses have been specifically implicated in human diseases; MCPyV is associated with Merkel cell carcinoma (*3*), and TSPyV has been linked to trichodysplasia spinulosa (*5*). Immunosuppression is a likely cofactor in both diseases. The potential pathogenicity of the other 5 novel polyomaviruses is unknown. As a first step toward exploring their disease potential, we sought to define their prevalence in immunocompromised transplant recipients. To this end, we established a longitudinal cohort of children undergoing transplantation at St. Louis Children's Hospital, St. Louis, Missouri, USA.

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The Study

We recruited 32 patients who were scheduled to receive transplants (2 lung, 11 liver, 5 heart, 2 kidney, 1 liver/lung, and 11 bone marrow transplants) during October 2008–April 2010. The Human Research Protection Office of Washington University in St. Louis approved this study. The mean age of enrolled patients was 5.8 years, and the median age was 3.1 years. Thirty patients received transplants and were studied for 1 year after transplantation. We collected 716 clinical specimens (160 nasopharyngeal swab, 169 urine, 122 fecal, 265 plasma) during 265 patient visits. We collected 298 specimens from patients during symptomatic episodes, which were defined as having ≥1 of the following: fever, respiratory symptoms, or gastrointestinal symptoms. We collected clinical data using a questionnaire and the medical records.

Fecal material was diluted 1:6 in phosphate-buffered saline and filtered through 0.45-μm membranes. For all specimens, we extracted total nucleic acids using an Ampliprep Cobas extractor (Roche, Branchburg, NJ, USA). We used published real-time PCRs for WUPyV (*9*), KIPyV (*9*), TSPyV (*5*), MCPyV (*10*), BKPyV (*11*), and JCPyV (*12*) (Table 1). We developed assays for HPyV6, HPyV7, and HPyV9 using Primer Express software (Applied Biosystems, Carlsbad, CA, USA) (Table 1). To assess the performance of each assay, we used serial dilutions (5 to 5×10^6 copies/reaction) of a plasmid containing the target sequence. All 3 assays demonstrated a sensitivity of ≈5 copies/reaction and yielded linear curves with R^2 values >0.99.

Each of the 25-μl quantitative PCRs included 5 μL of extracted sample, 12.5 pmol of each primer, and 4 pmol of probe. The MCPyV primers and probe were used as described (*10*). We tested samples in a 96-well plate format, with 8 water negative controls and 1 positive control/plate. Reactions were cycled as recommended using either an ABI 7500 real-time thermocycler (Applied Biosystems) or a CFX96 real-time thermocycler (BioRad, Hercules, CA, USA). The threshold of all plates was set at a standard value, and samples were counted as positive if their cycle threshold was <37.00.

All 716 specimens were tested for each virus (Table 2). The most frequently detected virus was BKPyV, which was found primarily in urine as expected. JCPyV was detected in 1 plasma sample. HPyV6, HPyV7, MCPyV, and TSPyV were detected in specimen types not previously reported. HPyV6 and TSPyV were detected in fecal samples and

Table 1. Real-time PCR assays to detect human polyomaviruses in children undergoing transplants, United States, 2008–2010*

Virus	Target	Primers, 5′ → 3′	Probe, 5′ → 3′
WUPyV	NCCR	WU-C-4824-F: GGCACGGCGCCAACT WU-C-4898-R: CCTGTTGTAGGCCTTACTTACCTGTA	WU-C-4861-TM: FAM- TGCCATACCAACACAGCTGCTGAGC-TAMRA-3′
KIPyV	LTAg	KI-B-4603-F: GAATGCATTGGCATTCGTGA KI-B-4668-R: GCTGCAATAAGTTTAGATTAGTTGGTGC	KI-B-4632-TM: FAM- TGTAGCCATGAATGCATACATCCCACTGC-TAMRA
TSPyV	LTAg	LTF: TGTGTTTGGAAACCAGAATCATTTG LTR: TGCTACCTTGCTATTAAATGTGGAG	LTP: FAM-TTCTTCTTCCTCCTCATCCTCCACCTCAAT- TAMRA
MCPyV	LTAg	LT.1F: CCACAGCCAGAGCTCTTCCT LT.1R: TGGTGGTCTCCTCTCTGCTACTG	LT probe: FAM-TCCTTCTCAGCGTCCCAGGCTTCA- TAMRA
HPyV6	VP1	ES011F: GCCTGGAAGGGCCTAGTAAAG ES012R: ATTGGCAGCTGTAACTTGTTTTCTG	ES024: FAM- ACCAACCATCTGTTGCAGGCATTAAAGCTA-TAMRA
HPyV7	VP1	ES017F: GGTCCAGGCAATACTGATGTAGCTA ES018R: TCTGCAACCCAGAGCTCTACTG	ES025: FAM-CCTGCAAGCCCTCAGAAAGCAAGTAAATG- TAMRA
HPyV9	LTAg	ES026F: GAAGACCCTGATCCTGAGGAAGA ES027R: CTCTGGAGTATTAGGTTCAGGCTTCT	ES031: FAM-TGGATCATCCCAGAGTTCATTTACCTGCA- TAMRA
BKPyV	LTAg	BK-Deg-F: AGCAGGCAAGRGTTCTATTACTAAAT BK-Deg-R: GARGCAACAGCAGATTCYCAACA	Bk-Deg-P: FAM- AAGACCCTAAAGACTTTCCYTCTGATCTACACCAGTTT- TAMRA
JCPyV	VP2/3	JL1 (F): AAGGGAGGGAACCTATATTTCTTTTG JL1 (R): TCTAGCCTTTGGGTAACTTCTTGAA	JL1 (P): FAM- CTCATACACCCAAAGTATAGATGATGCAGACAGCA- TAMRA

*WUPyV, WU polyomavirus; NCCR, non-coding control region; KIPyV, KI polyomavirus; LTAg, large T antigen; TSPyV, trichodysplasia spinulosa polyomavirus; MCPyV, Merkel cell polyomavirus; HPyV, human polyomavirus; VP, virion protein; BKPyV, BK polyomavirus; JCPyV, JC polyomavirus.

nasopharyngeal swab samples, and HPyV7 was detected in a nasopharyngeal swab and urine. One fecal sample was positive for MCPyV. Because HPyV6, HPyV7, and MCPyV have been previously detected in skin, we cannot rule out the possibility that their presence in specimens could have been caused by shedding from skin.

We collected 2 serial nasopharyngeal samples that were positive for KIPyV from patient 3001 (Table 2), a 1-year-old child who had received a bone marrow transplant as treatment for Fanconi anemia. The first sample, a nasopharyngeal swab obtained 1 month after transplant, had low levels of KIPyV. To determine the viral load of the second nasopharyngeal swab specimen collected 2 months later, we reanalyzed the sample in triplicate; on the basis of extrapolation of the standard curve run in parallel, we estimated the viral load to be 1.3×10^9 genome copies/mL of nasopharyngeal swab transport media. This patient's course was complicated by graft-versus-host disease of the gut and skin, renal failure requiring dialysis, and recurrent pulmonary hemorrhage. The patient was critically ill and had experienced multiorgan failure at the time of the second sampling. Other microbiological test results were negative at that time, including PCR for Epstein-Barr virus, cytomegalovirus, human herpesvirus-6, and adenovirus in the blood; aspergillus antigen detection in blood; and bacterial cultures of blood, tracheal aspirate, urine, and peritoneal fluid. The fecal specimen collected at this time was negative for KIPyV; plasma and urine were not available for this study. The patient died of acute respiratory failure and extensive pulmonary hemorrhage 24 days after collection of this specimen. Despite the frequent detection of KIPyV in respiratory specimens, no studies have definitively linked infection with respiratory disease. Titers of KIPyV were high in the nasopharyngeal swab sample from this patient 3 weeks before respiratory failure. Although this observation does not necessarily implicate KIPyV infection as a contributing factor in the death of the patient, it suggests a poorly controlled KIPyV infection in the respiratory tract.

Three specimens collected from patient 4001, a 13-year-old heart transplant recipient, were positive for TSPyV (Figure), but the patient did not have trichodysplasia spinulosa. At 1 week after transplant, the nasopharyngeal swab and fecal samples were positive for TSPyV. At 1 month after transplant, the nasopharyngeal swab sample was again positive for TSPyV, with a viral load of $\approx 2.3 \times 10^4$ genome copies/mL of transport media. There is currently only 1 TSPyV sequence in GenBank (accession no. GU989205). We used 4 primer pairs to amplify the complete genome of TSPyV from the nasopharyngeal swab taken 1 month after transplant. PCR products were cloned, and the complete genome was sequenced to 3× coverage (GenBank accession no. JQ723730) and compared with the other TSPyV sequence. There were 5 nt substitutions: 3 in noncoding regions and 2 synonymous mutations.

Table 2. Polyomaviruses detected among specimens from children undergoing transplants, USA, 2008–2010*

Virus	Specimen type	Transplant	C_t	Patient ID	Date of collection, time elapsed from transplant
HPyV6	Feces	BMT	32.19	3011	2012 Jun 6, 1 mo after
HPyV6	NP	Heart	36.13	4005	2010 Nov 25, 7 mo after
HPyV6	Feces	Lung	36.95	5001	2010 Aug 17, 1 mo after
HPyV7	NP	Liver	34.57	1002	2009 Jun 16, 7 mo after
HPyV7	Urine	Liver	36.54	1002	2009 Jul 15, 8 mo after
HPyV9	Urine	Liver	36.72	1009	2010 Feb 9, 1 wk after
KIPyV	NP	BMT	16.28	3001	2009 Jul 7, 3 mo after
KIPyV	NP	BMT	36.07	3001	2009 May 19, 1 mo after
KIPyV	NP	BMT	33.37	3008	2009 Nov 12, before
KIPyV	NP	BMT	31.04	3009	2010 Jul 3, 6 mo after
MCPyV	NP	BMT	36.29	3011	2010 Apr 15, before
MCPyV	Feces	BMT	34.56	3011	2010 Jul 2, 2 mo after
TSPyV	NP	Heart	32.98	4001	2009 May 29, 1 wk after
TSPyV	NP	Heart	30.74	4001	2009 Jun 18, 1 mo after
TSPyV	Feces	Heart	33.89	4001	2009 May 29, 1 wk after
WUPyV	NP	BMT	36.62	3005	2009 Jul 15, before
WUPyV	NP	BMT	28.81	3007	2009 Nov 6, 2 mo after
JCPyV	Plasma	BMT	36.12	3011	2010 Aug 24, 3 mo after
BKPyV	Urine	BMT	15.83	3010	2010 Apr 15, 1 mo after
BKPyV	Urine	Kidney	36.67	2022	2010 Jul 1, 10 mo after
BKPyV	Urine	BMT	30.80	3011	2010 Aug 24, 3 mo after
BKPyV	Urine	Heart	25.84	4001	2009 Aug 14, 2 mo after
BKPyV	Urine	Heart	35.89	4003	2009 Dec 23, 2 mo after
BKPyV	Urine	Heart	24.37	4001	2009 Sep 23, 4 mo after
BKPyV	Urine	Liver	28.56	1010	2009 Nov 23, 1 wk after
BKPyV	Urine	Lung	33.13	5002	2011 May 3, 1 y after
BKPyV	Urine	Lung	25.25	5002	2011 Feb 8, 10 mo after
BKPyV	Urine	Kidney	9.97	2002	2010 Mar 4, 6 mo after
BKPyV	Urine	BMT	30.10	3009	2010 Mar 5, 2 mo after
BKPyV	Urine	Liver	22.89	1001	2009 Jan 7, 3 mo after
BKPyV	Urine	Kidney	34.41	2002	2010 May 13, 8 mo after
BKPyV	NP	Kidney	35.93	2002	2010 Mar 4, 6 mo after
BKPyV	Feces	Kidney	33.15	2002	2010 Mar 4, 6 mo after
BKPyV	Feces	Liver	33.33	1001	2008 Dec 18, 2 mo after
BKPyV	Feces	Liver	34.84	1001	2009 Jan 7, 3 mo after

*C_t, cycle threshold; ID, identification; HPyV, human polyomavirus; BMT, bone marrow transplant; NP, nasopharyngeal; KIPyV, KI polyomavirus; MCPyV, Merkel cell polyomavirus; TSPyV, trichodysplasia spinulosa polyomavirus; WUPyV, WU polyomavirus; JCPyV, JC polyomavirus; BKPyV, BK polyomavirus

Although serologic studies have demonstrated that ≈70% of adults in Europe have been infected by TSPyV (*13*), its mode of transmission is unknown. The detection of TSPyV in nasopharyngeal swab and fecal samples raises the possibility that it may be transmitted by a respiratory or fecal–oral route. Furthermore, in the current study, 2 sequential nasopharyngeal swab samples taken 20 days apart were positive for TSPyV, suggesting it may persist for extended periods in the respiratory tract, at least in immunosuppressed persons.

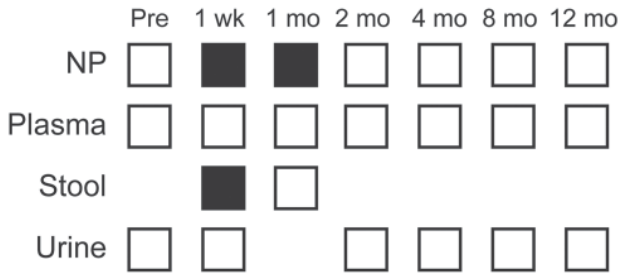

Figure. Samples tested for TSV (trichodysplasia spinulosa polyomavirus) during May–June 2009 from patient 4001, a 13-year-old heart transplant recipient at St. Louis Children's Hospital, St. Louis, Missouri, USA. Samples tested at each time point are indicated by white squares. Black squares represent positive samples. NP, nasopharyngeal.

Conclusions

Our goals were to establish a longitudinal repository of different specimen types from transplant recipients and to define the prevalence of polyomaviruses in these patients. We detected all 9 polyomaviruses in at least 1 specimen. Although the prevalence of each virus was generally low, TSPyV, HPyV6, HPyV7, and MCPyV were detected in specimen types not previously reported. These observations expand understanding of the recently identified polyomaviruses and the tissue and organ systems they may infect and suggest possible modes of transmission. Further studies to define their possible roles in human diseases are needed.

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Preventing Maritime Transfer of Toxigenic *Vibrio cholerae*

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Organisms, including *Vibrio cholerae*, can be transferred between harbors in the ballast water of ships. Zones in the Caribbean region where distance from shore and water depth meet International Maritime Organization guidelines for ballast water exchange are extremely limited. Use of ballast water treatment systems could mitigate the risk for organism transfer.

Cholera is an acute diarrheal illness caused by toxigenic strains of the bacterium *Vibrio cholerae* serogroups O1 and O139. *V. cholerae*, like other vibrios, is found commonly in marine and estuarine environments, living freely or on surfaces, such as plants and animal shells, and in intestinal contents of marine animals (*1*). *V. cholerae* infection is typically acquired by ingestion of contaminated water or food (*2*).

Ballast water is collected in ships to regulate their stability; the discharge of ballast water can transfer toxigenic *V. cholerae* O1 from one harbor to another. (*3*). During 1992, shellfish in Mobile Bay, Alabama, on the US coast of the Gulf of Mexico, were contaminated with an epidemic strain of toxigenic *V. cholerae* O1 from Latin America, although no human illnesses were reported (*4*). *V. cholerae* transfer by cargo ship was documented when the same strain was isolated from ballast and other nonpotable water samples collected from 5 cargo ships from ports in Latin America that arrived in the US Gulf of Mexico (*5*).

