EMERGING INFECTIOUS DISEASES®

August 2014

Vector-borne Diseases

James Abbott McNeill Whistler (1834–1903) Man at Table beneath Mosquito Net (from Sketchbook), 1854–1855
Black ink on manilla-colored wove paper, 4 5/16 x 3 7/16 in. (11 x 8.7 cm). Metropolitan Museum of Art, New York, NY

EMERGING INFECTIOUS DISEASES®

Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30333, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

∞ Emerging Infectious Diseases is printed on acid-free paper that meets the requirements of ANSI/NISO 239.48-1992 (Permanence of Paper)

EMERGING INFECTIOUS DISEASES

August 2014

On the Cover

James Abbott McNeill Whistler (1834–1903)

Man at Table beneath Mosquito Net (from Sketchbook), 1854–1855

Black ink on manilla-colored wove paper, 4 5/16 x 3 7/16 in. (11 x 8.7 cm)

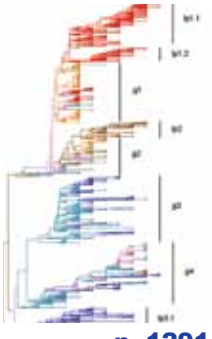
Metropolitan Museum of Art, New York, NY, USA

About the Cover p. 1429

Synopsis

Medscape EDUCATION ACTIVITY

Research

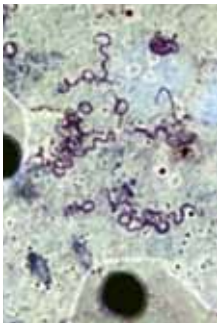


p. 1291

p. 1329

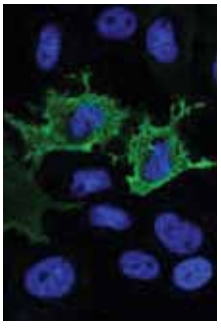
Dispatches

EMERGING INFECTIOUS DISEASES
August 2014



p. 1337

p. 1341

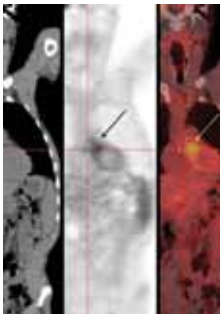


Commentary

Letters

EMERGING INFECTIOUS DISEASES

August 2014



p. 1396

In Memoriam

Book Review

About the Cover

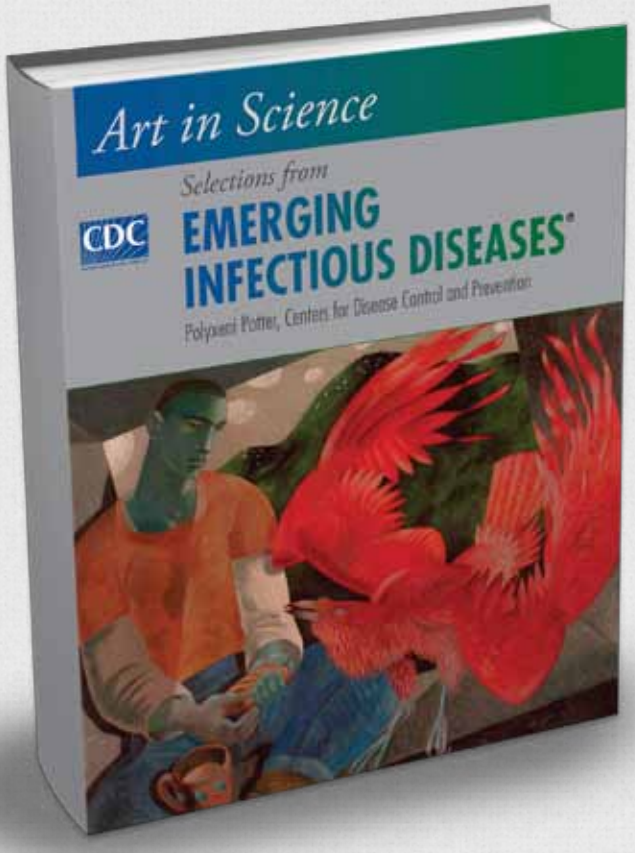
Etymologia

Correction

Online Report

Preparedness for Threat of Chikungunya in the Pacific

http://dx.doi.org/10.3201/eid2008.130696



NOW AVAILABLE

Art in Science: **Selections from EMERGING INFECTIOUS DISEASES**

[Hardcover]

The journal's highly popular fine-art covers are contextualized with essays that address how the featured art relates to science, and to us all.

Available at http://bit.ly/16sJI6R Use promo code 32327 for an additional 20% off

This collection of 92 excerpts and covers from **Emerging Infectious Diseases** will be of interest to readers of the journal or to anyone who wishes to reach across the aisle between art and science.

Leptospirosis-Associated Hospitalizations, United States, 1998–2009

Rita M. Traxler,[1] Laura S. Callinan,[1] Robert C. Holman, Claudia Steiner, and Marta A. Guerra

Medscape EDUCATION ACTIVITY

Medscape, LLC is pleased to provide online continuing medical education (CME) for this journal article, allowing clinicians the opportunity to earn CME credit.

This activity has been planned and implemented in accordance with the Essential Areas and policies of the Accreditation Council for Continuing Medical Education through the joint providership of Medscape, LLC and Emerging Infectious Diseases. Medscape, LLC is accredited by the ACCME to provide continuing medical education for physicians.

Medscape, LLC designates this Journal-based CME activity for a maximum of 1.0 ***AMA PRA Category 1 Credit(s)™***. Physicians should claim only the credit commensurate with the extent of their participation in the activity.

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

Release date: July 16, 2014; Expiration date: July 16, 2015

Learning Objectives

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

1. Analyze the clinical presentation of leptospirosis
2. Evaluate the epidemiology of leptospirosis-associated hospitalizations in the United States
3. Distinguish the time of year when leptospirosis-associated hospitalizations are most common
4. Compare the clinical impact of leptospirosis-associated hospitalizations vs non-leptospirosis infectious disease hospitalizations

CME Editor

Claudia Chesley, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Claudia Chesley has disclosed no relevant financial relationships.*

CME Author

Charles P. Vega, MD, Associate Professor and Residency Director, Department of Family Medicine, University of California, Irvine. *Disclosure: Charles P. Vega, MD, has disclosed the following financial relationships: Served as an advisor or consultant for McNeil Pharmaceuticals.*

Authors

Disclosures: ***Rita M. Traxler, MHS; Laura S. Callinan, MPH; Robert C. Holman, MS; Claudia Steiner, MD, MPH;*** *and* ***Marta A. Guerra, DVM, PhD, MPH,*** *have disclosed no relevant financial relationships.*

A small percentage of persons with leptospirosis, a reemerging zoonosis, experience severe complications that require hospitalization. The number of leptospirosis cases in the United States is unknown. Thus, to estimate the hospitalization rate for this disease, we analyzed US hospital discharge records for 1998–2009 for the total US population by using the Nationwide Inpatient Sample. During that time, the average annual rate of leptospirosis-associated hospitalizations was 0.6 hospitalizations/1,000,000 population. Leptospirosis-associated hospitalization rates were higher for persons >20 years of age and for male patients. For leptospirosis-associated hospitalizations, the average age of patients at admission was lower, the average length of stay for patients was longer, and hospital charges were higher than those for nonleptospirosis infectious disease–associated hospitalizations. Educating clinicians on the signs and symptoms of leptospirosis may result in earlier diagnosis and treatment and, thereby, reduced disease severity and hospitalization costs.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (R.M. Traxler, L.S. Callinan, R.C. Holman, M.A. Guerra); and Agency for Healthcare Research and Quality, Rockville, Maryland, USA (C. Steiner)

DOI: http://dx.doi.org/10.3201/eid2008.130450

[1]These authors contributed equally to this article.

Leptospirosis is a bacterial zoonotic infection caused by pathogenic serovars in the genus *Leptospira* (*1*). Approximately 10% of infections in humans result in clinical disease characterized by abrupt onset of fever, headache, muscle aches, and gastrointestinal involvement (*2*,*3*). Some infected persons can experience biphasic illness, in which more severe symptoms begin after a short recovery period (*2*,*3*). A total of 10%–15% of patients with clinical disease experience severe leptospirosis, characterized by multiple organ involvement (e.g., renal and liver failure, pulmonary distress and hemorrhage, cardiac arrhythmia), and a high rate of death (*2*,*3*). Severe infections comprise the majority of reported cases, but these cases underrepresent the incidence of disease (*4*).