To reduce the risk for transfer of invasive species and pathogens between harbors by introduction of contaminated ballast water, the International Maritime Organization (IMO) adopted the International Convention for the Control and Management of Ships' Ballast Water and Sediments (BWM Convention) on February 13, 2004 (*6*). Regulation D-2 of the convention establishes numeric ballast water discharge standards, to be phased in with a start date of January 1, 2012, that limit the concentration of viable organisms and human pathogens (including toxigenic *V. cholerae*, *Escherichia coli*, and intestinal enterococci). The limit for toxigenic *V. cholerae* is <1 CFU/100 mL or <1 CFU/g (wet weight) zooplankton samples. In the interim, the BWM Convention requires that, whenever possible, ships conduct ballast water exchange >200 nautical miles from the nearest land and in water >200 m deep. If these requirements cannot be met, the exchange should be performed as far from the nearest land as possible, but at a minimum >50 nautical miles from the nearest land and in water >200 m deep. When these requirements cannot be met, areas may be designated where ships can conduct ballast water exchange. Ballast water exchange is based on the principles that 1) organisms from coastal areas will not survive in the open ocean and 2) fewer organisms (including fewer human pathogens) will be taken up in the open ocean, and these will be less likely to adapt to coastal waters.

A cholera epidemic emerged in Haiti in October 2010; lack of safe water and sanitation infrastructure and the devastation caused by the January 2010 earthquake contributed to its spread (*7*). Concerns were raised that cholera could be transferred from Haiti to other countries through contamination of coastal waters by ship ballast water. Ship traffic to Haiti (233 vessel calls in Port-au-Prince in 2008) consists predominantly of cargo vessels with destinations in the United States, other Caribbean islands, and Latin America (*8*). During an assessment of cholera contamination of fresh and marine water sources in Haiti during the epidemic conducted by the US Centers for Disease Control and Prevention (CDC), US Food and Drug Administration, and the Haitian Ministry of Health and Population, water and seafood collected from harbors at Port-au-Prince and St. Marc were tested for viable *V. cholerae* and for the cholera toxin gene (*ctx*A) (*9*). Toxigenic *V. cholerae* O1 serotype Ogawa indistinguishable from the outbreak strain was isolated from seafood samples from Port-au-Prince. Although *V. cholerae* was not isolated from marine water samples, the *ctx*A gene was detected in broth cultures of seawater samples from both harbors, suggesting that harbor waters were contaminated with toxigenic *V. cholerae*.

The Study

To further evaluate the risk for cholera transfer through ballast water under existing management approaches, we applied the IMO ballast water exchange depth and distance criteria to the Caribbean region. Buffers of 50 and 200 nautical miles were generated on the basis of the Global Self-Consistent, Hierarchical, High-Resolution Shoreline

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Figure. Zones in the Caribbean region where distance from shore and water depth meet International Maritime Organization guidelines for ballast exchange. To exchange ballast >200 nautical miles from shore in water 200 m deep, ships must travel 280 nautical miles northeast of Haiti (A) or to the Gulf of Mexico (B). To exchange ballast at the minimum 50 nautical miles from shore in water >200 m deep, ships must travel >90 nautical miles northeast (C) or 50 nautical miles south (D) of Haiti or conduct the exchange in an area <45 nautical miles wide approximately equidistant from Haiti, Cuba, and Jamaica (E). Light gray shading indicates distance from land is <50 nautical miles and/or seawater depth is <200 m. Medium gray shading indicates distance from land is >50 nautical miles but <200 nautical miles, and seawater depth is >200 m. Dark gray shading indicates distance from land is >200 nautical miles and seawater depth is >200 m.

Database (version 2.1; www.ngdc.noaa.gov/mgg/shorelines/gshhs.html) and overlaid on bathymetric data from the ETOPO1 Global Relief Model (www.ngdc.noaa.gov/mgg/global/global.html) (*10*,*11*). We acquired these datasets through the National Oceanic and Atmospheric Administration's National Geophysical Data Center (www.ngdc.noaa.gov).

Mapping indicates that waters around Haiti where the IMO guidelines can be followed are extremely limited (Figure). To exchange ballast >200 nautical miles from shore in water 200 m deep, ships must travel 280 nautical miles northeast of Haiti (Figure, A) or to the Gulf of Mexico (Figure, B). To exchange ballast at the minimum 50 nautical miles from shore in water >200 m deep, ships must travel >90 nautical miles northeast (Figure, C) or 50 nautical miles south (Figure, D) of Haiti or conduct the exchange in an area <45 nautical miles wide approximately equidistant from Haiti, Cuba, and Jamaica (Figure, E).

After discussions with staff at CDC and the Pan American Health Organization, the Director General of the Haitian Ministry of Health and Population issued 2 memoranda addressing ballast water management. The first, issued November 10, 2011, asked ship captains not to exchange ballast water in Haitian harbors. The second, issued November 15, 2011, reminded ship captains to adhere to IMO guidelines for ballast water exchange. However, ships operating in the Caribbean Sea face practical difficulties in conducting ballast water exchange at the recommended distances without making large deviations from usual routes. The BWM Convention recommends that designated ballast water exchange areas should be on or close to existing maritime routes and does not require that ships deviate course or delay voyages to comply with ballast water exchange guidelines (*6*). Ballast water exchange at sea also presents operational and safety challenges to ships and might not be completely effective in preventing spread of aquatic organisms (*12*).

As an alternative management measure, ballast water treatment systems are being developed to meet the BWM Convention's numeric discharge standards, and several systems are available (*13*). These systems combine filtration with nonchemical (e.g., UV light, shear, heat) and chemical biocides to remove or kill organisms (*14*). Ballast water treatment systems are designed to achieve the BWM Convention efficacy levels, and their future use in the Caribbean region would likely be a more effective management approach than ballast water exchange.

The Haiti cholera outbreak spread to the neighboring Dominican Republic, and cholera cases associated with travel to Haiti were recognized in the United States (*7*), but there is no evidence that *V. cholerae* was transferred by ship ballast water in these instances. The Wider Caribbean Region comprises 28 island and continental countries with coasts on the Caribbean Sea, the Gulf of Mexico, and adjacent waters of the Atlantic Ocean. This area is at elevated risk for transfer of contaminated ballast water because of the high volume of cargo trade in the region and has been prioritized by the Global Ballast Water Management Program (http://globallast.imo.org/index.asp), a program administered by IMO, the Global Environment Facility, and the United Nations Development Program to assist developing countries implement ballast water management measures (*15*).

Conclusions

A comprehensive regional strategic plan has been developed to promote ratification of the BWM Convention and to facilitate its implementation within the Wider Caribbean Region through regional cooperation, training, communication, compliance monitoring and enforcement, and promotion of national-level legislation, task forces, action plans, and sustainable resources to support activities (*15*). Implementation of this plan will help protect public health by reducing the likelihood that *V. cholerae* and other pathogens will be transferred by ballast water.

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A Natural History of Infective Endocarditis, Preceded by Decompensated Chronic Liver Disease and Severe Community-acquired Pneumonia

Indictment in Four Parts and with Right of Reply

Nancy L. Merridew

ONE. Trespass

His stalwart skin, sallow armor,
harbored memories and marks
of a difficult life.
His grave affliction, already perilous,
he endured alone—
ostracized by family and society.
His swollen belly, crazed by Medusa,
pouted taut as artificial breaths
stretched rankled lungs.
His seasoned vessels, illicit euphoria's labyrinth,
adorned with plastic portals
infused antidote.
His cloying blood, claret lately poisoned,
delivered assassin to lair—
Duke in silent carriage alighted at the chamber.

TWO. Malice Aforethought

His haughty foe, ignoble despite the Criteria,
stole a vantage of supreme command
at the Greatest Vessel door.
His insistent heartbeat, two sounds once discreet,
in rhythmic phrases murmured
remarks of furtive progress.
His ardent doctor, genius clinician,
lifted stethoscope to precordium—
message received.
His pliant pulses, company accommodated—
Watson struck and shuddered,
Corrigan thumped and ebbed.
His teeming veins, mercurial blood extracted,
yielded Duke's identity
for precise retaliation.
His shriveled Liver, nominally ironic,
sacrificed to intoxicated sanctuary—
Child-Pugh C proscribed surgical reprieve.
His adept assailant, though exposed for battle,
abetted by fickle liver
retained advantage for the coup.

THREE. Progressive Assault

His heart, excavated by festering mercenaries,
inexorably leached
their venomous arsenal.
His body, conscripted slave to advancing legion,
appointed satellite outposts—
Streptococcal Empire rising.
His scent, previously acrid,
drifted metallic—
life corroding.
His brain, shadowed by grim delirium;
encephalopathic, embolic, prophetic—
entombed within cranial vault.
His eyelids, fluttering shrouds,
veiled infinite irises, icteric globes and
retinas maligned by immunity.
His urine, once flaxen,
oozed vermillion—
tributary of beckoning Rubicon.
His skin, chameleon canvas—
flushed lesions and jaundice
defaced faded tattoos.
His fingers, pulps mottled carmine,
uniquely etched tips
reached to probe oblivion.
His nail-beds, fleshy Rosetta,
signaled the secret
in flecked hieroglyphs.
His palms, unaccustomed to prayer,
denied redemption—
pocked, they too bore stigmata.

FOUR. Homicidal Ascendancy

His surgical saviors, glinting mirage,
could offer no cure
except hastened demise.
His thwarted physicians, ashen surrender—
our elixirs no match
for the adversary.

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His recalcitrant Duke, relentless,
accomplished unsavory
feats of cardiac passage.
His core engine, chambers thus connected,
ramped to fuel abscessed conduit—
blood roared relentlessly.
His slumped neck, kinetically possessed—
terminally mutilated muscle's
futile beats rocketed and ricocheted.
His uncanny figure, cadaveric,
inevitably bloated;
Starling forces prevailed.
His sodden lungs, emancipated from machines,
hissed and spluttered
beneath swelling king tide.
His dying breaths, labored then whispered,
dim light obscured final chest fall—
curtains drawn, despite the summer sun.
His only visitor, a community case worker,
absent that day.
Alone when he succumbed to the darkness.

Audi Alteram Partem

Virulent villain—do you admire your conquest?

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Trypanososma brucei rhodesiense Sleeping Sickness, Uganda

To the Editor: The past 2 decades have heralded notable success in efforts to control sleeping sickness (human African trypanosomiasis [HAT]) in Africa. HAT is a neglected tropical disease with major public health and economic effects in sub-Saharan Africa, and its effects on livestock productivity and development are considered major constraints to alleviating poverty in this region (*1*,*2*). Because of concerted and coordinated continental control efforts, its incidence has steadily decreased.

Despite these successes, concern has increased recently regarding potential convergence of the 2 causes of HAT (*Trypanosoma brucei gambiense* and *T. brucei rhodesiense*). These organisms differ in transmission and how infections are diagnosed and treated, and control, and have never coincided in the same area. Uganda is the only country with endemic distributions of these 2 trypanosome species, and convergence there represents a major public health concern, given the potential for overlapping infections to compromise treatment and control programs and spread into neighboring countries (*3*,*4*).

Risk for convergence led to an international emergency intervention. In 2006, an international public–private partnership, Stamp Out Sleeping Sickness (SOS), was established to control spread of this disease in central Uganda (*5*). However, despite the continental effect of convergence of the 2 causes of HAT, little is known about trends in incidence and epidemiology of HAT in central Uganda. We report results of data analysis for HAT caused by *T. b. rhodesiense* during 2000–2009.

This study was approved by the ethics review board for human subjects at McGill University (Montreal. Quebec, Canada). We obtained data on case-patients given a diagnosis of *T. b. rhodesiense* HAT at a HAT treatment unit in Uganda. These diagnoses were reported to the National Sleeping Sickness Control Program of the Ministry of Health (*4*,*6*). Data were assigned locations by parish, and analyses focused on spatiotemporal trends in case occurrence. The final cleaned dataset contained 2,501 reported cases of presumed *T. b. rhodesiense* HAT.

In the past 10 years in Uganda, 140 cases of fatal *T. b. rhodesiense* HAT have been reported. However, given estimates of underreporting and cessation of active surveillance, actual deaths are likely > 1,700 (170 deaths/year) (*6*). Notably, mortality rates have increased from an average of 5% in the early 2000s to ≈10% in later years, and rates have been higher in recently affected districts. This pattern is predominantly driven by higher mortality rates in newly affected SOS districts in central Uganda, in which diagnostic and treatment delays are higher, and from which an increasing proportion of HAT cases are originating.

Patients in SOS intervention districts were more likely to report cases in late stages of the disease ($p<0.01$, by χ^2 test). The mortality rate was >3-fold higher for persons with late-stage cases (8.1%) than for those with early-stage cases (2.4%) ($p<0.01$, by χ^2 test). Given that central Uganda is the critical zone for convergence and intervention, such evidence of presumed diagnosis and treatment delay is cause for concern.

The SOS phase 1 intervention period (2006–2008) coincided with a period of reduced reported prevalence of HAT (Figure). The monthly average was 27 cases/month before the intervention and 10 cases/month after the intervention ($p<0.01$, by Mann-Whitney test). However, a substantive component of reduction in incidence occurred in districts not included in the SOS intervention program. This pattern may reflect reporting bias caused by a transition in Uganda in 2005 to a period of passive surveillance and underfunding for national reporting. Therefore, it remains unclear to what extent increased international attention and SOS intervention have contributed to HAT prevention and control. The absence of a clear increase in incidence after reinstatement of national data acquisition in 2008 provides an early indication that interventions may be contributing to the decrease in, or at least to stabilization of, geographic spread in central Uganda.

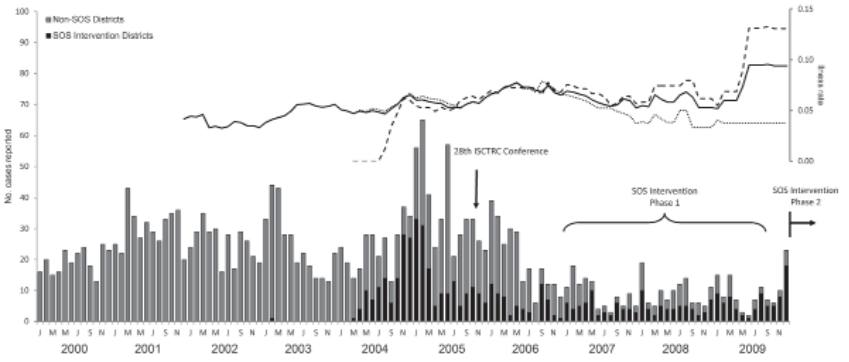

Figure. Human African trypanosomiasis cases and deaths by month, Uganda, 2000–2009. Bars indicate cases in districts in the Stamp Out Sleeping Sickness (SOS) intervention region and outside the SOS region. Solid line indicates overall 24-month moving average of deaths, dashed line indicates 24-month moving average of deaths in SOS intervention districts, and dotted line indicates 24-month moving average of deaths in non-SOS districts. ISCTRC, International Scientific Council for Trypanosomiasis Research and Control; J M M J S N, Jan, Mar, May, Jul, Sep, Nov.

HAT data indicate seasonality of this disease; incidence is higher during January, February, and March (p = 0.04, by Mann-Whitney test). Seasonality of HAT incidence has been noted elsewhere and linked to seasonal influences on tsetse habitat suitability. We propose that seasonality of cattle trading may also play a role because cattle purchases increase before the Christmas season, which promote pathogen spread and increased transmission. This finding is consistent with research highlighting the role of livestock markets in the spread of *T. b. rhodesiense* in central Uganda and would further support a body of literature suggesting, as espoused by the SOS initiative, that control of animal reservoirs of the disease is a critical component of intervention measures (*2*,*7*–*9*). Implementation and enforcement of regulations for treatment of cattle before sale at markets would also contribute to limiting spread (*9*,*10*);

Interventions in districts in central Uganda in which convergence is predicted have been slow and incomplete. If convergence has occurred, this finding indicates that a specific region in Africa has had concurrent infection with both causes of HAT, with implications for prevention, treatment, and control. Since 2000, Uganda has had continued northward spread of *T. b. rhodesiense* infections, reducing the distance with TbG to <100 km, which we believe is a conservative estimate. Reinstatement of active surveillance of HAT and support for central data collection in Uganda are long overdo and warranted immediately.

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Rickettsia felis in *Aedes albopictus* Mosquitoes, Libreville, Gabon

To the Editor: *Rickettsia felis*, an emerging pathogen first identified in the cat flea (*1*), has been detected in other fleas, ticks, mites, and booklice (*2*). *R. felis* can be cultured in mosquito cell lines derived from *Anopheles gambiae* and *Aedes albopictus* (Asian tiger) mosquitoes (*2*), so its compatibility with mosquitoes in nature can be suspected. In sub-Saharan Africa, *R. felis* bacteremia in humans is common, especially during the rainy season, when mosquitoes proliferate. We tested anthropophilic mosquitoes for the presence of *R. felis* DNA (*3*–*5*).

During December 2008–January 2010, we randomly selected female *Ae. albopictus* and *Ae. aegypti* mosquitoes (96 each) from specimens obtained by human-landing collections from 4 sites in Libreville, Gabon (*6*). Specimens were collected during the rainy season (mid-January–end of May and end of September–mid-December); no parity data were available.

We extracted 192 DNA samples from homogenate (abdomen, wings, legs) of each nonengorged, host-seeking, adult mosquito by using the BioRobot 8000 (QIAGEN S.A.S., Courtaboeuf, France) and QIAamp Media MDx Kit (QIAGEN) according to the manufacturer's instructions. Samples were screened by quantitative real-time PCR (qPCR) targeting the biotin synthase (*bio*B) gene (*4*). Positive results were confirmed by qPCR-based molecular detection targeting the *orf*B gene, which codes for a transposition helper protein. This qPCR used a set of primers not previously used in our laboratory (R_fel.OrfB_F: 5′-CCCTTTTCGTAACGCTTTGCT-3′ and R_fel.OrfB_R: 5′-GGGCTAAA CCAGGGAAACCT-3′) and the probe

R_fel.OrfB_P: 6-FAM-TGTTCCGGT T T T A A C G G C A G A T A C C C A - TAMRA. Specificity of the qPCR was tested in silico and on the 31 *Rickettsia* spp. from our laboratory. The final qPCR reaction mixture contained extracted DNA (5 μL) and mix (15 μL) that contained master mix (10 μL) from the QuantiTect Probe PCR Kit (QIAGEN, Hilden, Germany), each primer (0.5 μL, 20 pmol), probe (0.5 μL, 62.5 nmol), and RNase-free water (3.5 μL). Amplification and sequence detection were performed in a CFX96 Touch thermocycler (Bio-Rad, Marnes-la-Coquette, France) as follows:15 min at 95°C followed by 40 cycles of 1 s at 95°C, 40 s at 60°C, and 40 s at 45°C.

Test results for all *Ae. aegypti* homogenates were negative for *R. felis* DNA. Of the 96 *Ae. albopictus* specimens, 3 (3.1%) had positive test results for the *R. felis* species–specific real-time qPCR and the confirmatory qPCR, with mean cycle thresholds ± SDs of 37.34 ± 1.7 (*bio*B gene; mean copies/mosquito 5×10^2 [minimum 1.2×10^2, maximum 1.4×10^3]) and 33.64 ± 1.4 (*orf*B gene; mean copies/mosquito 5×10^2 [minimum 1.5×10^2, maximum 1×10^3). One of the 3 samples was collected in January and 2 in March. The samples came from 3 different districts of Libreville (Akebe Poteau, Alibandeng, Camp des Boys) and were tested by nested PCR targeting the citrate synthase (*glt*A) gene (*7*). *Rickettsia montanensis* DNA was used as a positive control. Sequencing was performed as described (*7*), and ChromasPro version 1.34 (Technelysium Pty Ltd., Tewantin, Queensland, Australia) was used to analyze sequence data. Sequences of the *bio*B (120/120) and *glt*A (1,230/1,230) amplicons at the nucleotide level were 100% homologous to sequences for *R. felis* URRWXCal2 (GenBank accession no. CP000053). The *glt*A fragment sequence was deposited in GenBank (accession no. JQ674484). Mosquitoes were considered positive for *R. felis* when the qPCR result was ≤35 cycle thresholds for 1 target gene and the additional DNA sequence was successfully amplified. No sample in this study was positive for only 1 target gene or had a qPCR threshold ≥35 cycle thresholds for both genes.

Contamination is a critical problem for the PCR-based identification of microbes. However, the validity of the data we report is based on strict laboratory procedures and controls that are commonly used in the World Health Organization Reference Center for Rickettsial Diseases, including rigorous positive and negative controls to validate the test. Each positive qPCR result was confirmed by another *R. felis*–specific qPCR (*orf*B) not previously used in our laboratory (to avoid contamination with other amplicons).

Ae. albopictus mosquitos are native to Southeast Asia, colonizing rural and peri-urban sites. In Gabon, *Ae. albopictus* was the vector for outbreaks of chikungunya and dengue virus infections (*6*). Our study indicates that mosquitoes can carry *R. felis*, and the prevalence and load (1.8%–70% and 1.3×10^3–1.6×10^7, respectively) detected in mosquitoes in this study are consistent with the low-end range of those detected in cat fleas, the confirmed biological vector and reservoir (*8,9*).

We investigated the presence of *Rickettsia* spp. in mosquitoes neglected as possible vectors of rickettsial diseases (*2*). Other *Aedes* spp. and other genera of mosquitoes should be tested. The role of mosquitoes as *Rickettsia* spp. vectors remains to be demonstrated in additional studies that use the Mitchell criteria. These studies should include the use of cell culture to isolate or detect *R. felis* in salivary glands of specimens from wild-caught mosquitoes, PCR, immunofluorescence, and the fluorescence in situ hybridization technique; demonstration of infection of a mosquito after experimental feeding on a bacteremic host or bacterial suspension; and demonstration of the transmission of bacteria to a vertebrate through the bite of a mosquito (*10*).

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Bartonella spp. Infection Rate and *B. grahamii* in Ticks

To the Editor: Bacteria of the genus *Bartonella* are transmitted by arthropods and are often implicated in human disease. Even though ticks are known to transmit a variety of pathogens, vector competences for transmission of *Bartonella* spp. by ticks were speculative (*1*) until recently, when in vivo transmission of *B. birtlesii* by *Ixodes ricinus* ticks was demonstrated in mice (*2*). This finding suggests that this tick species, which is common in Europe, may also transmit zoonotic *Bartonella* spp. Evidence of possible tick transmission of bartonellae to humans under natural conditions was provided by Eskow et al. (*3*) and Angelakis et al. (*4*), who identified *Bartonella* spp. in tissue samples of patients who were recently bitten by ticks. We determined the prevalence of *Bartonella* spp. in questing *I. ricinus* ticks in the city of Hanover, Germany, which is nicknamed The Green Metropolis and was selected the German Capital of Biodiversity in 2011.

During April–October 2010, we collected 2,100 questing ticks, consisting of 372 adults (177 female and 195 male), 1,698 nymphs, and 30 larvae, from 10 recreation areas in Hanover. Tick DNA was extracted by using the NucleoSpin 8 Blood kit (Macherey-Nagel, Düren, Germany). Plasmid DNA constructed from *B. henselae* reference strain ATCC49793 containing the 249-bp target sequence of the *gltA* gene was used as positive control. *Bartonella* spp. in ticks was detected by quantitative PCR (qPCR) by using the Mx3005 Multiplex Quantitative PCR System (Stratagene, Heidelberg, Germany) according to the protocol described by Mietze et al. (*5*), with minor modifications. Samples positive by qPCR were verified by gel electrophoresis. *Bartonella* species were differentiated by sequencing (Eurofins MWG Operon, Ebersberg, Germany), and obtained sequences underwent BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparison to published sequences.

On the basis of the amplicon-specific melting temperature and DNA bands representing the specific size of 249-bp after gel electrophoresis, results of qPCR showed 100 (4.76%) infected *I. ricinus* ticks (Table). Positive results did not vary by developmental tick stages; 4.84% (18/372) adult ticks (5.08% [9/177] female and 4.62% [9/195] male), 4.71% (80/1,698) nymphs, and 6.67% (2/30) larvae were infected (Table). Because *Bartonella* spp. do not seem to be transmitted transovarially (*6*), it is likely that larvae had interrupted blood meals and thus did not take up enough blood to develop into the nymphal stage.

Seasonal changes in *Bartonella* spp. infection rates resulted in a higher peak in May (38/300 [12.67%]) than in the other months (Table). For sampling locations, infection rates for grassy sampling location 6 (4/210 [1.90%] infected ticks) differed significantly (Bonferroni-Holm adjusted $p<0.001$; *<0.0011) from that of densely wooded sampling location 9 (22/210 [10.48%] infected ticks).

Sequencing of the *gltA* fragment resulted in *Bartonella* species identification for 56/100 positive samples; 52 of these samples (from 38 nymphs, 13 adults, and 1 larva) were identified as infected with *B. henselae*. In 51 samples (92.86%), maximum identity with the BLAST top hit sequence was 99% because of

Table. Seasonal distribution of *Ixodes ricinus* ticks infected with *Bartonella* spp., Hanover, Germany, 2010*

Ticks	April	May	June	July	August	September	October	Total
No. infected ticks/no. tested (%)	5/300 (1.67)	38/300 (12.67)	7/300 (2.33)	10/300 (3.33)	5/300 (1.67)	17/300 (5.67)	18/300 (6.00)	100/2,100 (4.76)
No. (%) adults positive	1/88 (1.14)	8/48 (16.67)	0/39	0/41	2/56 (3.57)	3/53 (5.66)	4/47 (8.51)	18/372 (4.84)
No. (%) females	1/32 (3.13)	3/19 (15.79)	0/20	0/17	1/32 (3.13)	2/28 (7.14)	2/29 (6.90)	9/177 (5.08)
No. (%) males	0/56	5/29 (17.24)	0/19	0/24	1/24 (4.17)	1/25 (4.00)	2/18 (11.11)	9/195 (4.62)
No. (%) nymphs	3/203 (1.48)	30/248 (12.10)	7/261 (2.68)	10/259 (3.86)	3/244 (1.23)	14/240 (5.83)	13/243 (5.35)	80/1,698 (4.71)
No. (%) larvae	1/9 (11.11)	0/4	ND	ND	ND	0/7	1/10 (10.00)	2/30 (6.67)

*ND, testing not done.

nucleotide substitutions in position 198 (T→C) and in position 136 (A→G) of the 249-bp fragment. The remaining sample showed 100% identity with *B. henselae* strains Brazil-1 and 45-00249 (GenBank accession nos. HQ012580 and GQ225709).

Four of the 56 successfully sequenced samples (7.14%; all samples from nymphs) showed the sequence pattern of *B. grahamii*. One sample revealed 100% identity with *B. grahamii* (GenBank accession no. EU014266); the remaining 3 samples showed an identity of 98% with the *B. grahamii* strain Hokkaido-1 (GenBank accession no. AB426652) and 99% (T→C in position 93) with a sequence described as *B. grahamii*–like (GenBank accession no. AY435122). Sequences obtained in this study (deposited in GenBank under accession nos. JQ770304 and JK758018) support the genetic variability of *Bartonella* spp., as demonstrated by others (*5*,*7*,*8*).

It remains unclear whether ticks are involved in transmission of pathogenic *Bartonella* spp. to humans under natural conditions. However, the total prevalence rate of 4.76% (100/2,100) questing *I. ricinus* ticks infected with *B. henselae* and *B. grahamii* highlights the need for public awareness and draws attention to the possibility of an infection with zoonotic *Bartonella* spp. after a tick bite (*3*,*4*). *B. henselae*, the predominantly identified species, has been associated with cat scratch disease, peliosis hepatis, and bacillary angiomatosis in humans. Eskow et al. (*3*) also connected chronic symptoms of Lyme disease to co-infections with *Borrelia burgdorferi* and *B. henselae*. *B. grahamii* has been associated with neuroretinitis and ocular artery thrombosis in humans (*9*,*10*). The potential risk for zoonotic *Bartonella* spp. infection in urban recreation areas should not be underestimated.

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Human Parvovirus 4 Viremia in Young Children, Ghana

To the Editor: Establishment of viremia is a characteristic feature of infection with human parvovirus 4 (PARV4). In northern Europe, PARV4 (human partetravirus) is primarily transmitted by blood-borne routes (*1*,*2*). In other areas (southern Europe, western Africa, South Africa, Asia) infection seems to be more widespread, suggesting alternative modes of virus acquisition (*3*–*6*).

We reported PARV4 genotype 3 viremia in young children with unknown parenteral blood exposure from the rural Ashanti region of Ghana (*7*). In that study, 2 (2.1%) of 94 children (median age 14.9 months) and 22 (11.9%) of 185 children (median age 24.0 months) were virus positive (ages of the 2 virus-positive children from the younger cohort 14.9 and 15.6 months). Because the number of infants was small in that study, we extended our investigations on PARV4 viremia to an additional

cohort of 15-month-old children from the same study group.

Plasma samples from 361 randomly selected children (191 girls) were tested. Specimens were collected during January–December 2004 during a trial of intermittent preventive malaria treatment in the rural Afigya Sekyere District, Ashanti Region, Ghana (*7*). Plasma samples were analyzed because of limited availability of whole blood samples. Median age of children was 14.9 months (range 13.8–17.5 months, interquartile range 14.5–15.2 months).

Nucleic acid was prepared from 200-µL plasma samples by using the NucliSENS EasyMAG system (bioMérieux, Nürtingen, Germany). All samples were analyzed by using 2 real-time PCRs and primers described elsewhere (*7,8*). The limit of detection was ≈200 plasmid copies/mL. Strict precautions were applied during plasma handling and amplification to avoid false-positive results.

PARV4 genotype 3 DNA was detected in plasma of 32 (8.9%) of 361 children. Viral load ranged from ≈200 copies/mL to 3.0×10^4 copies/mL (Figure). Median viral load was 453 copies/mL. Nucleotide sequencing of screening PCR amplicons and additional genomic regions amplified from 6 plasma samples identified the viruses as PARV4 genotype 3 (GenBank accession nos. JN183933–JN183938). There was no association between history of fever, anemia, or erythema in children with or without PARV4 viremia ($p>0.05$, by χ^2 test).

PARV4 viremia status was already known for 78 children 24 months of age (*7*). These data enabled comparison of viremia at 2 time points (24 months and 15 months of age). Of these 78 children, 10 showed viremia (viral load range 4.0×10^2–1.4×10^4 copies/mL) and 3 (3.8%) had viremia at both time points and identical viral nucleotide sequences (time between bleedings 8.7 months for 2 children and 9.0 months for 1 child). However, only short genomic regions (780 nt for 1 child, 599 nt for a second child, and 95 nt for a third child) could be amplified and sequenced because of low viral loads. Four children had positive results in the first sample, and 3 had positive results in the second sample.

Because comparison of large and contiguous parts of the viral genomes within each sample pair was not possible and serologic data were lacking, PARV4 positivity over a 9-month period can be interpreted by 3 hypotheses. First, detection of PARV4 DNA over time might represent long-term viremia after infection, similar to observations in human parvovirus B19 infection. Second, demonstration of PARV4 during widely spaced intervals might indicate endogenous reactivation of viremia. Third, exogenous reinfection might have occurred.