Leptospirosis has historically occurred in persons who have contact with fresh water following heavy rains and in persons who work outdoors, with animals, or in wet environments contaminated with animal urine (*2*,*3*,*5*). The disease occurs more frequently in adult men than in children or women (*4*,*6*), and it is most prominent during warm and rainy seasons (*2*,*3*). In the United States, new groups at risk for leptospirosis have emerged, including residents in urban areas (*7*) and participants in freshwater sports (*8*,*9*).

In most places worldwide, leptospirosis is considered a reemerging human and animal disease (*1*,*5*). However, the disease was not considered nationally notifiable during 1995–2012, so whether human leptospirosis is reemerging in the United States is unknown (*10*). During those years, leptospirosis was reportable in many states; among them, California and Hawaii showed reemergence of the disease (*11*,*12*). In addition, a report describing a higher than expected death rate among leptospirosis-infected persons in Puerto Rico suggested that, on the basis of the average death rate, many more clinical cases of leptospirosis should have been reported (*13*). The fewer than expected number of reported cases might have resulted from underreporting or from a lack of disease recognition. The findings in those reports indicate the potential reemergence of leptospirosis as a public health problem in the United States.

To increase our knowledge of this neglected disease in the United States, we used national hospital discharge data for 1998–2009 to estimate the number of persons in the US population with symptomatic leptospirosis requiring hospitalization. We also used the discharge data to evaluate trends of leptospirosis-associated hospitalizations during the study period and to compare hospitalizations for leptospirosis with those for other infectious diseases.

Methods

We analyzed the general US population hospital discharge data for 1998–2009 from the Nationwide Inpatient Sample (NIS) (*14*). The Healthcare Cost and Utilization Project (HCUP), sponsored by the Agency for Healthcare Research and Quality (Rockville, MD, USA), produces NIS in collaboration with participating states (*15*). NIS is the largest all-payer inpatient care database in the United States and is a nationally representative sample of hospitals that includes a 20% sample of participating US community hospitals. Participating hospitals are short-term, nonfederal general and specialty hospitals sampled annually from up to 44 states. The overall design objective of NIS is to select a sample of hospitals that accurately represents the US population (*15*).

We calculated national estimates of the number of hospitalizations in the United States by using the HCUP weighting method (*15*,*16*). SEs and 95% CIs for rates were calculated by using SUDAAN software (http://www.rti.org/sudaan/). If the relative SE (i.e., SE/no. of estimated hospitalizations) of an estimate was >0.30 or if unweighted counts were <10.0, data were suppressed because the estimate was considered unreliable (*15*,*16*). The unit of analysis was a hospitalization; birth-associated hospitalizations were excluded from the analysis.

For analysis, we selected hospitalizations during 1998–2009 with an International Classification of Diseases, 9th revision, Clinical Modification, code (ICD-9-CM code) for leptospirosis (i.e., code100) listed as any 1 of up to 15 diagnoses on the hospitalization record (*17*). We calculated annual and average annual leptospirosis-associated hospitalization rates (per 1,000,000 persons) for the study period by using the annual number of weighted leptospirosis-associated hospitalizations and the corresponding annual census population overall and by sex, age group, and census region. Denominators were estimated by using the annual bridged race population estimates for 1998–2009 from the National Center for Health Statistics, US Centers for Disease Control and Prevention (*18*,*19*). Regions, defined by HCUP, were based on the US census regions (Northeast, South, Midwest, and West) (*20*), which do not include US territories. Leptospirosis-associated hospitalizations were compared with nonleptospirosis infectious disease–associated hospitalizations, which were defined as hospitalizations for a first-listed infectious disease, as defined in previous studies (*21*) with updates as appropriate, other than leptospirosis. We calculated rate ratios to compare rates between groups (*22*,*23*). Hospitalizations were not examined by patients' race/ethnicity because these data were missing in 19% of the records.

For patients with leptospirosis-associated hospitalizations, we calculated the mean and median age at admission overall and by sex. We examined seasonality for leptospirosis-associated hospitalizations by month of patient admission during the study period. We calculated the mean and median hospital charges for leptospirosis-associated hospitalizations overall, and we calculated the mean and median length of stay by the age and sex of patients and by region.

Table 1. Leptospirosis-associated hospitalizations and hospitalization rates by selected demographic characteristics, United States, 1998–2009*

Characteristic	No. leptospirosis-associated hospitalizations (SE)†	Hospitalization rate (95% CI)‡
Total	1,994 (126)	0.6 (0.5–0.6)
Patient age group		
0–19	287 (41)	0.3 (0.2–0.4)
20–59	1,260 (95)	0.7 (0.6–0.7)
≥60	441 (53)	0.7 (0.6–0.9)
Patient sex, age group, y		
M	1,401 (105)	0.8 (0.7–0.9)
0–19	190 (32)	0.4 (0.3–0.5)
20–59	934 (80)	1.0 (0.8–1.1)
≥60	277 (42)	1.1 (0.8–1.4)
F	587 (57)	0.3 (0.3–0.4)
0–19	97 (22)	0.2 (0.1–0.3)
20–59	326 (42)	0.3 (0.3–0.4)
≥60	164 (31)	0.5 (0.3–0.7)
Region of residence		
Northeast	261 (37)	0.4 (0.3–0.5)
Midwest	387 (48)	0.5 (0.4–0.6)
South	780 (68)	0.6 (0.5–0.7)
West	565 (87)	0.7 (0.5–0.9)

*SEs and 95% CIs were calculated by using SUDAAN software (http://www.rti.org/sudaan/).
†Numbers in subgroups do not total 1,994 because of missing values in the Nationwide Inpatient Sample.
‡Rate per 1,000,000 persons in corresponding population.

Age, hospital charges, and length of stay for leptospirosis-associated hospitalizations were also compared with those for nonleptospirosis infectious disease–associated hospitalizations. We performed *t*-tests in SUDAAN to determine whether leptospirosis-associated hospitalization charges and lengths of stay differed significantly by sex and region (*24*).

Results

During 1998–2009 in the United States, the average annual rate of leptospirosis-associated hospitalizations was 0.6 hospitalizations/1,000,000 population (95% CI 0.5–0.6) (Table 1); the annual rate did not change over the period (Figure 1). Regional average annual rates ranged from 0.4 hospitalizations/1,000,000 population (95% CI 0.3–0.5) in the Northeast to 0.7 hospitalizations/1,000,000 population (95% CI 0.5–0.9) in the West (Table 1).

The mean age of US patients with leptospirosis-associated hospitalizations was significantly younger than that for US patients with nonleptospirosis infectious disease–associated hospitalizations (43.2 y [SE 1.1] vs. 52.1 y [SE 0.2]; p<0.001) (median ages are shown in Table 2). The mean age of female patients with leptospirosis-associated hospitalizations was slightly older than that for male patients, but the difference was not statistically significant (45.7 y [SE 2.2] vs. 42.1 [SE 1.2]; p = 0.15).

The leptospirosis-associated hospitalization rate for adults 20–59 years of age (0.7 hospitalizations/1,000,000 corresponding population, 95% CI 0.6–0.7) and ≥60 years of age (0.7 hospitalizations/1,000,000 corresponding population, 95% CI 0.6–0.9) differed from the rate for persons 0–19 years of age (0.3 hospitalizations/1,000,000 corresponding population, 95% CI 0.2–0.4) (Table 1).

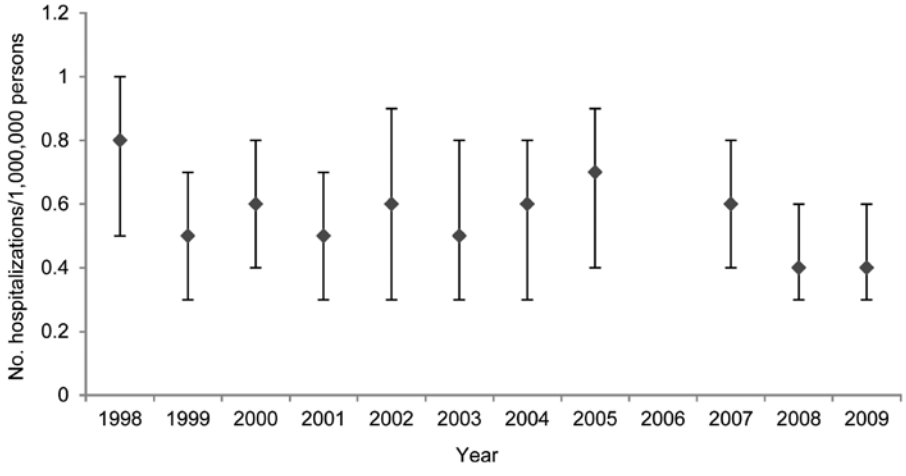


Figure 1. Yearly rate of leptospirosis-associated hospitalizations, United States, 1998–2009. Vertical bars indicate 95% CIs. The rate for 2006 is not included because it was unstable (relative SE >0.3).