PARV4 viremia was detected in a study in the United Kingdom among 110 PARV4-negative persons with hemophilia screened over 5 years for PARV4 viremia and seroconversion (IgG and IgM) (*9*). Nine patients who seroconverted were identified, and 1 had PARV4 viremia (genotype 1) over an 8-month period. Viral loads for this patient were low ($<10^3$ copies/mL), a finding similar to ours for the 3 children. However, negative IgM results in the person with hemophilia suggest that the sampling window might have missed the acute infection.

Comparison of results of our study with those of our previous study (*7*) showed 2 differences. First, frequency of viremia in children tested previously at 15 months of age was lower (2.1%, 2/94) than in the children in this study (8.9%). Second, median viral loads differed by nearly 1 $\log_{10}$, with the higher concentrations in the previous study analyzing EDTA whole blood. Whether these differences were caused by the relatively small number of children included by or by the fact that whole blood samples were compared with plasma samples

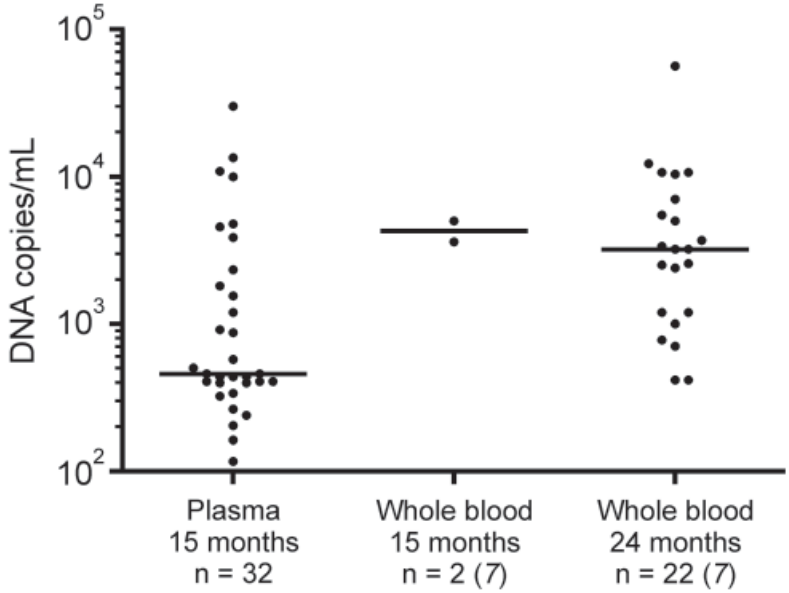

Figure. Parvovirus 4 DNA loads in virus-positive plasma specimens from children compared with those in whole blood samples previously tested (*7*), Ghana. Virus concentrations are given on a log scale on the y-axis. Each dot represents 1 specimen. Horizontal lines represent median values for each sample group. Children whose plasma was tested had a median age of 15 months, and children whose whole blood was tested had a median age of either 15 or 24 months. Viral load data (i. e., median viral load and range) for the 2 groups of whole blood samples have been reported (*7*) and were included for comparison with plasma data from this study.

remains to be clarified. However, our previous hypothesis that prenatal or perinatal transient infection was an unlikely mode of virus acquisition needs to be modified because PARV4 infection in newborns has recently been demonstrated (*10*).

Although we lacked IgM and IgG serologic data to interpret our findings, our study suggests that PARV4 genotype 3 infection might be characterized by viral persistence, reactivation, or reinfection. Additional longitudinal studies, including serologic testing for short intervals, are needed to determine the pathogenesis and potential public health role of PARV4 infection.

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Multidrug-Resistant *Salmonella enterica*, Democratic Republic of the Congo

To the Editor: *Salmonella enterica* serotype Typhi and the nontyphoid *S. enterica* (NTS) are leading causes of bacteremia in sub-Saharan Africa, but little information is available from central Africa (*1,2*). We describe an epidemic increase of *S. enterica* bacteremia in Kisantu in southwestern Democratic Republic of the Congo (DRC).

The Hospital of Saint Luc in Kisantu is a 274-bed referral hospital serving a community of 150,000 inhabitants. As part of an ongoing microbiological surveillance study in DRC (*3*), we identified pathogens grown from blood cultures (BacT/ALERT; bioMérieux, Marcy L'Etoile, France) and assessed them for antimicrobial drug susceptibility (Vitek II system; bioMérieux) (*4*) and serotype (Sifin, Berlin, Germany). We determined MICs for nalidixic acid, ciprofloxacin, and chloramphenicol using the Etest macromethod (bioMérieux). For salmonella isolates, we defined decreased ciprofloxacin susceptibility as an isolate with an MIC >0.064 mg/L (*5*) and multidrug resistance (MDR) as co-resistance of the isolate to ampicillin, chloramphenicol, and trimethoprim/sulfamethoxazole (*6*). Screening for mutations causing decreased ciprofloxacin susceptibility included assessment of the quinolone resistance–determining regions of the *gyrA*, *gyrB*, and *parC* genes and the plasmid-mediated *qnrA*, *qnrB*, and *qnrS* genes (*7*). Multilocus variable-number tandem-repeat analysis was performed on a subset of 37 *S. enterica* ser. Enteritidis isolates (*8*).

The pathogens isolated were *S. enterica* ser. Typhi (n = 17, 14.4%), Enteritidis (n = 79, 67.0%), and Typhimurium (n = 22, 18.6%). The increased incidence of *S. enterica* bac-

teremia was caused by an increased incidence of *S. enterica* ser. Enteritidis infection from 1 and 2 isolates reported in 2008 and 2009, respectively. The rate of infection by serotypes Typhi and Typhimurium had remained constant during this period.

During September 2010–May 2011, the proportion of pathogens isolated from blood cultures increased to 53.2% (197/370), compared with 19.7% (63/319) and 25.2% (85/328) for 2008 and 2009, respectively (p<0.001). *S. enterica* isolates represented 59.9% (118/197) of pathogens, compared with 53.3% (70/131) and 30.9% (84/272) for the same months during 2008–2009 and 2009–2010, respectively (p<0.001). Of 118 *S. enterica* samples isolated, 89 (75.4%) were isolated from specimens from children <5 years old and 17 (15.3%) from children 5–10 years old. Clinical signs and symptoms were nonspecific; malaria and gastrointestinal infection were the leading diagnoses on admission. Data for in-hospital deaths (retrieved for 87 patients) revealed case-fatality rates of 23.0% (17/74) for children <5 years old, compared with 1 in 10 patients 5–10 years old. Because of the retrospective nature of the study, it was not possible to assess population incidence rates, and we had no estimates of the number of children who were referred to the hospital but died before reaching the emergency department. There was no apparent geographic clustering, but the epidemic coincided with the onset of the rainy season, which had started late and had unusually heavy rainfall.

All NTS isolates were MDR; 1 (1.3%) *S. enterica* ser. Enteritidis and 1 (4.5%) *S. enterica* ser. Typhimurium isolate had additional decreased ciprofloxacin susceptibility. Most (16/17, 94.1%) *S. enterica* ser. Typhi isolates were resistant to amoxicillin and trimethoprim/sulfamethoxazole, 4 (23.5%) and 7 (41.2%) were MDR and had decreased ciprofloxacin susceptibility, respectively. Three combinations of resistance genes encoding decreased ciprofloxacin susceptibility were found (Table). No resistance to cefotaxime was observed. Multilocus variable-number tandem-repeat analysis typing of the *S. enterica* ser. Enteritidis isolates revealed 2 major profiles (differing in 3 tandem repeats in 1 locus) and 3 minor profiles (differing from the major profiles by 1 tandem repeat at 1 and 2 loci, respectively). Considering the long sample period, we concluded that 1 clonal type had caused the infections in which *S. enterica* ser. Enteritidis was isolated.

A recent literature review of bacteremia reported aggregated data from 16 studies from eastern (Kenya, Tanzania), western (the Gambia), and southern Africa (Malawi, Mozambique) (*1*). *S. enterica* ser. Typhi and NTS represented 0–42% and 9%–84%, respectively, of associated pathogens. The study reported that most NTS isolated were of the serotypes Enteritidis and Typhimurium. A sequential occurrence of disease caused by NTS serotypes similar to that in this study was recorded in Malawi (*6*).

The reservoir of NTS in sub-Saharan Africa remains unclear; person-to-person and zoonotic transmissions have been postulated (*1*,*2*). The coincidence of the onset of the epidemic described in this study with the start of the rainy season is a known phenomenon and might be related to increased incidences of malaria and malnutrition (*9*) and to contamination of the surface waters caused by floods after heavy rainfalls (*2*).

Case-fatality rates in this study were similar to those reported previously from sub-Saharan Africa, describing mortality rates up to 27% (*2*), irrespective of the serotype involved. The MDR rates among NTS and among *S. enterica* ser. Typhi in this study are among the highest reported in sub-Saharan Africa (*10*). The finding of decreased ciprofloxacin susceptibility among isolates in a rural area in DRC highlights the need for surveillance of antimicrobial drug resistance of *S. enterica* isolates.

Table. MICs and genetic analysis of *Salmonella enterica* isolates with decreased ciprofloxacin susceptibility, Democratic Rebublic of the Congo

Serotype	MIC, mg/L		No. isolates	Mutations
	Nalidixic acid	Ciprofloxacin		
Enteritidis	128	0.094	1	Asp82-Asn in gyrA + *qnrB*
Typhimurium	>256	0.125	1	Asp87-Tyr in gyrA + *qnrB*
Typhi	>256	0.19–0.25	7	Ser83-Phe in *gyrA*

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Co-Circulation and Persistence of Genetically Distinct Saffold Viruses, Denmark

To the Editor: Cardioviruses are positive-sense, single-stranded RNA viruses of the family *Picornaviridae*, genus *Cardiovirus*. Until recently, cardioviruses were primarily known for their ability to infect rodents. In 2007, findings of a retrospective study of undiagnosed enteric illnesses in the United States were published, including results from analysis of a fecal sample from an infant girl whose symptoms were diagnosed as fever of unknown origin in 1981. The novel human cardiovirus that was isolated was designated Saffold virus (SAFV) (*1*). Eight genotypes of SAFV have been described (*1*–*4*), and a ninth was recently isolated in Nigeria (O. Blinkova, unpub. data). Serologic studies indicate that infection with SAFV genotypes 2 and 3 generally occurs in early life (*5*), although the clinical significance of these findings remains unclear.

The first SAFV infection in Denmark was recorded in 2009 (*6*). To elucidate the molecular epidemiology of SAFV, we performed a 3-year surveillance study of SAFV in Denmark. During 2009–2011, we tested 1,393 fecal samples from 454 children. Surveillance included collection of fecal samples from children at 6, 10, and 15 months of age; additional fecal samples were collected when the children had gastroenteritis. Most of the SAFV-positive samples reported in this study were obtained from a randomized trial in the pediatric department of University Hospital of Holbaek (Holbaek) on the effect of probiotic therapy on the incidence of infection during early childhood (M. Gyhrs, unpub. data). The study was approved by the local ethics committee; Den Regionale Videnskabsetiske Komité for Region Sjaelland, Denmark.

Nucleic acids were extracted from 200-µL fecal suspension (10% in phosphate-buffered saline) by using the Cobas AmpliPrep Total Nucleic Acid Isolation Kit (Roche Diagnostics, Ltd., Mannheim, Germany) on the MagnaPure LC instrument (Roche Diagnostics). We used 5 µL of extracted nucleic acid for reverse transcription PCR (RT-PCR) (total volume 25 µL) using the OneStep RT-PCR Kit (QIAGEN, Hilden, Germany). The samples were tested for SAFV by using real-time RT-PCR primer/probe, and all positive samples were genotyped by partial sequencing of the viral protein (VP) 1 gene (*6*). Overall, 38 (2.8%) of the clinical samples were positive for SAFV (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0793-Techapp.pdf), all of which fell into genotype 2 (SAFV-2), which is most prevalent in Western nations. Of these samples, 31 had sequence information of sufficient length for additional analyses. All SAFV-2 sequences were submitted to GenBank (accession nos. JX048000–JX048030).

To determine the evolutionary history of strains of SAFV identified in persons in Denmark, we combined the VP1 sequences collected here with all others available on GenBank. We aligned sequences as described using MUSCLE software (*7*), then checked the alignments using manual calculations. We performed phylogenetic analysis using the

maximum likelihood method as described in PhyML 3.0 (*8*), on the basis of the best-fit GTR+Γ nucleotide model as determined by jModelTest (*9*). Phylogenetic robustness was determined by using 1,000 bootstrap replicates.

Our phylogenetic analysis places the strains isolated in Denmark within the SAFV-2 group (online Technical Appendix). These SAFV-2 strains were further subdivided into 2 strongly supported clusters: DK-A, which comprised viruses isolated during 2009–2011, demonstrating probable persistence in Denmark during this period; and DK-B, a smaller group that included viruses from United States, Germany, and the Netherlands, indicating widespread viral gene flow (Figure). The DK-B strains were identified in samples collected during 2009–2010, supporting probable sustained viral persistence within Denmark. Within DK-B, strain 115883 is phylogenetically distinct from the other DK-B viruses.

We next measured the selection pressures acting on these lineages through the mean number of nonsynonymous (d_N) to synonymous (d_S) nucleotide substitutions per site using the single-likelihood ancestor counting, fixed effects likelihood, and random effects likelihood methods available in the Datamonkey HyPhy package as described (*10*). The DK-A and DK-B groups differed significantly in selection pressure: DK-A, d_N/d_S ratio = 0.195 (95% CI 0.105–0.328); and DK-B, d_N/d_S ratio = 0.033 (95% CI 0.015–0.062), which indicates stronger purifying selection on the DK-B group. Ancestral state reconstruction, performed by using Datamonkey (*10*), revealed that the ancestors of DK-A and DK-B differ only at aa 135 in VP1: Val in DK-A and Ala in DK-B. Notably, aa 135 was positively selected in DK-A (random effects likelihood: $d_N/d_S = 3.53$, Bayes factor = 50: fixed effects likelihood: $d_N/d_S >>1$; cutoff p = 0.1), with more tentative evidence for adaptation at aa 135 in DK-B: d_N/d_S ratio >>1 by using fixed effects likelihood (p = 0.2). The functions of aa 135 in VP1, and what it means for the fitness of SAFV, merit further consideration.

We conclude that SAFV-2 has been introduced into Denmark in 3 groups: DK-A, viral strain 115883 and strains of DK-B reported in Denmark; all have recently co-circulated in this country. We have demonstrated the entry and persistence of 3 phylogenetically distinct lineages of SAFV-2 in Denmark. That SAFV-2 can persist between years suggests that it might be common, yet underreported, in Denmark, which provides the opportunity for spread to additional localities. Increased awareness of improved laboratory protocols for SAFV detection is needed among clinicians in Denmark and neighboring countries.