Table 2. Numbers of leptospirosis-associated and nonleptospirosis infectious disease–associated hospitalizations by selected variables, United States, 1998–2009

Variable, characteristic	Median no. (25th, 75th quartiles) hospitalizations
Patient age, y	
Infection type	
Leptospirosis-associated	42.1 (27.8, 57.4)
Nonleptospirosis infectious disease	56.4 (30.4, 76.2)
Sex of patient	
M	40.8 (27.0, 55.4)
F	44.2 (30.2, 60.6)
Length of hospital stay, d, by hospitalization type	
Leptospirosis-associated	4.1 (2.4, 7.5)
Nonleptospirosis infectious disease–associated	3.3 (1.7, 6.0)
Hospital charges, US dollars, by leptospirosis-associated hospitalizations	
Total	19,768 (10,444, 37,422)
Sex of patient	
M	18,577 (11,161, 34,855)
F	24,093 (9,279, 44,960)
Patient age group, y	
<20	17,815 (9,253, 33,780)
20–59	18,942 (10,700, 35,046)
≥60	24,578 (10,230, 58,103)

The leptospirosis-associated hospitalizations rate for male patients was 2.5 times the rate for female patients (95% CI 1.9–3.1, p<0.001).

A high proportion of leptospirosis-associated hospitalization admissions occurred during June–September (41.2% [SE 2.7%]) (Figure 2). The mean length of stay for patients with leptospirosis-associated hospitalizations was longer than that for patients with nonleptospirosis infectious disease–associated hospitalizations (6.9 days [SE 0.4] vs. 5.6 days [SE 0.01]; p<0.001). The mean and median lengths of stay were not statistically significantly different by the age or sex of patients or by region.

For 1998–2009, the estimated hospital charges for leptospirosis-associated hospitalizations totaled US $76,013,667 (SE US $7,908,317). The mean charge for leptospirosis-associated hospitalizations was US $39,181 (SE US $3,493); this amount was significantly higher than the mean charge for nonleptospirosis infectious disease–associated hospitalizations (US $26,871 [SE US $198], p<0.001). The mean leptospirosis-associated hospitalization charges did not differ significantly between male patients (US $39,427 [SE US $4,231]) and female patients (US $38,605 [SE US $6,119]) or by age group (<20 years of age, US $40,659 [SE US $8,545]; 20–59 years of age, US $33,606 [SE US $3,351]; ≥60 years of age, US $53,533 [SE US $10,566]).

Leptospirosis-specific diagnoses were listed on the hospital records as follows (the percentage of records listing each diagnosis is shown in parentheses): unspecified leptospirosis (73% [SE 2.5%]); leptospirosis icterohemorrhagica (17% [SE 1.9%]); leptospirosis meningitis (6% [SE 1.3%]); and other specific *Leptospira* spp. infections (4% [SE 1.0%]). The most common diagnoses listed for leptospirosis-associated hospitalizations were volume depletion (23.8% [SE 2.1%]); thrombocytopenia, unspecified (18.7% [SE 2.0%]); acute kidney failure, unspecified (18.3% [SE 2.0%]); and fever and other physiologic disturbances of temperature regulation (13.1% [SE 1.7%]) (Table 3). Frequently performed procedures included spinal tap (20.5% [SE 2.1%]), venous catheterization (10.2% [SE 1.4%]), hemodialysis (7.4% [SE 1.3%]), and transfusion of packed cells (6.2% [SE 1.3%]) (Table 4).

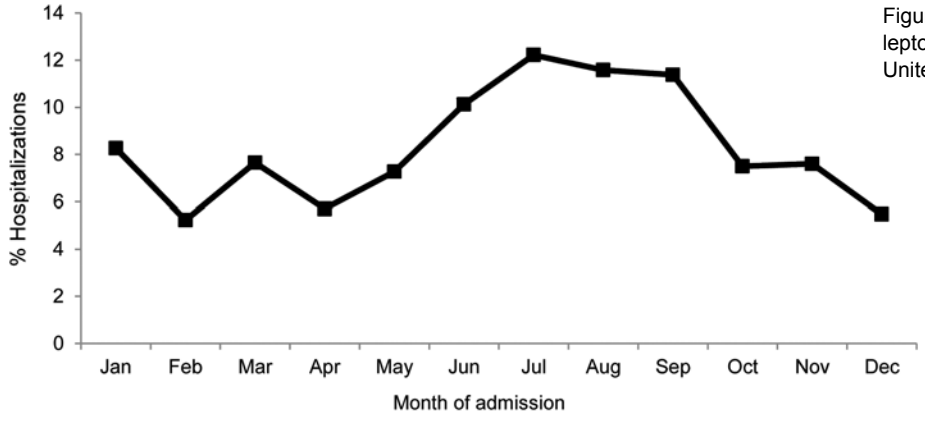


Figure 2. Monthly percentages of leptospirosis-associated hospitalizations, United States, 1998–2009.

Table 3. Selected diagnoses listed on leptospirosis-associated hospitalization discharge records, United States, 1998–2009*

Diagnosis†	ICD-9-CM code	No. (SE) discharge records	% (SE) discharge records
Volume depletion	276.5	475 (53)	23.8 (2.1)
Thrombocytopenia, unspecified	287.5	373 (50)	18.7 (2.0)
Acute kidney failure, unspecified‡	584.9	365 (48)	18.3 (2.0)
Fever and other physiologic disturbances of temperature regulation	780.6	261 (36)	13.1 (1.7)
Hyposmolality and/or hyponatremia	276.1	249 (37)	12.5 (1.6)
Hypopotassemia	276.8	203 (34)	10.2 (1.5)
Acute and subacute necrosis of liver‡	570	148 (28)	7.4 (1.4)
Jaundice, unspecified, not of newborn‡	782.4	124 (25)	6.2 (1.2)
Atrial fibrillation†	427.31	99 (24)	5.0 (1.1)
Acute respiratory failure‡	518.81	93 (21)	4.7 (1.1)

* ICD-9-CM, International Classification of Diseases, 9th revision, Clinical Modification (*17*).
†Discharge records may contain >1 listed diagnosis.
‡Diagnoses commonly associated with leptospirosis.

Discussion

The current incidence of leptospirosis in the United States is unknown because national surveillance of the disease ceased after 1994 (*10*). Reports of reemergence and increased incidence of leptospirosis in US states and globally (*1*,*5*,*11*,*12*), expanded number of risk groups (*7*,*8*), and a higher than expected death rate among reported case-patients in Puerto Rico (*13*) are raising concern that human leptospirosis infections may be on the rise in the United States. Thus, existing data must be used to estimate the number of cases nationwide. NIS is an available dataset that can be used to estimate the number of leptospirosis case-patients requiring hospitalization, evaluate trends of leptospirosis-associated hospitalizations, and compare parameters of leptospirosis-associated hospitalizations with those of nonleptospirosis infectious disease–associated hospitalizations.

The findings from our study indicate that the number of symptomatic patients with leptospirosis requiring hospitalization may be low in the United States. In addition, the findings do not indicate an increase in leptospirosis-associated hospitalizations over the study period, 1998–2009. However, the average annual leptospirosis-associated hospitalization rate of 0.6 hospitalizations/1,000,000 population likely represents only a proportion of all clinically diagnosed leptospirosis cases during 1998–2009, and the rate represents a much smaller proportion of all *Leptospira* spp. infections (*2*,*3*,*12*). Several studies have found that 70%–90% of patients with reported leptospirosis cases are hospitalized (*5*,*12*,*25*); however, an active surveillance study identified 5 times more leptospirosis cases than had been identified through passive surveillance, of which only 30% of the actively identified patients were hospitalized (*26*). Two studies of active case-finding following common-source outbreaks in the United States reported that 6% and 32% of the patients, respectively, were hospitalized (*8*,*9*). Although not directly comparable, the US leptospirosis incidence rate for 1994 (calculated from data in the Nationally Notifiable Diseases Surveillance System, http://wwwn.cdc.gov/nndss/) was 0.2 hospitalizations/1,000,000 population (*27*). The differences between the percentage of hospitalized patients identified from passive and active surveillance and between the leptospirosis-associated hospitalization rate and the 1994 leptospirosis incidence rate could indicate underrecognition of cases and underreporting of cases to the Nationally Notifiable Diseases Surveillance System.

Male patients were more likely than female patients to have a leptospirosis-associated hospitalization. The difference in disease occurrence between sexes has been established in the literature (*2*,*3*). Although the cause for this difference is not clear, it has often been ascribed to higher rates of exposure to *Leptospira* spp. among the male population (*2*,*3*); this higher exposure is reflected in labor statistics and in the demographics of recreational activities associated with leptospirosis outbreaks (*8*,*9*,*28*). A few studies have demonstrated increased hospitalization rates, disease severity, and leptospiremia among male patients, which may indicate greater susceptibility for severe disease in male patients (*25*,*29*). More research is needed to determine the reason for this disparity; however, it is likely multifactorial.

We found that persons ≥20 years of age were more likely than younger persons to have a leptospirosis-associated

Table 4. Most frequent procedures listed on leptospirosis-associated hospitalization discharge records, United States, 1998–2009*

Procedure†	ICD-9-CM code	No. (SE) discharge records	% (SE) discharge records
Spinal tap	03.31	408 (46)	20.5 (2.1)
Venous catheterization, not elsewhere classified	38.93	203 (32)	10.2 (1.4)
Hemodialysis	39.95	147 (27)	7.4 (1.3)
Transfusion of packed cells	99.04	124 (26)	6.2 (1.3)
Venous catheterization for renal dialysis	38.95	107 (23)	5.4 (1.2)

*ICD-9-CM, International Classification of Diseases, 9th revision, Clinical Modification (*17*).
†Discharge records may contain >1 listed procedure.

hospitalization. A lower incidence of infection in children has been widely reported (*6*,*12*,*25*,*26*,*30*,*31*); the difference is likely due to increased environmental exposure to the bacteria among adults (*3*,*4*).

Because the incubation period for leptospirosis is 1–2 weeks (range 2–30 days), the month of hospital admission for infected persons closely approximates the month of exposure to the pathogen (*2*). The distribution of hospitalizations by admission month in our study reflects the seasonality of leptospirosis infections (*2–4*). The predominance of leptospirosis cases in summer and fall has been linked to increased environmental exposure to the bacteria through contaminated water and soil during warm months (*2*,*4*) and through flooding events associated with hurricanes (*6*,*9*).

In our study, the median length of hospital stay for patients with leptospirosis-associated hospitalizations was 4.1 days; other studies have reported median lengths of stay of 5–10 days (range 1–46 days) (*5*,*26*). The higher hospital charges and longer lengths of stay for patients with leptospirosis-associated hospitalizations, compared with those for nonleptospirosis infectious disease–associated hospitalizations, likely result from the need for intensive care, supportive therapies, and invasive procedures that may be associated with the more severe form of leptospirosis. Support from an intensive care unit was required for 33%–64% of leptospirosis patients (*5*,*32*). In the presence of renal dysfunction and failure, which have been reported in 26%–47% of leptospirosis patients (*25*,*31–34*), fluid replacement therapy and dialysis are indicated to improve clinical outcome (*2*,*3*). The presence of hemorrhagic conditions, including hematuria, hematemesis, and hemoptysis, ranges from 9.1% to 81.5% in patients hospitalized for leptospirosis (*30*,*31*,*35*), and these conditions often require blood transfusions (*2*). These therapies and procedures increase the cost of patient care (*36*), and such increases may be reflected in our study findings. Improved awareness among clinicians of the clinical signs and symptoms of leptospirosis may lead to earlier diagnosis and treatment of the disease, which may reduce disease severity and, thus, hospitalization charges (*36*).

In NIS, aggregate demographic data for hospitalizations with an ICD-9-CM code of 100 (leptospirosis) are similar to data reported in the literature for patients with leptospirosis, but the leptospirosis-specific diagnoses are lower than expected. The NIS data are likely a valid representation of leptospirosis patients, although they may overrepresent the number of patients hospitalized after the initial febrile phase has ended. However, the use of the NIS dataset has limitations. Data from ≥44 US states are included in the NIS each year (*14*); the incidence of leptospirosis may or may not be higher in the states not included in the annual sample. This limitation is especially pertinent to US territories, where leptospirosis is often an endemic disease (*6*,*37*). For these reasons, the overall rate of leptospirosis-associated hospitalization may have been underestimated. However, in 1998, the annual NIS sampling frame comprised 67% of all US hospitalizations, but by 2009, 95% of all hospitalizations were included. Also, there is potential for misdiagnosis or for miscoding on hospitalization records. The diagnoses (ICD-9-CM codes) are physician-based; neither laboratory confirmation of the diagnosis nor the reason for the hospitalization is included in the hospital discharge records.

Leptospirosis outbreaks have occurred in temperate and tropical areas of the United States, typically following flooding events (*8*,*9*,*11*,*12*). As flooding events occur, infections may go unrecognized, particularly when other concurrent febrile illness outbreaks are occurring (*6*,*12*,*13*). The reinstatement of leptospirosis as a nationally notifiable condition in the United States has enabled the establishment of leptospirosis surveillance and the collection of case data (*10*). These data will be used to calculate the national incidence of reported leptospirosis cases in the United States, clarify the current epidemiology of the disease, and possibly assess the benefit of earlier diagnosis and treatment on patient outcomes. In addition, changes in health outcomes may be reflected in future analyses of hospitalization data. Educating clinicians on the clinical signs and symptoms of leptospirosis and the importance of case reporting is needed; it may reduce possible underrecognition and underreporting of the disease.

Acknowledgments

We thank the states that voluntarily participate in the Healthcare Cost and Utilization Project to produce the Nationwide Inpatient Sample and Jason Mehal for his valuable assistance with the final revisions to this article.

Ms Traxler is an epidemiologist in the Bacterial Special Pathogens Branch at the US Centers for Disease Control and Prevention. Her research interests include surveillance and epidemiology of bacterial zoonotic diseases.

References

1. World Health Organization. Leptospirosis: an emerging public health problem. Wkly Epidemiol Rec. 2011;86:45–50.
2. Guerra MA. Leptospirosis. J Am Vet Med Assoc. 2009;234:472–8, 430. http://dx.doi.org/10.2460/javma.234.4.472
3. Levett PN. Leptospirosis. Clin Microbiol Rev. 2001;14:296–326. http://dx.doi.org/10.1128/CMR.14.2.296-326.2001
4. Katz AR, Ansdell VE, Effler PV, Middleton CR, Sasaki DM. Leptospirosis in Hawaii, 1974–1998: epidemiologic analysis of 353 laboratory-confirmed cases. Am J Trop Med Hyg. 2002;66:61–70.
5. Hartskeerl RA, Collares-Pereira M, Ellis WA. Emergence, control and re-emerging leptospirosis: dynamics of infection in the changing world. Clin Microbiol Infect. 2011;17:494–501. http://dx.doi.org/10.1111/j.1469-0691.2011.03474.x