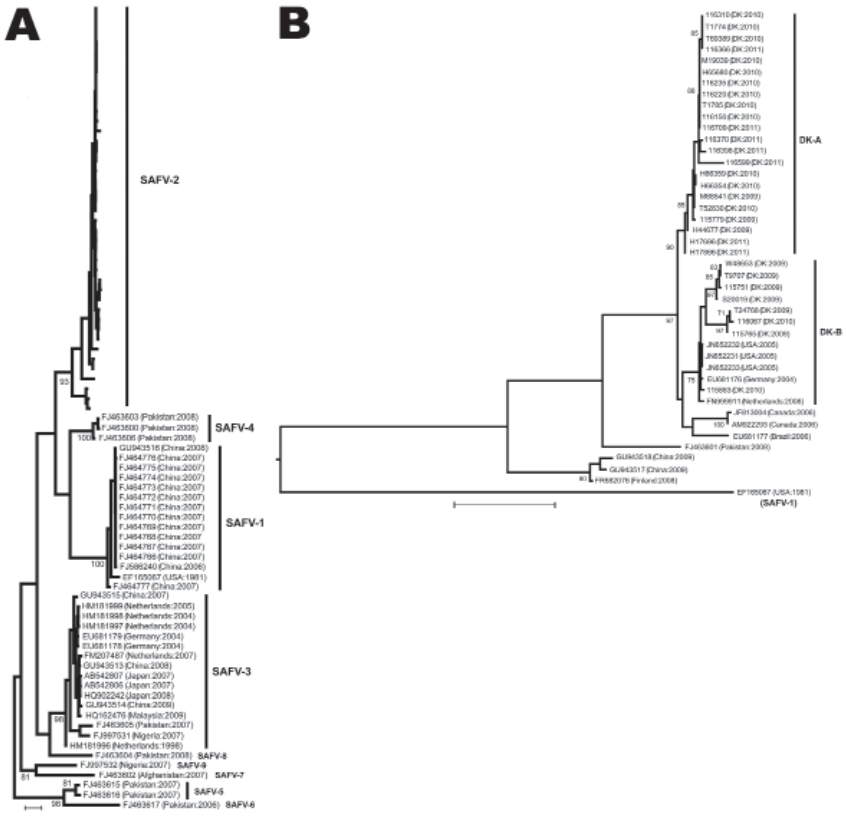

Figure. Phylogenetic analyses of Saffold viruses (SAFVs). Phylogenetic analysis of SAFV-2. Strains from Denmark are named by using the isolation numbers assigned for the study, then the country of origin and year of sampling in parentheses. The 2 subgroups (DK-A and -B) are shown. The tree is rooted by using SAFV-1 outgroup sequence accession no. EF165067. Bootstrap values >70% are shown. DK, Denmark; US, United States. Scale bars represent 0.2 nucleotide substitutions per site.

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Pathogenic *Leptospira* spp. in Bats, Madagascar and Union of the Comoros

To the Editor: Leptospirosis is a zoonosis of global distribution; incidence rates are particularly high in tropical areas (*1*). Leptospirosis is a major public health problem on islands in the southwestern Indian Ocean, particularly La Réunion, Mayotte, and the Seychelles (where incidence rates are among the highest in the world) (*1*). In contrast, no human case has been reported on the nearby islands of Madagascar and Union of the Comoros. However, the recent demonstration of pathogenic *Leptospira* spp. in small mammals introduced to Madagascar suggests possible transmission from free-living animals to humans (*2*).

In addition to the fact that incidence rates vary among humans, clinical bacterial isolates from different islands belong to different serogroups and serovars and show diverse molecular features (*3,4*). This diversity might be correlated with that of the reservoir hosts; the islands in the southwestern Indian Ocean are a hot spot of biodiversity with extraordinary levels of vertebrate endemism. Most studies investigating wild-animal reservoirs of *Leptospira* spp. on the islands in the southwestern Indian Ocean have focused on small mammals that had been introduced to the islands (*2*,*5*), although bats infected with pathogenic *Leptospira* spp. have been identified in other regions (*6*). Whether bats are a reservoir of *Leptospira* spp. on these islands remains unknown. Therefore, we looked for this bacterium in bats from Madagascar and Union of the Comoros and characterized associated genetic diversity.

As part of an ongoing program aimed at identifying viral and bacterial infectious agents in island wild fauna, 129 insectivorous and frugivorous bats were tested for *Leptospira* spp. The bats belonged to 12 species: 9 from Madagascar (*Mormopterus jugularis*, *Otomops madagascariensis*, *Triaenops furculus*, *T. menamena*, *Miniopterus gleni*, *Miniopterus griffithsi*, *Miniopterus mahafaliensis*, *Myotis goudoti*, *Hypsugo anchietae*) and 3 from Union of the Comoros (*Rousettus obliviosus*, *Chaerephon pusillus*, *Miniopterus griveaudi*). Bats were captured in mist nets or harp traps at 8 sites in Madagascar and 6 sites in Union of the Comoros. Organs were collected in the field and immediately stored in liquid nitrogen.

Total nucleic acids were extracted from a pool of kidney, spleen, and lung tissue by using the Biorobot EZ1 and EZI Virus Mini Kit version 2.0 (QIAGEN, Les Ulis, France). Reverse transcription was then performed with GoScript reverse transcriptase (Promega, Charbonnières-les-Bains, France) to obtain cDNA. We screened pathogenic *Leptospira* spp. with a probe-specific real-time PCR (*7*). The 25 positive samples were subsequently subjected to a PCR procedure that amplified fragments from 682 to 1,293 bp of the 16S rRNA gene (depending on the amplification success) by using published primers (*8–10*). Resulting PCR products from 7 samples were sequenced and compared with available sequences in GenBank by using phylogenetic construction with PhyML 3.0.

Of the 12 bat species tested, 11 were positive for *Leptospira* spp. (the only *H. anchietae* bat tested was negative). Among 52 bats from Madagascar, 18 (34.6%) were infected; detection rates were often high, e.g., 8 (80%) of 10 *T. menamena* bats. In contrast, among 77 bats from Union of the Comoros, only 9 (11.7%) were infected. *Leptospira* spp. seem to be ubiquitous in the study areas; infected bats were found at 7 of 8

sites in Madagascar and 3 of 6 sites in the Union of the Comoros. Of the 7 *Leptospira* spp. sequences obtained from bats in this study, 3 were closely related to *L. borgpetersenii*, 1 grouped with *L. interrogans*, and 3 were not associated with any described species (Figure). *L. interrogans* and *L. borgpetersenii* were identified from *R. obliviosus* bats from the same cave in the Union of the Comoros, and the *L. borgpetersenii* sequence was closely related to that identified from the *O. madagascariensis* bats, which are endemic to Madagascar. Potentially pathogenic *Leptospira* spp. were found in bats of a wide variety of species in Madagascar and Union of the Comoros, at most study sites, and at levels notably higher than those reported from similar studies in other regions (*6*). Some of the bats that were *Leptospira* spp.–positive by PCR, particularly the genera *Mormopterus* and *Chaerephon*, often occupy synanthropic day roost sites. For example, we sampled 1 positive colony of *C. pusillus* bats in a school attic (Pomoni, Anjouan, Union of the Comoros), and bat scats were visible on the floor within the classroom.

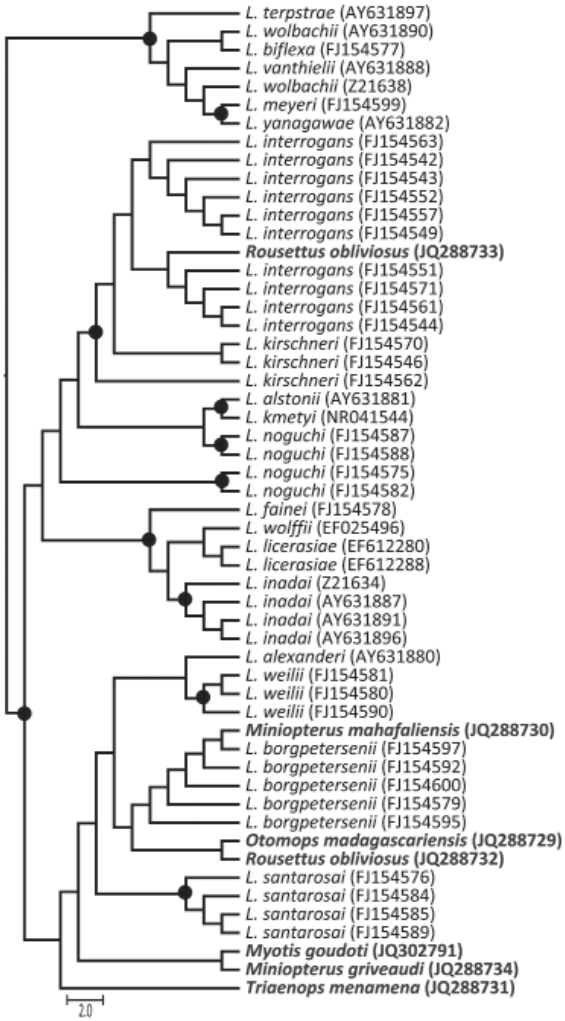

Figure. Maximum-likelihood phylogenetic tree of *Leptospira* spp.16s rDNA in bats from Madagascar and the Union of the Comoros. The dendrogram was constructed with a fragment of 641 bp, with the TIMef+I+G substitution model, and with 1,000 replications. Only bootstrap supports >70% are shown (circles). The precise geographic information of the sampled bats can be accessed through the GenBank accession numbers indicated in parentheses at branch tips. Host bat species for the sequences generated in this study are shown in **boldface**. Scale bar indicates number of nucleotide substitutions per site.

Bats from Madagascar and Union of the Comoros harbor a notable diversity of *Leptospira* spp.; this finding is in accordance with the diversity found in a comparable investigation of bats in the Amazon region (*6*). Although leptospirosis in humans is suspected only on the islands associated with this study (*10*), incidence among humans in Mayotte, part of the Union of the Comoros archipelago, has been shown to be high and mainly associated with *L. borgpetersenii* (*3*). The use of more polymorphic markers combined with the sequencing of clinical isolates should provide better characterization of *Leptospira* spp. diversity and the potential role of bats in human leptospirosis.

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West Nile Virus Meningoencephalitis Imported into Germany

To the Editor: West Nile virus (WNV) is a single-stranded RNA virus in the family *Flaviviridae* that is transmitted to humans by mosquitoes. Approximately 80% of WNV infections in humans are asymptomatic, whereas ≈20% of infected persons experience fever, often accompanied by a rash. Less than 1% of infections are manifested as neuroinvasive disease, such as meningoencephalitis, polyradiculoneuritis, and polio-like flaccid paralysis (*1*). WNV is endemic in Africa, southern Asia, and northern Australia, and only sporadic cases or small epidemics are seen in Europe (*2*). In 1999, WNV emerged in North America. By 2010, ≈1.8 million persons had become infected, with 12,852 reported cases of meningoencephalitis and 1,308 deaths (*2*). In Europe, the last notable outbreak of WNV infection occurred in Greece in 2010; 197 persons were infected, and 33 died (*3*). The Czech Republic, Denmark, France, and the Netherlands reported laboratory-confirmed WNV infections in travelers returning from North America (*1*).

We report a case of WNV meningoencephalitis in a 28-year-old German woman, who sought treatment the emergency department of a hospital in Potsdam, Germany, on September 7, 2011. She had a 3-day history of fever of up to 40°C and mental confusion. Six days before admission, she had returned from a 2-week holiday trip to Ottawa, Ontario, Canada. She had spent most of her time in the city of Ottawa.

The patient's medical history was unremarkable. She was in a reduced general condition because of a severe encephalitic syndrome characterized by somnolence, meningism, fever, and mental confusion. Laboratory investigations revealed leukocytosis with 15.000 leukocytes/μL (reference range 4,400–11,300 leukocytes/μL) and elevated C-reactive protein of 14.8 mg/L (reference <3 mg/L). Cerebrospinal fluid (CSF) analysis on the day of admission showed pleocytosis, 430 cells/μL (72% granulocytes, 27% lymphocytes. and 1% monocytes); elevated levels for total protein, 1,023 mg/L (reference range 150–450 mg/L); an albumin level of 637 mg/L (normal range 0–350 mg/L); and a moderately elevated level of the albumin quotient of 20 (reference range <6.5). The CSF/serum diagrams demonstrated a moderate disturbance of the blood–CSF barrier and a substantial intrathecal IgM synthesis of 27.6%

(6.15 mg/L) but no intrathecal IgA or IgG synthesis. Results of magnetic resonance imaging of the brain were unremarkable. No parenchymal lesions were found.

Antimicrobial drug therapy was initiated with ceftriaxone and ampicillin. Acyclovir was administered empirically for herpes simplex encephalitis until this diagnosis was excluded. Molecular and serologic testing of serum and CSF samples revealed no acute infection with herpesviruses, enteroviruses, alphaviruses, orthobunyaviruses, and arenaviruses or with mycobacteria, *Borrelia* spp., *Toxoplasma gondii*, *Chlamydia* spp., *Leptospira* spp., and *Mycoplasma pneumoniae*. CSF and blood cultures were negative for fungi and bacteria, including mycobacteria. An encephalitic syndrome caused by N-methyl-D-aspartate antibodies was also excluded. On the basis of the patient's travel history, the clinical symptoms, and the initial laboratory findings, WNV infection was suspected. Indirect immunofluorescence assays and virus neutralization tests (VNT) for WNV and other flaviviruses were performed as described (*4*). IgM and IgG against WNV were detected in serum and in CSF by indirect immunofluorescence assay with an 8-fold (IgM) and 32-fold (IgG) increase in serum titer from day 4 to day 26 (Table). WNV IgG and WNV IgM titers were higher than the titers of antibodies against the other flaviviruses tested (Table), indicating that the antibodies resulted from a WNV infection. The serologic diagnosis was further substantiated by detection of WNV neutralizing antibodies at day 11(VNT titer 640). The VNT titer further increased to 2,560 on day 26 after onset of disease. Results of reverse transcription PCR were negative for WNV and members of genus *Flavivirus* in serum and CSF samples taken 4 days after disease onset. Attempts to isolate WNV from serum and CSF samples in cell culture failed as well. The patient recovered slowly and was discharged from the hospital in Potsdam on September 15, 2011. She was then referred to a neurologic rehabilitation center in Berlin and was discharged from there after 2 months with a characterization of *restitutio ad integrum* (i.e., full recovery, restoration to original condition).

We report a case of WNV infection imported into Germany that was unambiguously confirmed by laboratory testing. WNV meningoencephalitis was diagnosed on the basis of strict serologic criteria established by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (*5*). In 2011, 69 clinical cases of WNV infection were reported from the province of Ontario, although no cases in the city of Ottawa were reported (Public Health Agency of Canada; www.eidgis.com/wnvmonitorca). This imported case adds to these cases and suggests that travelers are at risk, even if they visit only Ottawa. Physicians in Germany should be aware of the risk for WNV infection among travelers returning from Canada, especially during late summer.

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Table. Results of indirect immunofluorescence assays performed on serum and CSF samples from patient with suspected WNV infection, Germany, 2011*

		Antibody titer in serum				Antibody titer in CSF	
Virus used as antigen	Ig class	Day 4	Day 6	Day 11	Day 26	Day 4	Day 6
WNV	IgG	320	1,280	5,120	10,240	20	40
WNV	IgM	160	160	1,280	1,280	10	20
SLEV	IgG	80	80	1,280	2,560	<10	<10
SLEV	IgM	20	20	40	<20	<10	<10
JEV	IgG	ND	<20	ND	1,280	ND	<10
JEV	IgM	ND	<20	ND	<20	ND	<10
DENV	IgG	ND	80	ND	640	ND	<10
DENV	IgM	ND	20	ND	<20	ND	<10
YFV	IgG	ND	<20	ND	ND	ND	<10
YFV	IgM	ND.	<20	ND	ND	ND	<10
TBEV	IgG	ND	<20	ND	ND	ND	<10
TBEV	IgM	ND	<20	ND	ND	ND	<10

*CSF, cerebrospinal fluid; WNV, West Nile virus; SLEV, St. Louis encephalitis virus; JEV, Japanese encephalitis virus; ; ND, not done; DENV, dengue virus; YFV, yellow fever virus; TBEV, tick-borne encephalitis virus. Titers <20 for serum and <10 for CSF are considered negative.

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Scarlet Fever Outbreak, Hong Kong, 2011

To the Editor: Scarlet fever is a notifiable disease in Hong Kong, Guangdong Province, and Macau in the People's Republic of China. All 3 areas reported substantial increases in cases during 2011 (Figure, panel A). In Hong Kong, individual data, including age, geographic location, date of notification, and travel history within the incubation period, were collected from all locally notified scarlet fever case-patients. As of December 31, 2011, a total of 1,535 cases (21.7 cases/100,000 population) were reported, which was ≈10× higher than the average number of annual cases reported during the preceding 10 years (*1*). Of those, 730 cases were laboratory confirmed; 46 cases were imported; and 2 cases, 1 each in a 7-year-old girl and a 5-year-old boy co-infected with chickenpox, resulted in death (*2*).