6. Bruce MG, Sanders EJ, Leake JA, Zaidel O, Bragg SL, Aye T, et al. Leptospirosis among patients presenting with dengue-like illness in Puerto Rico. Acta Trop. 2005;96:36–46. http://dx.doi.org/10.1016/j.actatropica.2005.07.001
7. Vinetz JM, Glass GE, Flexner CE, Mueller P, Kaslow DC. Sporadic urban leptospirosis. Ann Intern Med. 1996;125:794–8. http://dx.doi.org/10.7326/0003-4819-125-10-199611150-00002
8. Centers for Disease Control and Prevention. Update: leptospirosis and unexplained acute febrile illness among athletes participating in triathlons—Illinois and Wisconsin, 1998. MMWR Morb Mortal Wkly Rep. 1998;47:673–6.
9. Stern EJ, Galloway R, Shadomy SV, Wannemuehler K, Atrubin D, Blackmore C, et al. Outbreak of leptospirosis among Adventure Race participants in Florida, 2005. Clin Infect Dis. 2010;50:843–9. http://dx.doi.org/10.1086/650578
10. Council of State and Territorial Epidemiologists. Public health reporting and national notification for leptospirosis. 2012 [cited 2012 Dec 12]. http://c.ymcdn.com/sites/www.cste.org/resource/resmgr/PS/12-ID-02FINAL.pdf
11. Meites E, Jay MT, Deresinski S, Shieh WJ, Zaki SR, Tompkins L, et al. Reemerging leptospirosis, California. Emerg Infect Dis. 2004;10:406–12. http://dx.doi.org/10.3201/eid1003.030431
12. Katz AR, Buchholz AE, Hinson K, Park SY, Effler PV. Leptospirosis in Hawaii, USA, 1999–2008. Emerg Infect Dis. 2011;17:221–6. http://dx.doi.org/10.3201/eid1702.101109
13. Centers for Disease Control and Prevention. Notes from the field: investigation of leptospirosis underreporting—Puerto Rico, 2010. MMWR Morb Mortal Wkly Rep. 2012;61:421.
14. Agency for Healthcare Research and Quality. Healthcare Cost and Utilization Project (HCUP). Databases; last modified February 2013 [cited 2013 Mar 2]. http://www.hcup-us.ahrq.gov/databases.jsp
15. Agency for Healthcare Research and Quality. Healthcare Cost and Utilization Project (HCUP). NIS database documentation; last modified February 2013 [cited 2013 Mar 2]. http://www.hcup-us.ahrq.gov/db/nation/nis/nisdbdocumentation.jsp
16. Houchens R, Elixhauser A. US Agency for Healthcare Research and Quality. Final report on calculating nationwide inpatient sample (NIS) variances, 2001. HCUP methods series report #2003–2. June 2005 (revised 2005 June 6) [cited 2011 Dec 13]. http://www.hcup-us.ahrq.gov/reports/methods/CalculatingNISVariances200106092005.pdf
17. Public Health Service and Health Care Financing Administration. International classification of diseases, 9th revision, clinical modification, 6th ed. (CD-ROM). Washington (DC): US Department of Health and Human Services; 2005.
18. National Center for Health Statistics. Bridged-race intercensal estimates of the July 1, 1990–July 1, 1999, United States resident population by county, single-year of age, sex, race, and Hispanic origin. July 26, 2004 [cited 2011 Dec 13]. http://www.cdc.gov/nchs/nvss/bridged_race/data_documentation.htm#july1999
19. National Center for Health Statistics. Vintage 2009 bridged postcensal estimates of the resident population of the United States for July 1, 2000–July 1, 2009, by year, county, single-year of age (0 to 85+ years), bridged race, Hispanic origin, and sex. July 23, 2010 [cited 2011 Dec 13]. http://www.cdc.gov/nchs/nvss/bridged_race/data_documentation.htm#vintage2009
20. US Census Bureau. Census regions and divisions of the United States [cited 2012 Nov 20]. https://www.census.gov/geo/maps-data/maps/pdfs/reference/us_regdiv.pdf
21. Christensen KL, Holman RC, Steiner CA, Sejvar JJ, Stoll BJ, Schonberger LB. Infectious disease hospitalizations in the United States. Clin Infect Dis. 2009;49:1025–35. http://dx.doi.org/10.1086/605562
22. DeFrances CJ, Cullen KA, Kozak LJ. National hospital discharge survey: 2005 annual summary with detailed diagnosis and procedure data. Vital Health Stat 13. 2007;165:1–209.
23. Levy PS, Lemeshow S. Sampling of populations—methods and applications. New York: John Wiley & Sons, Inc.; 1991.
24. Lehmann E. Nonparametrics: statistical methods based on ranks. New York: Wiley; 1975.
25. Jansen A, Stark K, Schneider T, Schoneberg I. Sex differences in clinical leptospirosis in Germany: 1997–2005. Clin Infect Dis. 2007;44:e69–72. http://dx.doi.org/10.1086/513431
26. Sasaki DM, Pang L, Minette HP, Wakida CK, Fujimoto WJ, Manea SJ, et al. Active surveillance and risk factors for leptospirosis in Hawaii. Am J Trop Med Hyg. 1993;48:35–43.
27. Centers for Disease Control and Prevention. Summary of notifiable diseases, United States, 1994. MMWR Morb Mortal Wkly Rep. 1994;43:1–80.
28. US Bureau of Labor Statistics. Household data annual averages 2010 April 1, 2011 [cited 2011 Aug 30]. Employed persons by detailed industry, sex, race, and Hispanic or Latino ethnicity. http://www.bls.gov/cps/cpsaat18.pdf
29. Agampodi SB, Matthias MA, Moreno AC, Vinetz JM. Utility of quantitative polymerase chain reaction in leptospirosis diagnosis: association of level of leptospiremia and clinical manifestations in Sri Lanka. Clin Infect Dis. 2012;54:1249–55. http://dx.doi.org/10.1093/cid/cis035
30. Herrmann-Storck C, Saint-Louis M, Foucand T, Lamaury I, Deloumeaux J, Baranton G, et al. Severe leptospirosis in hospitalized patients, Guadeloupe. Emerg Infect Dis. 2010;16:331–4. http://dx.doi.org/10.3201/eid1602.090139
31. Daher EF, Lima RS, Silva Junior GB, Silva EC, Karbage NN, Kataoka RS, et al. Clinical presentation of leptospirosis: a retrospective study of 201 patients in a metropolitan city of Brazil. Braz J Infect Dis. 2010;14:3–10. http://dx.doi.org/10.1016/S1413-8670(10)70002-7
32. Spichler A, Athanazio D, Seguro AC, Vinetz JM. Outpatient follow-up of patients hospitalized for acute leptospirosis. Int J Infect Dis. 2011;15:e486–90. http://dx.doi.org/10.1016/j.ijid.2011.03.020
33. Katz AR, Ansdell VE, Effler PV, Middleton CR, Sasaki DM. Assessment of the clinical presentation and treatment of 353 cases of laboratory-confirmed leptospirosis in Hawaii, 1974–1998. Clin Infect Dis. 2001;33:1834–41. http://dx.doi.org/10.1086/324084
34. Vanasco NB, Schmeling MF, Lottersberger J, Costa F, Ko AI, Tarabla HD. Clinical characteristics and risk factors of human leptospirosis in Argentina (1999–2005). Acta Trop. 2008;107:255–8. http://dx.doi.org/10.1016/j.actatropica.2008.06.007
35. Paganin F, Bourdin A, Dalban C, Courtin JP, Poubeau P, Borgherini G, et al. Leptospirosis in Reunion Island (Indian Ocean): analysis of factors associated with severity in 147 confirmed cases. Intensive Care Med. 2007;33:1959–66. http://dx.doi.org/10.1007/s00134-007-0776-y
36. Galloway RL, Levett PN, Tumeh JW, Flowers CR. Assessing cost effectiveness of empirical and prophylactic therapy for managing leptospirosis outbreaks. Epidemiol Infect. 2009;137:1323–32. http://dx.doi.org/10.1017/S0950268808001751
37. Lau CL, Dobson AJ, Smythe LD, Fearnley EJ, Skelly C, Clements AC, et al. Leptospirosis in American Samoa 2010: epidemiology, environmental drivers, and the management of emergence. Am J Trop Med Hyg. 2012;86:309–19. http://dx.doi.org/10.4269/ajtmh.2012.11-0398

Address for correspondence: Rita M. Traxler, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop A30, Atlanta, GA 30329-4027, USA; email: rtraxler@cdc.gov

Independent Origin of *Plasmodium falciparum* Antifolate Super-Resistance, Uganda, Tanzania, and Ethiopia

Michael Alifrangis,[1] Sidsel Nag,[1] Mette L. Schousboe, Deus Ishengoma, John Lusingu, Hirva Pota, Reginald A. Kavishe, Richard Pearce, Rosalynn Ord, Caroline Lynch, Seyoum Dejene, Jonathan Cox, John Rwakimari, Daniel T.R. Minja, Martha M. Lemnge, and Cally Roper

Super-resistant *Plasmodium falciparum* threatens the effectiveness of sulfadoxine–pyrimethamine in intermittent preventive treatment for malaria during pregnancy. It is characterized by the A581G *Pfdhps* mutation on a background of the double-mutant *Pfdhps* and the triple-mutant *Pfdhfr*. Using samples collected during 2004–2008, we investigated the evolutionary origin of the A581G mutation by characterizing microsatellite diversity flanking *Pfdhps* triple-mutant (437G+540E+581G) alleles from 3 locations in eastern Africa and comparing it with double-mutant (437G+540E) alleles from the same area. In Ethiopia, both alleles derived from 1 lineage that was distinct from those in Uganda and Tanzania. Uganda and Tanzania triple mutants derived from the previously characterized southeastern Africa double-mutant lineage. The A581G mutation has occurred multiple times on local *Pfdhps* double-mutant backgrounds; however, a novel microsatellite allele incorporated into the Tanzania lineage since 2004 illustrates the local expansion of emergent triple-mutant lineages.