Group A *Streptococcus* (GAS), the bacterium that causes scarlet fever, is mainly transmitted by direct contact with saliva and nasal fluids from infected persons (*3*). Many children can also carry GAS or be asymptomatically infected (*4*). A recent study in China showed that GAS is commonly resistant to macrolides and tetracycline but sensitive to penicillin, chloramphenicol, cefradine, and ofloxacin (*5*). In Hong Kong, GAS *emm* type 12 dominated among the isolates cultured during 2011 (*6*). Most of the cases reported were in children <10 years of age (range 1 month–51 years; median 6 years [interquartile range 4–7 years]). The age distribution is similar to that reported during previous years (data not shown).

In the United Kingdom during the mid-19th century, scarlet fever epidemics were found to follow a 5- to 6-year cycle, but this pattern disappeared as incidence decreased (*7*). Annual scarlet fever notifications in Hong Kong remained low during 2001–2010 (<4 cases/100,000 population) and did not demonstrate any apparent long-term pattern. The recent increase in scarlet fever notifications might be attributable to antigenic drift, increase in virulence of GAS (*8*), or increased circulation of GAS. However, other than mandatory notification of medically attended case-patients, systematic laboratory testing of GAS isolates was not conducted in Hong Kong, and these possibilities could not be further investigated.

Notifications of scarlet fever usually peak during December–March in Hong Kong, but the outbreak in 2011 peaked in June (Figure, panel B). The rise in scarlet fever cases in Guangdong Province and Macau slightly preceded that in Hong Kong; cases in Guangdong peaked in April (Figure, panel A). Maximum cross-correlations between spline-interpolated weekly scarlet fever notifications in Guangdong and Macau and those in Hong Kong were found at 1- and 2-week lags, respectively ($\rho = 0.45$ and 0.58) (online Technical Appendix, wwwnc.cdc.gov/EID/pdfs/12-0062-Techapp.pdf).

In 2011, scarlet fever notification rates were elevated in all 4 regions of Hong Kong: New Territories East, New Territories West, Kowloon, and Hong Kong Island at 27.2, 21.7, 18.9, and 19.6 cases per 100,000 population, respectively. However, a distinctly higher proportion of imported cases before July 2011 (12 of 14, p value for exact binomial test = 0.01) were notified in New Territories East and New Territories West, where the main border crossings to mainland China are located. This finding suggests a link to the outbreak in Guangdong in these regions during the early phase of the local outbreak.

We estimated the instantaneous reproduction number (R_t), which measures the time-dependent frequency of transmission per single primary case (online Technical Appendix) (*9*). An R_t consistently >1 would indicate sustained local transmission. We estimated R_t on the basis of the daily scarlet fever notification data in different periods, adjusted for imported cases. For 19 cases (1.2% of all cases), we could not determine whether infection was local or imported. We estimated R_t in 2 different ways: either by assuming that all of these cases were local or by assuming that they all were imported, to represent possible extreme values of R_t. R_t fluctuated between 0.6 and 2.0 and was consistently >1 from mid-May through the end of June. R_t fell quickly to <1 beginning in early July after 2 fatal scarlet fever cases were reported on May 29 and June 21, which raised widespread concern in the community (Figure, panel C). Heightened surveillance, publicity, health education to the public (online Technical Appendix) were implemented by the Centre for Health Protection in early June and could have contributed to the reduction in transmissibility. The health education measures included guidance on pre-

vention and control measures, such as updates of antimicrobial drug resistance profile of GAS issued to all doctors and strengthening reporting of scarlet fever cases by child care centers and schools for prompt epidemiologic investigations,

In summary, we analyzed the notification data of scarlet fever and investigated spatiotemporal spreading patterns of the disease with certain time lags in Hong Kong, Macau, and Guangdong. The estimated R_t in 2011 indicated the potential for local transmission and persistence. Such a borderless spread indicates a critical need to enhance cross-border communication and timely sharing of epidemic information so that future disease control efforts can be made at multiple geographic levels.

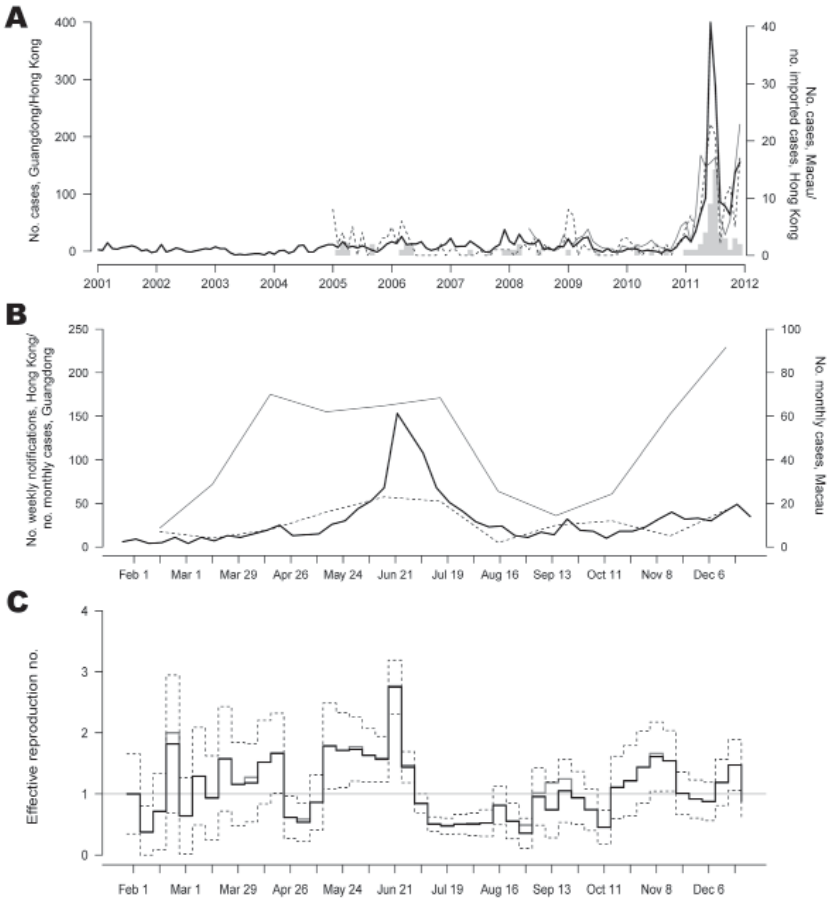

Figure. Trends in scarlet fever during outbreak in Hong Kong, Guangdong, and Macau, People's Republic of China, 2011. A) Monthly scarlet fever notifications in Hong Kong, Guangdong (data obtained from Department of Health Guangdong Province, www.gdwst.gov.cn/a/yiqingxx), and Macau (data obtained from Health Bureau, Government of the Macau Special Administrative Region (www.ssm.gov.mo/news/content/ch/1005/statistic.aspx). Vertical tick marks indicate January of each year. Data from Guangdong and Macau were available beginning in 2005. Black line indicates data from Hong Kong; gray line, data from Guangdong; broken line, data from Macau; gray bar, number of imported cases in Hong Kong, 2005-2011. B) Weekly notifications of scarlet fever cases in Hong Kong and monthly notifications in Guangdong and Macau. Black line indicates data from Hong Kong; gray line, data from Guangdong; broken line, data from Macau. C) Estimated instantaneous reproduction number (R_t) and 95% pointwise confidence intervals (CIs) based on scarlet fever notifications in Hong Kong, February–December, 2011. Black line indicates estimate calculated by grouping patients with unknown importation status with patients with imported cases; gray line, estimate calculated by grouping patients with unknown importation status with local case-patients; broken lines, the upper and lower limits of the 95% CIs for R_t. For better presentation, CIs are shown only for the former estimates. Horizontal line indicates the critical value of R_t, under which transmission of disease will not be sustainable.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Hand, Foot, and Mouth Disease Caused by Coxsackievirus A6

To the Editor: Coxsackievirus A6 (CVA6) is a human enterovirus associated with herpangina in infants. In the winter of 2012, we evaluated a cluster of 8 patients, 4 months–3 years of age, who were brought for treatment at Boston Children's Hospital (Boston, MA, USA) with a variant of hand, foot, and mouth disease (HFMD) that has now been linked to CVA6 (Table, Appendix, wwwnc.cdc.gov/EID/article/18/10/12-0813-T1.htm). During this same period, the Boston Public Health Commission's syndromic surveillance system detected a 3.3-fold increase in emergency department discharge diagnoses of HFMD. In the United States, HFMD typically occurs in the summer and early autumn and is characterized by a febrile enanthem of oral ulcers and macular or vesicular lesions on the palms and soles; the etiologic agents are most often CVA16 and enterovirus 71.

In contrast to the typical manifestation, the patients in the Boston cluster exhibited symptoms in late winter (Table, Appendix) and had perioral (Figure, panel A) and perirectal (Figure, panel B) papules and vesicles on the dorsal aspects of the hands and feet (Figure, panel C). Patients experienced a prodrome lasting 1–3 days, consisting of fever (8 patients), upper respiratory tract symptoms (4 patients), and irritability (7 patients). This prodrome was followed by the development of a perioral papular rash (8 patients), which was often impetiginized with secondary crusting; a prominent papulovesicular rash on the dorsum of the hands and feet (6 patients); and a perirectal eruption (7 patients). Half of the patients had intraoral lesions. Fever abated in most of the patients within a day after onset of the exanthem. The rash resolved over 7–14 days with no residual scarring. Samples from the oropharynx, rectum, and vesicles from these patients were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA) for analysis. Reverse transcription PCR and sequencing by using primers specific for a portion of the viral protein 1 coding region identified CVA6 (*1*) (Table, Appendix).

Outbreaks of HFMD caused by CVA6 have been described in Singapore, Finland, Taiwan, and most recently in Japan; most cases have occurred in the warmer months (*2*–*6*). Cases in the cluster described here are likely related to an emerging outbreak of CVA6-associated HFMD in the United States (*7*). The atypical seasonality of the outbreak, during the winter in Boston, could be related to the unusually mild temperatures in the winter of 2012.

Recent CVA6 outbreaks have been characterized by a febrile illness associated with an oral enanthem and lesions on the palms, soles, and buttocks. CVA6 infections in Taiwan during 2004–2009 were associated with HFMD in 13% of cases, with disease defined as oral ulcers on the tongue or buccal mucosa and vesicular rashes on the palms, soles, knees, or buttocks (*2*). In Singapore, where CVA6 accounted for 24% of HFMD cases, patients had oral lesions and <5 peripheral papules, placing them on a spectrum closer to the herpangina more typically observed in CVA6 infection (*8*).

The patients we report in this cluster most typically had perioral and perirectal papules in addition to vesicles on the dorsum of their hands. Two reports of CVA6-associated HFMD outbreaks describe cases that more closely resemble patients in the Boston outbreak. In a series from Finland in 2008, representative patients had both perioral lesions and vesicles on the dorsum of their hands (*6*). In a large series of patients with

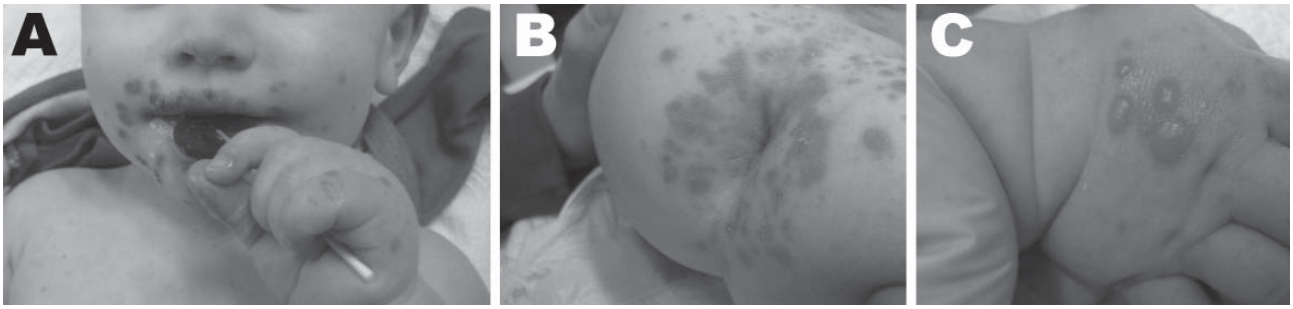

Figure. Manifestations of hand, foot, and mouth disease in patients, Boston, Massachusetts, USA, 2012. Discrete superficial crusted erosions and vesicles symmetrically distributed in the perioral region (A), in the perianal region (B), and on the dorsum of the hands (C). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/18/10/12-0813-F1.htm).

HFMD in Taiwan in 2010, patients with CVA6 had perioral lesions in addition to an enanthem (*3*).

Outbreaks of CVA6-associated HFMD in Finland, Taiwan, and Japan were associated with onychomadesis, with the loss of nails occurring 1–2 months after initial symptoms (*3*,*4*,*6*). The association between more typical HFMD and onychomadesis has additionally been described in the United States and Europe but without a link to specific serotype or with a small percentage of CVA6-associated cases (*9*). Cases from the Boston epidemic may fit into an emerging clinical phenotype of CVA6, and it will be interesting to see whether nail loss develops in those patients.

Given the numerous CVA6 outbreaks in multiple countries in 2008 and a US population that may be relatively naïve to this serotype, CVA6 is likely to spread throughout North America. Clinicians should be aware that, although standard precautions are routinely recommended for managing enteroviral infections in health care settings, contact precautions are indicated for children in diapers to control institutional outbreaks (*10*). In addition, the presence of perioral lesions and peripheral vesicles on the dorsum rather than palmar/plantar surface of the hands and feet represents a unique phenotype of HFMD that could be confused with herpes simplex or varicella-zoster virus infections. Because of the atypical presentation of CVA6-associated HFMD, clinical vigilance is needed to recognize emerging regional outbreaks. More detailed epidemiologic and genetic analyses will be required to characterize the role of CVA6 in US outbreaks of HFMD.

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Duffy Phenotype and *Plasmodium vivax* infections in Humans and Apes, Africa

To the Editor: Benign tertian malaria, caused by *Plasmodium vivax,* has long been considered absent, or at least extremely rare, in western and central Africa. In these regions, 95%–99% of humans are of the Duffy negative phenotype, a condition that is thought to confer complete protection against the parasite during the blood stages of its life cycle (*1*,*2*). Sporadic reports throughout the latter half of the 20th century, however, have hinted at the presence of the parasite in these regions, the most convincing of which were the steady and consistent numbers of non-African travelers who returned to their countries of origin infected with malarial parasites that were subsequently identified as *P. vivax* (*2*).

More recently, evidence has emerged regarding the transmission of *P. vivax* in regions of Africa where the local human population is predominantly Duffy negative (*3*–*6*). In 4 (3.5%) of 155 patients from western Kenya (*6*), 7 (0.8%) of 898 persons from Angola (*4*), and 8 (8.2%) of 97 persons from Equatorial Guinea (*4*), *P. vivax* parasites were detected in the blood of apparently Duffy-negative persons, suggesting that the parasite might not be as absolutely dependent on the Duffy receptor for erythrocyte invasion as previously thought. These findings are supported by a report from Madagascar (where the human population is composed of a mixture of Duffy-positive and Duffy-negative persons), in which 42 (8.8%) of 476 Duffy-negative persons who had symptoms of malaria were reported to be positive for *P. vivax* by both microscopy and PCR (*7*). The prevalence of *P. vivax* in Duffy-negative persons was significantly lower than its prevalence in Duffy-positive persons residing in the same area, suggesting that Duffy negativity is a barrier to the parasite to some degree. Given the extremely high rates of malaria transmission in western and central Africa, a *P. vivax* parasite that could efficiently invade Duffy-negative erythrocytes would, presumably, become highly prevalent very rapidly.