Controlling and reducing malaria requires a combination of vector control measures and administration of antimalarial drugs as prophylaxis or treatment (*1*). The widespread use of antimalarial drugs has resulted in the emergence of resistant *Plasmodium falciparum*, recurrently exposing persons in malaria-endemic regions to an unacceptably high risk for treatment failures (*2*).

Highly chloroquine-resistant parasites spread from Asia in the 1960s and led to devastating rates of malaria-related death in Africa starting in the late 1980s, gradually forcing affected countries to replace chloroquine with sulfadoxine–pyrimethamine (SP) (*3*–*5*). The effectiveness of SP did not last long. In fact, retrospective analysis indicated that pyrimethamine-resistant parasites were present in sub-Saharan Africa before SP was implemented as first-line treatment, probably because pyrimethamine as monotherapy had been used in Asia during the 1960's and 1970's (*6*–*8*). Resistance to sulfadoxine also soon emerged (*9*), and the combination of pyrimethamine- and sulfadoxine-resistant parasites led to severe and widespread SP treatment failure (*10*–*12*). As a consequence, affected countries were once again forced to change their drug policies (*13*) and have now adopted artemisinin-based combination therapies as first-line treatment for uncomplicated malaria. Yet, SP is still recommended for use as intermittent preventive treatment in pregnant women (SP-IPTp) and infants (SP-IPTi) (*14*,*15*). Also, seasonal malaria chemoprevention applies SP in combination with amodiaquine (*16*). Use of SP for prevention in many countries of sub-Saharan Africa, where clinical failure after SP treatment has been reported, underscores the need for effective surveillance of its protective efficacy and for monitoring of the development and spread of SP resistance in *P. falciparum* populations.

The molecular basis of SP resistance is a combination of single-nucleotide polymorphisms (SNPs) in 2 distinct

Author affiliations: Centre for Medical Parasitology at Department of International Health, Immunology, and Microbiology, Faculty of Health and Medical Science, University of Copenhagen and at Department of Infectious Diseases, Copenhagen University Hospital (Rigshospitalet), Copenhagen, Denmark (M. Alifrangis, S. Nag, M.L. Schousboe); Tanga Research Centre, Tanzania (D. Ishengoma, J. Lusingu, D.T.R. Minja, M.M. Lemnge); London School of Hygiene and Tropical Medicine, London, UK (H. Pota, R. Pearce, R. Ord, C. Lynch, J. Cox, C. Roper); Kilimanjaro Christian Medical University College, Moshi, Tanzania (R.A. Kavishe); Kilimanjaro Clinical Research Institute, Moshi (R.A. Kavishe); Médécins Sans Frontières, Humera, Ethiopia (S. Dejene); and Ministry of Health of Uganda, Kampala, Uganda (J. Rwakimari)

DOI: http://dx.doi.org/10.3201/eid2008.131897

[1]These authors contributed equally to this article.

genes coding for the target enzymes of SP. The enzymes dihydrofolate reductase (DHFR) and dihydropteroate synthetase (DHPS) are targeted by pyrimethamine and sulfadoxine, respectively (*17*). High-level pyrimethamine resistance is generally encoded by 3 mutations in the *Pfdhfr* gene, coding for substitutions: N51I, C59R, and S108N (*18*); the molecular basis of sulfadoxine resistance is caused by substitutions S/A436F, A437G, K540E, A581G, and A613S/T in a variety of combinations in DHPS (*19*).

The most prevalent genotype in eastern Africa is a combination of the *Pfdhfr* triple mutant (51I, 59R, and 108N, denoted as IRN) combined with the *Pfdhps* double mutant (S436, 437G, 540E, A581, and A613, denoted as SGEAA). Together, this combination of SNPs is referred to as the "quintuple" mutant *Pfdhfr*/*Pfdhps* genotype and is associated with high risk for SP treatment failure (*17*) and results in limited protective value of SP-IPTi (*20*). Accordingly, the World Health Organization (WHO) recommends that SP-IPTi should be implemented only when the prevalence of the K540E mutation (and thus the quintuple mutant) is <50% (*14*).

More recently, an alanine to glycine mutation at codon position 581 in *Pfdhps* has emerged that, in combination with the *Pfdhfr* triple-mutant allele IRN, was shown to confer higher level resistance (*21*). This combination, referred to as the "sextuple *Pfdhfr*/*Pfdhps* mutant genotype" or the "super-resistant genotype" (*22*), is associated with reduced SP-IPTp efficacy by 1) a reduction in the protection period of SP-IPTp from 4 weeks to 2 weeks (*23*); 2) increased parasitemia attributed to competitive facilitation (*23*); 3) increased risk for severe malaria in the offspring (*24*); and 4) low birthweight in newborns from mothers undergoing SP-IPTp in Tanzania (*25*). Consequently, WHO recommendations concerning the use of SP-IPTp base the threshold on 2 mutations: SP-IPTp should be discontinued if the prevalence of the K540E mutation is >95% and the A581G mutation is >10% (*20*). No threshold in the prevalence of molecular markers of resistance has been set with regard to seasonal malaria chemoprevention (*15*,*16*).

Maps collating all published data from molecular surveillance of *Pfdhfr* and *Pfdhps* mutations (*22*) indicate 3 main foci of super-resistant parasites: 1 in northern Tanzania (*26*); a second in southwestern Uganda, Rwanda, and bordering areas of Democratic Republic of Congo (*27*–*29*); and a third in western Kenya (*30*). Prevalence of A581G also is high in Ethiopia and northern Sudan, where it again occurs as the *Pfdhps* triple-mutant allele SGEGA but in combination with a *Pfdhfr* double-mutant allele 51I-108N.

Assessments of microsatellite variation linked to *Pfdhps* have shown that limited microsatellite diversity flanking the SGEAA double mutants compared with the SAKAA wild types. Two SGEAA lineages were discovered in eastern Africa: 1 prevailing in northeastern Africa (Ethiopia and Sudan) and the other throughout southeastern Africa. Both lineages derived from independent ancestry (*10*). Here we apply the same approach, using the same microsatellite loci, to determine the ancestry and possible relationship between the double SGEAA and triple SGEGA alleles in Ethiopia, Uganda, and Tanzania. By focusing on microsatellite variation linked to *Pfdhps*, we can explore whether the emergence of the SP-IPT-threatening SGEGA triple mutants in Ethiopia, Uganda, and Tanzania derive from local SGEAA alleles or are being imported.

Materials and Methods

Study Sites

Samples for the study were collected during 2004–2008. Study sites were in Uganda (2 sites), Tanzania (3 sites), and Ethiopia (1 site) (Table).

Sample Collection

Bufundi and Rikungiri, Uganda

Uganda implemented SP-IPTp in 2000 and has not implemented SP-IPTi or seasonal malaria chemoprevention. Finger-prick blood-spot samples were obtained from symptomatic patients of all ages after *P. falciparum* infection was confirmed by a Paracheck rapid test (Orchid Biomedical Systems, Chennai, India) during May–December 2005 at reference health facilities in Bufundi (Kabale District) (38 samples) and Kebisoni (Rukungiri District)

Table. Study sites and number of samples genotyped for super-resistant *Plasmodium falciparum*, eastern Africa*

	SGEAA		SGEGA		Total haplotype
Study site.	No. samples	Haplotype diversity	No. samples	Haplotype diversity	diversity
Ethiopia, Humera, Tigray	35	4	41	1	4
Uganda					
Bufundi, Kabale	24	5	14	3	7
Kebisoni, Rukungiri	27	5	14	3	7
Tanzania					
Hale, Tanga	21	6	15	3	8
Korogwe, Tanga	64	21	19	3	22
Magoda, Tanga	7	3	15	6	6

*By country, district, village. Samples from Ethiopia are from 2004; samples from Uganda are from 2005; and samples from Tanzania are from 2004 (Korogwe only), 2006 (Korogwe and Hale), 2007 (Korogwe only) and 2008 (Magoda only).

(41 samples). Blood spots were air dried on Whatman no. 3 filter paper (VWR–Bie & Berntsen, Herlev, Denmark), sealed in plastic bags with a desiccant, and stored at room temperature for molecular genotyping (*27*). The Uganda National Council for Science and Technology (UNSCT HS 35) and the ethics committee of the London School of Hygiene and Tropical Medicine (London, UK) gave scientific and ethical permission. Consent was obtained from all persons or their guardians before sample collection.