With the exception of the cases reported from Angola and Kenya, which lie outside the area where the proportion of the population with Duffy negativity is highest, the reports of the transmission of *P. vivax* within predominantly Duffy-negative populations all come from regions inhabited by chimpanzees and gorillas (i.e., Democratic Republic of the Congo [*3*], Uganda [*4*], and Equatorial Guinea [*5*]). During our seroepidemiologic study from the Democratic Republic of the Congo, in which *P. vivax* sporozoite–specific antibodies were detected in ≈10% of the population, we found that women were significantly more likely than men to have been exposed to *P. vivax* sporozoites (*3*). Women in this region typically spend more time than men near the forest fringe, where they work in crop fields. This forest is within the known habitat range of the chimpanzee *Pan troglodytes* and the gorilla, *Gorilla gorilla gorilla*, both of which have been reported to be natural hosts of the malaria parasite *P. schwetzi*, which is a *P. vivax*–like or *P. ovale*–like parasite that might also be unable to invade the erythrocytes of persons who are Duffy negative (*8*). These animals have recently been shown to be infected occasionally with parasites that have mitochondrial genomes closely resembling those of *P. vivax* (*9*,*10*).

We have argued that, given the high malaria transmission rates in sub-Saharan Africa, it is plausible that the 1%–5% of the human population who are Duffy positive might maintain the transmission of the parasite (*2*). The discovery of *P. vivax* parasites (or *P. vivax*–like parasites) in the blood of African great apes leads to a question: could nonhuman primates in Africa be acting as Duffy-positive reservoirs of *P. vivax* in regions where the human population is almost entirely insusceptible? This possibility warrants further investigation. Given the increasing rarity of the great apes, however, their capacity to act as zoonotic reservoirs could be limited. It would be informative, in any case, to determine how the regions that *P. vivax*–positive travelers visit during their stay in Africa correspond with the ranges of chimpanzees and gorillas.

If African great apes do, indeed, constitute a zoonotic reservoir of *P. vivax* parasites, what are the

repercussions for human health? Given that 95%–99% of humans possibly exposed to such a reservoir are Duffy negative, and therefore resistant to the parasite, these would appear to be slight. However, as humans encroach more frequently into ape habitats, the chances of humans encountering the parasite will increase. In the short term, the risks are probably limited to Duffy-positive persons who enter areas where apes are present, such as tourists and migrant workers.

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Rickettsia parkeri and *Candidatus* Rickettsia andeanae in Gulf Coast Ticks, Mississippi, USA

To the Editor: *Rickettsia parkeri*, a spotted fever group *Rickettsia* (SFGR) bacterium, is transmitted by *Amblyomma maculatum*, the Gulf Coast tick (*1*). The prevalence of *R. parkeri* in Gulf Coast ticks has been reported as <42% in the United States, which is higher than reported rates of *R. rickettsii* (the cause of Rocky Mountain spotted fever) in *Dermacentor* species ticks. Misdiagnosis among SFGR infections is not uncommon, and *R. parkeri* rickettsiosis can cause symptoms similar to those for mild Rocky Mountain spotted fever (*1*). We evaluated infection rates of *R. parkeri* and *Candidatus* Rickettsia andeanae, a recently identified but incompletely characterized SFGR, in Gulf Coast ticks in Mississippi, USA.

During May–September of 2008–2010, we collected adult Gulf Coast ticks from vegetation at 10 sites in Mississippi. We extracted genomic DNA from the ticks using the illustra tissue and cells genomicPrep Mini Spin Kit (GE Healthcare Life Sciences, Piscataway, NJ, USA). We tested amplifiable tick DNA by PCR of the tick mitochondrial 16S rRNA gene (*2*). We tested for molecular evidence of any SFGR species by nested PCR of *rompA* (rickettsial outer membrane protein A gene) (*1*). Samples positive for SFGR were subsequently tested by using species-specific *rompA* PCR for *R. parkeri* (*3*) and *Candidatus* R. andeanae (*4*). All PCRs included 1) a positive control of DNA from cultured *R. parkeri*– (Tate's Hell strain) or *Candidatus* R. andeanae–infected Gulf Coast ticks and 2) a negative control of water (nontemplate). PCR products were purified by using Montage PCR Centrifugal Filter Devices (Millipore, Bedford, MA, USA) and sequenced by using Eurofins MWG Operon (Huntsville, AL, USA). We generated consensus sequences using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/) alignment and identified the sequences using GenBank BLAST searches (www.ebi.ac.uk/Tools/clustalw2/).

Proportions of ticks infected with SFGR, by region and year, were compared separately by using Fisher exact test followed by pairwise comparisons with a Bonferroni

Table. PCR results for adult *Rickettsia parkeri*– and *Candidatus* Rickettsia andeanae–infected Gulf Coast ticks (*Amblyomma maculatum*) collected from 10 sites in Mississippi, USA, 2008–2010*

Location (no. collection sites)	No. ticks	No. (%; 95% CI) SFG *rompA*	No. (%; 95% CI) *R. parkeri* only	No. (%; 95% CI) *Candidatus* R. andeanae only	Expected no. (%) co-infected ticks	No. (%; 95% CI) co-infected ticks
North (4)	257	49 (19.1; 14.5–24.4)†	48 (18.7; 14.1–24)‡	0§	0.19 (0.07)	1 (0.4; 0–2.1)¶
Central (1)	38	4 (10.5; NA)	1 (2.6; NA)	2 (5.3; NA)	0.16 (0.42)	1 (2.6; NA)
South (5)	403	75 (18.6; 14.9–22.8)†	57 (14.1; 10.9–17.9)‡	8 (2.0; 0.9–3.9)§	2.99 (0.74)	10 (2.5; 1.2–4.5)¶
Total (10)	698	128 (18.3; NA)	106 (15.2; NA)	10 (1.4; NA)	3.65 (0.52)	12 (1.7; NA)

*The estimated value of co-infection caused by chance alone (E) was calculated by using the formula E = (a + b)(a + c) / (a + b + c + d) (*5*), where a = no. ticks infected with both *Rickettsia* species, b = no. ticks infected only with *R. parkeri*, c = no. ticks infected only with *Candidatus* R. andeanae, and d = no. ticks not infected with either *Rickettsia* species. SFG *rompA*, spotted fever group rickettsial outer membrane protein A gene. NA, not applicable.
†p = 0.9187.
‡p = 0.1275.
§p = 0.0257.
¶p = 0.0578 (comparison of prevalence from northern and southern sites only).

adjustment (PROC FREQ, SAS for Windows, V9.2; SAS Institute, Cary, NC, USA). For all analyses, $p<0.05$ was considered significant. An index of co-infection was calculated by using the formula IC = ([O – E]/N) × 100, in which IC is index of co-infection, O is number of co-infections, E is expected occurrence of co-infection caused by chance alone, and N is total number of ticks infected by either or both *Rickettsia* species. A χ^2 test was used to determine statistical significance (*5*).

A total of 707 adult Gulf Coast ticks were collected during the 3 years (350 in 2008, 194 in 2009, and 163 in 2010). Tick mitochondrial 16S rRNA gene was detected in 698 (98.7%), of which 128 (18.3%) were positive for SFGR DNA, comprising 106 (15.2%) positive only for *R. parkeri*, 10 (1.4%) positive only for *Candidatus* R. andeanae, and 12 (1.7%) co-infected with *R. parkeri* and *Candidatus* R. andeanae (Table). Positive test results from 22 ticks singly or co-infected with *Candidatus* R. andeanae were confirmed by sequencing.

Most (94.6%) ticks were from northern (n = 260) and southern (n = 409) Mississippi (online Technical Appendix, Figure, wwwnc.cdc.gov/EID/article/18/10/12-0250-F1.htm). No significant difference in the number of *R. parkeri*–infected ticks between northern and southern Mississippi was observed (p = 0.13) (Table). However, significantly more ticks were singly infected with *Candidatus* R. andeanae in southern sites than in northern sites (p = 0.03). The infection rate for co-infected ticks in southern sites was higher than that in northern sites (p = 0.06). Among the 3 collection years for northern and southern sites, only the prevalence of *R. parkeri* in singly infected ticks differed significantly (p = 0.01) (data not shown); the infection rate was significantly greater during 2010 than during 2009 (p = 0.003, $\alpha/3 = 0.02$). The overall index of co-infection with *R. parkeri* and *Candidatus* R. andeanae was 6.5, statistically higher than expected by chance alone (Table) ($p<0.0001$).

The overall prevalence of infection with SFGR species in Gulf Coast ticks sampled was 18.3%; 15.2% of ticks were singly infected with *R. parkeri*, and 1.7% were infected with *R. parkeri* and *Candidatus* R. andeanae. As reported, the frequency of *R. parkeri* in Gulf Coast ticks is generally high, ranging from ≈10% to 40% (*3*,*4*,*6*–*8*). We found approximately 1 *R. parkeri*-infected Gulf Coast tick for every 6 ticks tested, suggesting that infected Gulf Coast ticks are commonly encountered in Mississippi. Because Gulf Coast ticks are among the most common human-biting ticks in Mississippi (*9*), awareness of *R. parkeri* rickettsiosis should be increased in this state. We identified *Candidatus* R. andeanae in ≈3% of Gulf Coast ticks in Mississippi; this frequency is similar to those reported in other studies of Gulf Coast ticks in the southern United States (*4*,*6*). Our finding of co-infected Gulf Coast ticks is at a frequency significantly higher than expected from chance alone. The biologic role of co-infections of Gulf Coast ticks with *R. parkeri* and *Candidatus* R. andeanae remains to be determined.

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Attributing Cause of Death for Patients with *Clostridium difficile* Infection

To the Editor: Hota et al. report that for deceased patients who had *Clostridium difficile* infection (CDI), agreement is poor between causes of death reported on death certificates and those categorized by a review panel (*1*). Our data support the difficulty of attributing cause of death for patients with CDI.

In 2004 in Quebec, Canada, a mandatory CDI surveillance program was implemented. Deaths that occurred within 30 days after CDI diagnosis were classified as 1) directly attributable to CDI (e.g., toxic megacolon, septic shock), 2) having a CDI contribution (e.g., acute decompensation of chronic heart failure), or 3) unrelated to CDI (e.g., terminal cancer) (*2*). To determine accuracy of the surveillance classifications, we compared cause-of-death classification of 22 deceased CDI patients reported to surveillance by 1 hospital in 2007 with causes of death reported by 13 external reviewers who examined summaries of medical files of the deceased patients. Reviewers were 11 infectious disease and 2 public health physicians involved with CDI surveillance at their respective hospitals but not this hospital. The median (minimal, maximal) κ statistics for comparison of external reviews with surveillance classification were 0.495 (0.252, 0.607) for directly attributable, 0.182 (–0.091, 0.182) for contributed, and 0.321 (0.124, 0.614) for unrelated. Comparison within external reviewers yielded 0.697 (0.394, 1.0), 0.233 (–0.294, 0.703), and 0.542 (0.154, 0.909), respectively. Complete agreement was found for only 6 cases (4 directly attributable and 2 unrelated) (Figure).

Variation among reviewers suggested that categorizations reported to surveillance were inaccurate. Number of deaths among patients with CDI, regardless of the cause of death, seemed to better indicate CDI severity. Since 2008, only the crude numbers of deaths, not subjected to individual interpretation, have been reported to surveillance. A questionnaire addressing concurrent medical conditions, prognosis, level of care, and circumstances of death is being implemented in Quebec hospitals participating in CDI surveillance and should help determine the role of CDI in deaths.

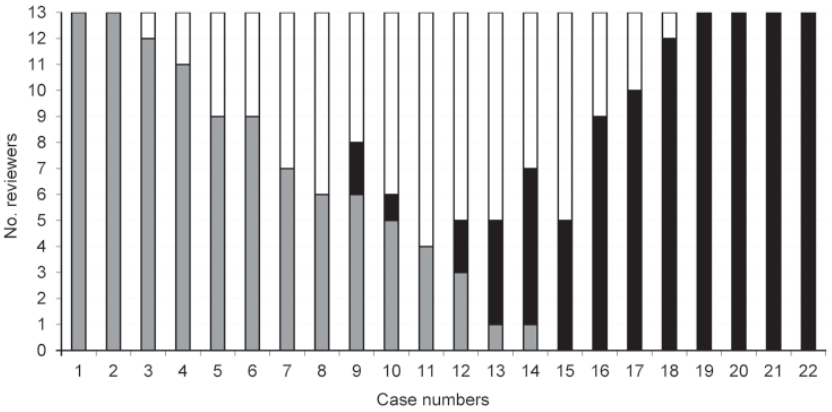

Figure. Classification of cause of death among 22 patients with *Clostridium difficile* infection (CDI), by 13 external reviewers, Quebec, Canada, 2007. Bars indicate the number of reviewers who assigned each category. Gray bars indicate that CDI was unrelated to death, white bars indicate that CDI contributed to death, and black bars indicate that death was directly attributable to CDI.

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Characterization of *Mycobacterium orygis*

To the Editor: In a recently published study, van Ingen et al. (*1*) described the molecular characterization and phylogenetic position of the oryx bacillus, a member of the *Mycobacterium tuberculosis* complex, and proposed a long overdue name for the organism: *Mycobacterium orygis*. The authors described oryx bacillus as a separate taxon; the aim was for this description to be used in the future to identify the subspecies. Thus, we thought it pertinent to provide additional information that would be useful in speciating isolates of the oryx bacillus.

In a recent study, we genotyped an isolate of oryx bacillus obtained from an African buffalo in South Africa (*2*). This isolate was typed by using 16S rDNA, *M. tuberculosis* complex–specific multiplex-PCR, regions-of-difference analyses, *gyrase B* gene single nucleotide polymorphism (SNP) analysis, spoligotyping, and mycobacterial interspersed repetitive units–variable number tandem repeat typing. We showed that, in addition to the markers described by van Ingen et al. (*1*), regions of difference 701 and 702 were also intact in *M. orygis*.

In addition, van Ingen et al. identified the *Rv2042*^{38} GGC mutation as a novel, useful genetic marker to identify *M. orygis*. However, such a marker already exists in the form of the very specific *gyrB*^{oryx} G to A SNP at position 1113, which was described by Huard et al. (*3*). On its own, SNP detection in the *gyrB* gene allows differentiation of at least 6 of the 9 *M. tuberculosis* complex species from each other (*M. canettii*, *M. tuberculosis*, *M. orygis*, *M. microti*, *M. caprae*, and *M. bovis*) (*3*). Thus, the SNP at position 1113 is more useful than the Rv2042^{38} mutation as a novel and distinct genetic marker to identify *M. orygis*.

Apart from this, we found that the sequence type (ST) 587 was not the only spoligotype specific for *M. orygis*. In our study, the variant type ST701 (annotated as *M. africanum* in the spolDB4 database) (*4*) is also an *M. orygis*–specific type and exactly matches that of a previous isolate of the oryx bacillus (SB0319) from the *M. bovis* spoligotype database (*5*). This spoligotype differs from ST587 by the presence of spacer 18, and the spoligotype was not found in the extensive sample set of van Ingen et al. (*1*).