Hale, Korogwe, and Magoda, Tanzania

Tanzania implemented SP-IPTp in 2001 and has not implemented SP-IPTi or seasonal malaria chemoprevention. Samples were obtained from 3 different settings in Tanga region. From Hale (36 samples), finger-prick blood-spot samples were taken from symptomatic children 6–59 months of age who attended Hale Health Centre during July–August 2006 as previously described (*21*). The study protocol was approved by the Ethics Review Committees of the National Institute for Medical Research, Tanzania, and the London School of Hygiene and Tropical Medicine and was registered as a clinical trial with the National Institutes of Health (http://www.clinicaltrials.gov, identifier NCT00361114). From Korogwe (83 samples), finger-prick or venous blood samples were obtained on filter paper from children and adolescents <20 years of age from Mkokola and Kwamasimba villages. Samples were collected in March 2004, May 2006, and May 2007, as described (*26*). The Medical Research Coordinating Committee of the National Institute for Medical Research and Ministry of Health, Tanzania, granted ethical clearance for the study. All participants or their parents or guardians provided informed consent. Samples from Magoda villages (22 samples) were collected from children <5 years of age in June 2008 as part of a cross-sectional assessment of malaria prevalence (*31*).

Humera, Ethiopia

Ethiopia has adopted neither of the WHO recommendations regarding use of SP as prophylaxis. Samples were collected from patients of all ages who attended Kahsay Abera Hospital in Humera during January–April 2004 and who had symptomatic uncomplicated malaria (*10*). The patients were enrolled in an in vivo efficacy trial, comparing artemether–lumefantrine therapy with SP therapy, which was conducted by staff of Kahsay Abera Hospital and the Mekele Regional Health Bureau. Finger-prick blood-spot samples were taken from patients before treatment after they gave written informed consent to participate in the study, and genetic analysis was conducted in support of the drug efficacy evaluation. The Ethical Clearance Committee of the Tigray Health Research Council and the external Ethics Review Board used by Médecins sans Frontières gave ethical permissions for the study.

Genotyping

Sample collection at the different study sites was not standardized because the samples derived from independent studies. However, all samples consisted of finger-prick blood spots stored on filter paper, and parasite DNA was extracted by using the Chelex method (*32*).

Point mutations in samples from Hale, Tanzania, were determined by direct sequencing (*21*). Sequencing was performed by using the ABI-3730 automatic sequencer (Applied Biosystems, Foster City, CA, USA), and samples were analyzed with Applied Biosystems BigDye V. 3.1 (Applied Biosystems).

For all other samples, the polymorphic region of *Pfdhps* was PCR-amplified before sequence-specific oligonucleotide probing (SSOP) for mutations at codons 436, 437, 540, 581, and 613 by using primers and PCR conditions described elsewhere (*33*). SSOP-genotyping of samples from Uganda and Ethiopia was conducted according to an SSOP–dot-blot method (*10*); genotyping of samples from Korogwe and Magoda in Tanzania was conducted according to an SSOP-ELISA method (*33*).

Only samples containing the *Pfdhps* SGEAA or SGEGA alleles were included for further analysis; other alleles, such as wild-type or single-mutant alleles, were excluded. In general, only a single sequence was detected at every codon, but if the sequence analysis detected a mixture, these samples were handled as mixed infections. Mixed infections, in turn, were further analyzed only if 1 allele was substantially in the majority (i.e., a 2:1 signal ratio between the dominant genotype and the minor genotype) and a majority SNP could be confidently determined at all codon positions (*33*).

Microsatellite Analysis

Analysis was performed on 3 *Pfdhps*-linked microsatellites located 0.8 kb (marker [m.] 0.8), 4.3 kb (m. 4.3), and 7.7 kb (m. 7.7) downstream of the coding position 437 of *Pfdhps*, located on chromosome 8 (*34*). Microsatellites were amplified by seminested PCR as described previously (*34*), and products were run with GeneScan-500 LIZ Size Standards (Applied Biosystems) in an ABI 3730 DNA analyzer (Applied Biosystems) and analyzed by using Genemapper software (Applied Biosystems). If >1 microsatellite allele was detected in any given sample, the peak height ratio was used to determine the majority allele for that locus. If the major allele did not have a peak height of at least double the height of the minor allele, the sample was excluded from further analysis.

Microsatellite haplotypes were constructed by combining alleles detected in each of the 3 microsatellite loci. Samples with missing data were not included.

Results

A total of 300 samples with either *Pfdhps* double-mutant (SGEAA) or triple-mutant (SGEGA) alleles were

subjected to microsatellite analysis, and 277 (92.3%) of these gave conclusive results. Microsatellite haplotypes associated with the *Pfdhps* double- and triple-mutant alleles are listed in full (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/8/13-1897-Techapp1.pdf), where the haplotypes are ranked hierarchically according to allele size, first at the 0.8-kb locus, then at 4.3-kb locus, and finally at the 7.7-kb locus, and each unique haplotype was assigned a number.

Diversity of Microsatellite Composition among SGEAA Samples

SGEAA alleles from Ethiopia were associated with 4 different microsatellite haplotypes (Figure, panel A; Table). Haplotype 4 (notation 121–114–98, refers to fragment size 121 bp at the 0.8-kb locus, 114 bp at the 4.3-kb locus, and 98 bp at the 7.7-kb locus) predominated and was found in 30 (85.7%) of the 35 SGEAA alleles sampled. Of the remaining 3 microsatellite haplotypes, haplotypes 7 and 21 were each found twice; 11 was found once. Haplotype 11 (131–104–107) was most common in the samples from Uganda and Tanzania. No samples from Tanzania or Uganda were Ethiopia haplotype 4. The Uganda SGEAA alleles were associated with 8 different microsatellite haplotypes (Figure, panel A; online Technical Appendix Table, 5 haplotypes found at each site); the most common haplotype, haplotype 11 (131–104–107), was found in 82.4%, (42/51) of samples. The 92 SGEAA samples collected from 3 study sites in Tanzania exhibited 24 different microsatellite haplotypes (Figure, panel A; online Technical Appendix Table). Korogwe exhibited the greatest diversity by having 21 haplotypes among 64 SGEAA sample. As in Uganda, most of the Tanzania SGEAA alleles were associated with haplotype 11 (53 [57.6%]). Among the remaining 39 samples were 23 alternative but related haplotypes.

Diversity of Microsatellite Composition among SGEGA Samples

Of 41 SGEGA samples from Ethiopia, only 1 microsatellite haplotype was present: haplotype 4 (121–114–98) (Figure, panel B; online Technical Appendix Table). The 28 SGEGA samples from Uganda were associated with 3 microsatellite haplotypes; all 3 combinations were represented in the 2 sites (Figure, panel B; online Appendix Table). Of these SGEGA samples, 24 (85.7%) were haplotype 11 (131–104–107). The 2 less common haplotypes, haplotypes 18 (131–104–125) and 14 (131–104–113), are evidently related to haplotype 11, differing by 1 allele at the 7.7-kb locus. Of 49 SGEGA samples collected from Tanzania, we found 7 microsatellite haplotypes (Figure, panel B; online Technical Appendix Table). This finding indicates less diversity than was associated with SGEAA alleles: Hale (3 haplotypes), Korogwe (3 haplotypes), and Magoda (6 haplotypes). Of the Tanzania SGEGA samples, 18 (36.7%) were haplotype 11, the same haplotype common among Tanzania and Uganda SGEAA samples and among Uganda SGEGA samples. Of the remaining 28 SGEGA samples from Tanzania, 24 (49.0% of total Tanzania SGEGA samples) were haplotype 18 (131–104–125), a haplotype found only twice in association with SGEAA samples from Tanzania. This haplotype was found twice among Uganda SGEGA samples and never among Uganda SGEAA samples.

Discussion

Although SP is no longer recommended as first-line treatment for *P. falciparum* infection, it is widely recommended for prevention and possibly still available over the counter for self-treatment. Presumably, therefore, SP has continued to exert selective pressure on already resistant parasites, which might explain the continuing emergence of the triple-mutant *Pfdhps* allele (SGEGA), which is currently being described for certain regions of eastern Africa.