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Epsilonproteobacteria in Humans, New Zealand

To the Editor: Cornelius et al. (*1*) addressed the potential of *Campylobacter ureolyticus* as an emerging pathogen by conducting a molecular study on 128 diarrheal specimens and 49 fecal samples from healthy volunteers**.** Reporting the identification of *C. ureolyticus* in 12 (24.5%) of 49 healthy volunteers, a number that they compared with our finding of 349 (23.8%) from *Campylobacter* spp.–positive samples (*2*), the authors concluded that *C. ureolyticus* species "are unlikely causes of diarrhea," an assertion with which we take issue.

This interpretation does not take into account that our screening involved 7,194 symptomatic patients: a sample size 40× greater than that of Cornelius et al. In this context, the likely carriage rate for *C. ureolyticus* is 1.15%. Also, our assay, which has a limit of detection in the picomolar range, is likely comparable with, if not greater than, that of Cornelius et al. (*1*).

Accounting for variations in geographic location and detection methods, a detection rate of 24.5% in healthy volunteers (overall detection rate 14.7%) is high in contrast to our reported rate of 1.15%. One possible explanation for this discrepancy is that Cornelius et al. "did not specifically exclude volunteers who had had gastrointestinal disturbances in the 10 days before sampling," Campylobacter can be shed in feces for <4 weeks after infection. Also, Cornelius et al. (*1*) noted the possibility of "genetically distinct but phenotypically indistinguishable genomospecies differing in their pathogenic potential" to account for the presence of the emerging pathogen *C. concisus* in healthy volunteers and patients with diarrheal illness. This may also apply for *C. ureolyticus*.

We reported a strong seasonal prevalence of *C. ureolytcius* and a bimodal age distribution (*2*). The lack of any related details from Cornelius et al. may undermine their reported detection rates. These factors strongly suggest that the statement, "these species are unlikely causes of diarrhea," should, at the very least, be taken under advisement.

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In Response: In response to the letter by Bullman et al. (*1*), a major aspect of our study (*2*) was to compare epsilonproteobacterial populations in healthy persons and those who have diarrhea. We have not examined as many diarrheal samples as Bullman et al. (*3*). However, in contrast with their study, we have examined samples from persons with no evident disease manifestations. Because the presence of an agent during disease is not proof of causation, we believed that a baseline for comparison was needed. *Campylobacter ureolyticus* was found in a greater proportion of samples from healthy persons (24%) than samples from persons who had diarrhea (11%) ($p = 0.041$, by χ^2 test).

Samples from healthy persons were tested on 2 occasions: 18 samples in September 2007 (New Zealand spring) at the Institute of Environmental Science and Research, Christchurch, in the workplace, and 31 samples in June 2009 (New Zealand winter), at Christchurch Hospital under the guidance of a clinician. We have no reason to believe any of the workplace samples were provided when volunteers had diarrhea, particularly considering our workplace guidelines and staff characteristics. In each testing round, 6 fecal samples had positive test results for *C. ureolyticus*. These periods equate to the peak and trough periods described by Bullman et al. (*3*). We were unable to provide many details regarding sampling in our paper because of space constraints.

Considering our baseline comparisons of healthy persons with those who had diarrhea, we affirm our con-

clusions are reasonable and that *C. ureolyticus* is an unlikely cause of acute diarrheal disease. Similarly, *C. ureolyticus* (previously classified as *Bacteroides ureolyticus*) has been detected in patients with Crohn's disease and in controls (*4*). However, different subtypes or undescribed subspecies may be pathogenic: some strains exhibit certain pathogenic characteristics in vitro (*5*) and others yield amplified fragment length polymorphism profiles that are visually quite distinct from others (*6*). Host factors also cannot be discounted.

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Mori Sosen (1747–1821) *Monkey Performing the Sanbasō Dance* (Dated 1800, the first day of the Monkey Year) Scroll painting, ink on paper (49.5 cm × 115.6 cm) Pacific Asia Museum Collection, Pasadena, California, USA, Gift of Mr. and Mrs. Bruce Ross www.pacificasiamuseum.org

Human minus Three Pieces of Hair

Polyxeni Potter

At age 61, Mori Sosen changed the first character of his name to one meaning "monkey." So close had he become to the subject of his paintings. To learn how to paint the animals convincingly, he lived for a time in the mountains, their natural environment, not relying as others before him on copying their images from Chinese art. His mastery in depicting the Japanese macaque earned him the title "undisputed master" from Dutch orientalist Robert van Gulik (1910–1967). In his book, Gibbon in China, van Gulik translated Confucian scholar Kimura Kenkadō's account of the animal's arrival in Japan. "In the winter of the sixth year of the era (1809), a gibbon was shown in Osaka …. Although we have heard the word 'gibbon' since olden times and seen pictures of him, we never had seen a live specimen, and therefore a large crowd assembled to see this gibbon. Generally he resembled a large macaque, and figure and fur are very similar." Mori Sosen created a graphic record of this sensational event.

Not much is known about his life but that he grew up with and around artists, started his training with his father Mori Jokansai, and lived most of his life in Osaka. Mori Sosen's legacy is his painting of animals, particularly monkeys, their personalities and attitudes as well as their coats and the movement of their muscles underneath. So well did he depict the nature of monkeys that he was accused of being their descendent. He established, with his brother Shūhō, a school of animal painting along the lines of the *Maruyama-Shijō* school in Kyoto. Shūhō's son studied there under Maruyama Ōkyo, an expert in a style influenced by Western realism and direct observation. The *Shijō* school promoted synthesizing this modern development with the local trend toward the decorative and stylized.

Monkey Performing the Sanbasō Dance, on this month's cover, showcases both Mori Sosen's favorite subject matter and his artistic style, a blend of realism and expressiveness. The action is set against a vacant background, the viewer drawn toward the main figure. Bold deliberate strokes outline the facial features, right hand and both feet of the animal, and folds in the kimono. Smudged strokes from a dry brush draw the ruffled texture of the fur against the black cap and smooth, mostly unpainted surface of the clothing. The monkey, mouth pursed with concentration, eyes fixed on some point outside the painting, holds a fan in one hand, and with the other, it raises a cluster of bells. The right leg is lifted in a dance step, while the left, toes curled inward for better balance, is rigid. During the Edo period (1603–1868), Kabuki theater programs began at dawn with a dance. In the final of three scenes in this dance, "the bell-tree," the dancer would shake a wand covered with small bells. Along these lines, the monkey in Mori Sosen's work

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lifts the bells in performing the Sanbasō, a dance celebrating the New Year, the first day of the Monkey Year.

Macaques, more than 20 species of *Macaca*, a genus of Old World monkeys mostly found in Asia, occupy a geographic range second only to that of humans in its extent. Their habitat varies from near desert to rainforest, from sea level to snow-covered mountain tops. The Japanese macaque, also known as snow monkey, which has been reported at an elevation of 3,180 m, representing the northernmost nonhuman primate population in the world, has gained some notoriety for visiting a hot spring in Nagano to find comfort from the cold in winter.

Culturally, this monkey has been a metaphor, a polysemic symbol throughout Japanese history—now a mediator between gods and humans, now a scapegoat, now a clown. Because of its unique role as similar to yet different from humans, the Japanese macaque was used to define what it means to be human and alternatively what it means to be a monkey: "human minus three pieces of hair," to the Japanese. This definition satisfied both the affinity between humans and monkeys and the animal's local status just below grade.

The monkey's role as mediator between gods and humans was long lived and well established. It implied possession of supernatural powers, which were often expressed in ritual dances with music. The monkey was believed a guardian that could cure disease in horses and secure good crops as mediator between the Mountain Deity and humans. This status diminished gradually. The monkey was secularized and demoted, becoming the object of ridicule, a scapegoat, for lacking (even if only by 3 pieces of hair) the essence of humanness. Though a monkey dance performance still likely showcases human superiority, the powerful metaphorical presence persists, despite the animal's virtual disappearance from everyday human contact outside the zoo.

Around the world, the status of macaques and their connection with humans continues to evolve. The Japanese tradition that the monkey was a scapegoat for a human victim of smallpox or of another disease, which persisted for centuries, is no longer held. In more recent times the animals have served instead as models for human disease, providing through their own infections or experimental studies, insight into pathogenic mechanisms, treatments, and vaccine approaches for human infectious agents, among them, hepatitis B, influenza virus, flaviviruses, *Plasmodium* spp. Some infections (HIV-2, *P. knowlesii*) have been transmitted from nonhuman primates to humans, suggesting that the role of these primates as "mediators" persists, but some, including measles and tuberculosis, can go both ways, with infected humans compromising the health of nonhuman primates and because of the infections in the monkeys, an employee health vaccination program was launched, potentially preventing tetanus among workers.

In this issue of the journal, a colony of Japanese macaques saw a mass die-off attributed to severe soil contamination by *Clostridium tetani* in the facility maintaining the animals. In China, *Cryptosporidium* spp., *Giardia duodenalis*, and *Enterocytozoon bieneusi* organisms were detected in free range rhesus monkeys in a popular public park. Most genotypes and subtypes detected were anthroponotic, which indicates that these animals, after becoming infected from exposure to infected humans, may have become reservoirs for human cryptosporidiosis, giardiasis, and microsporidiosis. In Afghanistan, bites from macaques may have exposed US troops and presumably the Afghans to serious infections, among them rabies, B virus, and tetanus. In Africa, nonhuman primates may be acting as a zoonotic reservoir of *P. vivax* in regions where the human population is almost entirely refractory. If so, with human encroachment into nonhuman primate habitats, the chances of susceptible humans encountering the parasite will increase.

As it lifts up the bells to ring in the New Year 1800, Mori Sosen's beloved monkey in the flawless kimono continues the age-old dance celebrating our phylogenetic closeness. Because of this closeness, humans and nonhuman primates are susceptible to many of the same infections, minus three pieces of hair or not.

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EMERGING INFECTIOUS DISEASES

Upcoming Issue

Investigation of Outbreaks Complicated by Universal Exposure

Seroprevalence of Chikungunya, Dengue, and Rift Valley Fever Viruses after Febrile Illness Outbreak, Madagascar

Sources of Dengue Importation to Areas at Risk for Epidemic Spread, Australia

Risk for Travel-associated Legionnaires' Disease, Europe, 2009

Phylogeography of Dengue Virus Serotype 4, Brazil, 2010–2011

Invasive Pneumococcal Disease and 7-Valent Pneumococcal Conjugate Vaccine, the Netherlands

Infectious Disease Mortality Rates, Thailand, 1958–2009

Unchanged Severity of Influenza A(H1N1)pdm09 Infection in Children during First Postpandemic Season

Antigenic Diversity of Enteroviruses and Nonpolio Acute Flaccid Paralysis, India, 2007–2009

Litchi Harvest–associated Acute Encephalitis in Children, Northern Vietnam, 2004–2009

Lack of Evidence for Zoonotic Transmission of Schmallenberg Virus

Nasopharyngeal Bacterial Interactions in Children

Mycoplasmosis in Ferrets

Coccidioidomycosis-associated Deaths, United States, 1990–2008

Lack of Cross-protection against *Bordetella holmesii* after Pertussis Vaccination

Epidemic Myalgia in Adults and Human Parechovirus Type 3 Infection, Yamagata, Japan, 2008

HIV Infection and Geographically Bound Transmission of Drug-Resistant Tuberculosis

Complete list of articles in the November issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

October 22–26, 2012
4th ASM Conference on Beneficial Microbes
San Antonio, TX, USA
http://conferences.asm.org

October 23–24, 2012
"Emerging Viruses: Disease Models and Strategies for Vaccine Development"
A symposium in honor of CJ Peters, MD
Galveston, TX, USA
http://www.utmb.edu/wrce

October 24–26, 2012
European Scientific Conference on Applied Infectious Disease Epidemiology (ESCAIDE)
Edinburgh, Scotland, UK
http://www.escaide.eu

October 27–31, 2012
APHA 140th Annual Meeting & Expo
San Francisco, CA, USA
http://www.apha.org/meetings/AnnualMeeting

November 11–15, 2012
ASTMH 61st Annual Meeting
Atlanta Marriot Marquis
Atlanta, GA, USA
http://www.astmh.org

December 1–7, 2012
ASLM's First International Conference
Cape Town, South Africa
http://www.aslm.org

2013

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

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Article Title:

Methicillin-Resistant *Staphylococcus aureus* Sequence Type 239-III, Ohio, USA, 2007–2009

CME Questions

1. You are seeing a 65-year-old woman admitted with newly-diagnosed methicillin-resistant *Staphylococcus aureus* (MRSA) bacteremia. Which of the following strains of MRSA are most common in the United States?

A. USA 100 and MRSA ST239-III
B. USA 300 and MRSA ST239-III
C. USA 100 and USA 300
D. USA 600 and MRSA ST239-III

2. As you evaluate this patient, what should you consider regarding clinical characteristics of MRSA ST239-III in the current study?

A. Most cases of MRSA ST239-III were diagnosed in community hospitals
B. Ninety-five percent of cases of MRSA ST239-III were healthcare associated
C. There were no cases of MRSA ST239-III associated with implanted medical devices
D. Nearly all cases of MRSA ST239-III were bloodstream infections

3. Which of the following statements regarding the treatment and prognosis of MRSA ST239-III in the current study is most accurate?

A. There was broad susceptibility to nearly all antimicrobials tested
B. Rates of resistance to vancomycin and linezolid were approximately 90%
C. Rates of treatment failure of MRSA ST239-III were significantly higher compared with those associated with USA 100 and USA 300
D. Over 20% of cases of infection with MRSA ST239-III were fatal

4. Which of the following statements regarding molecular typing of MRSA ST239-III infections in the current study is most accurate?

A. There was a single repetitive element PCR (rep-PCR) pattern
B. There were 9 different pulsed-field gel electrophoresis (PFGE) patterns
C. Traditional PFGE testing could identify all of the bacteria subtypes
D. The cluster groupings from PFGE, rep-PCR, and dru methods were essentially identical

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

Earning CME Credit

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Article Title:

Epidemiology of Foodborne Norovirus Outbreaks, United States, 2001–2008

CME Questions

1. You are an infectious disease expert consulting to a US public health office regarding prevention and reducing the impact of norovirus outbreaks. Based on the study by Dr. Hall and colleagues, which of the following statements about general characteristics and outcomes of US foodborne norovirus outbreaks during 2001–2008 is most likely to appear in your report?

A. On average, about 1 norovirus outbreak occurred every week
B. The increasing trend that began in the 1990s in the number of reported foodborne norovirus outbreaks continued through 2008
C. Norovirus outbreaks have been linked to an estimated average number of 10,324 illnesses, 1,247 health care provider visits, and 156 hospitalizations each year
D. No deaths have been attributed to norovirus

2. Based on the study by Dr. Hall and colleagues, which of the following statements about sources of US norovirus outbreaks is most likely correct?

A. Ground meat was often implicated
B. The most common single source was shellfish
C. Most foods were likely contaminated during production and processing
D. Contact with food handlers during preparation was cited in 82% of outbreaks as a possible contributor to contamination

3. Based on the study by Dr. Hall and colleagues, which of the following statements about recommended interventions to reduce the frequency and impacts of foodborne norovirus outbreaks would most likely be correct?

A. Food shipping plants are the best target for intervention
B. Food handlers preparing ready-to-eat foods should adhere to handwashing and gloving recommendations and to ill worker exclusion policies
C. A certified kitchen manager is unnecessary in most delis and restaurants
D. Analytic methods to detect norovirus in foods are well established

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES®

September 2012

Maternal and Child Health

LE TRESOR (Coree) Paul Jacoulet

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

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Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit figures in black and white. If you wish to have color figures online, submit both in black and white and in color with corresponding legends. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .jpeg or .tif 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.

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Types of Articles

Perspectives. Articles should not exceed 3,500 words and 40 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 and 40 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. This section comprises 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.

Research. Articles should not exceed 3,500 words and 40 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 40 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.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references but no abstract, figures, or tables. Include biographical sketch.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

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.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

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.

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.