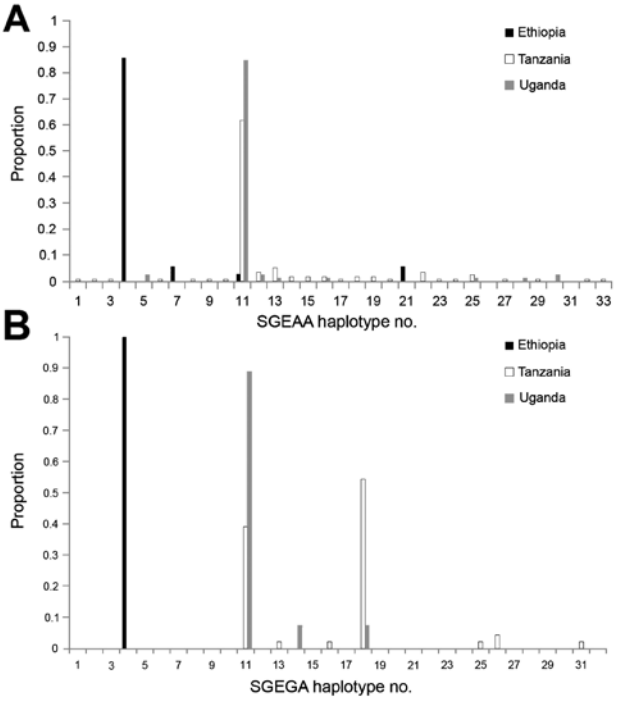


Figure. Proportion of microsatellite haplotypes linked to SGEAA and SGEGA, eastern Africa. Microsatellite haplotypes associated with the *Pfdhps* double-mutant allele SGEAA (A) and *Pfdhps* triple-mutant allele SGEGA (B) in Ethiopia, Tanzania, and Uganda. Haplotype numbering (x-axis) refers to a unique combination of microsatellite allele sizes at the 3 loci linked to *dhps* (specific microsatellite allele combinations are listed in the online Technical Appendix Table [http://wwwnc.cdc.gov/EID/article/20/8/13-1897- Techapp1.pdf]. Proportion (y-axis) is the number of alleles associated with each microsatellite haplotype expressed as a proportion of the total number of alleles sampled in each country for which the associated microsatellite haplotype could be determined.

An increased prevalence of the A581G mutation has been well documented in eastern Africa in recent years; it increased from 12% in 2003 to 56% in 2007 at study sites in Korogwe, Tanzania (*26*). In Kabale and Rukungiri, Uganda, samples from 2005 showed a high prevalence of the A581G mutation at 45% and 46%, respectively (*27*); studies during 2005–2006 in Rukara and Mahesha, Rwanda, observed prevalences of 60% and 29%, respectively (*28*). A study in 2010 in Huye District, Southern Province, Rwanda, reported a prevalence of 63% (*35*). In eastern Sudan, a study found an increase in the prevalence of the A581G mutation from 14% in 2003 to 34% in 2012 (*36*). In Kenya, Kisumu, a study showed an increase in the A581G mutation from 0% in 1999–2000 to 85% in 2003–2005 (*30*). More recently, in Nyanza Province, western Kenya, the prevalence of the A581G increased from 0% to 5.3% from 2008–2009 (*37*).

In this study, we investigated the origins of triple-mutant *Pfdhps* alleles by analyzing the microsatellite diversity flanking *Pfdhps*. We sampled both SGEAA double mutants and SGEGA triple mutants in 3 populations at the key moment: when the SGEGA triple mutant had emerged but had not yet replaced the SGEAA double mutant. At this time, double- and triple-mutant alleles were present in similar numbers in the areas, but SP-sensitive alleles were very rare.

In Ethiopia, both SGEAA and SGEGA alleles were associated with haplotype 4 (121–114–98), indicating a shared ancestry that has evolved independently from SGEAA and SGEGA alleles from Uganda and Tanzania. The SGEAA in these 2 countries were associated with lineage 11 (131–104–107), the same microsatellite haplotype previously shown to be associated with the double-mutant alleles throughout Tanzania, Kenya, Uganda, Mozambique, and Zambia (*10*).

In Ethiopia and Uganda, we found evidence that the most prevalent SGEAA haplotype locally had given rise to SGEGA haplotypes in the same area; the most common SGEAA haplotype was also the most common SGEGA haplotype in both countries (haplotypes 4 and 11, respectively). However, in Tanzania, the microsatellite haplotype most commonly associated with SGEGA (haplotype 18 [131–104–125], 25/49 samples) was not identical to that most commonly associated with SGEAA (haplotype 11 [131–104–107]), because only 2 SGEAA samples were haplotype 18. This finding leads us to speculate that the A581G mutation has emerged on at least 2 occasions in Tanzania.

We found that the microsatellite diversity associated with both SGEAA and SGEGA haplotypes in Ethiopia samples was less than in the SGEAA and SGEGA samples from sites in Uganda and Tanzania. The high level of homozygosity among microsatellite haplotypes in Ethiopia might be due to a high degree of selective pressure, which in turn might be assisted by population bottlenecks brought about by a narrow malaria transmission season and limited exchange of parasites, with neighboring regions resulting from limited migration. The Ethiopia samples originate from the Tigray District near the Eritrean border. Despite some migration of refugees from Eritrea to Ethiopia, a substantial spread to and from the Tigray District in Ethiopia during the years before sample collection is doubtful because of the continued presence of forces at the border during the cease fire succeeding the Eritrean–Ethiopian war initiated in 2000. Double- and triple-mutant alleles from northeastern and eastern Sudan also are associated with haplotype 4 (121–114–98) (*10*) and represent greater diversity than what we present from Ethiopia, which supports the view that the parasite populations in these 2 countries are linked (*10*,*38*).

Tanzania is a capital of trade and emigration for sub-Saharan Africa. The Tanzam highway (running from Tanzania through Zambia) is one of the most trafficked roads on the African continent, and higher diversity and sharing of common microsatellite haplotypes among the Ugandan and Tanzanian populations were therefore expected. A recent publication about the correlation between human population movement and malaria movement in Uganda, Tanzania, and Kenya (*39*) illustrates that the major human population movement and malaria movement in Tanzania originates from central Dodoma and directs northward and westward. In this regard, an early selection of a haplotype in the Tanga region (northeast) compared with other areas of Tanzania, can be speculated to be plausible because of the larger levels of parasite migration to northern and western parts of the country, diluting to some extent the newly selected haplotypes in these areas.

In conclusion, we provide evidence that the A581G mutation can arise on various SGEAA ancestral backgrounds, of which we have shown 3 different cases (haplotypes 4, 11, and 18), from areas previously known to represent 2 distinct parasite lineages. Our microsatellite analysis is consistent with reports that the SGEGA triple-mutant alleles are undergoing rapid expansion, and we found evidence of spread of the Tanzania SGEGA haplotype (haplotype 18) as far as southwestern Uganda, which illustrates the potential for dispersal of super-resistant *P. falciparum* malaria throughout the region. Given the rate of increase and the ability of double-mutant allele lineages to acquire the super resistance–conferring A581G mutation independently, it is vital for the continuing effectiveness of prophylaxis with SP that more comprehensive surveillance for the A581G mutation be used to track emerging super-resistant malaria in Africa.

Acknowledgments

Médecins Sans Frontières was responsible for the in vivo study in Humera, Ethiopia, and made samples available for this

study. We thank Ambachew Medhin, Ato Assefaw, and Manica Balasegaram for their participation in this research project. We thank Tarekegn A. Abeku for his work in the Uganda study. Collaboration partners in Tanga, Tanzania, are acknowledged for long-term collaboration resulting in many studies, as well as the samples and study presented here. We thank Ulla Abildtrup for excellent technical assistance in performing some of the SSOP-ELISA analyses.

The study was financially supported by the Gates Malaria Partnership and Danish International Development Agency.

Dr Alifrangis is an associate professor at the faculty of Health and Medical Sciences of the University of Copenhagen, His primary research interest is the use of molecular markers as tools to monitor and possibly hinder emergence and spread of drug resistance in malaria-endemic regions.

References

1. World Health Organization. World malaria report 2012 [cited 2014 May 23]. http://www.who.int/malaria/publications/world_malaria_report_2012/report/en/index.html
2. Aubouy A, Fievet N, Bertin G, Sagbo JC, Kossou H, Kinde-Gazard D, et al. Dramatically decreased therapeutic efficacy of chloroquine and sulfadoxine–pyrimethamine, but not mefloquine, in southern Benin. Trop Med Int Health. 2007;12:886–94. http://dx.doi.org/10.1111/j.1365-3156.2007.01859.x
3. Harinasuta T, Suntharasamai P, Viravan C. Chloroquine-resistant falciparum malaria in Thailand. Lancet. 1965;2:657–60. http://dx.doi.org/10.1016/S0140-6736(65)90395-8
4. Trape JF, Pison G, Preziosi MP, Enel C, Desgrées du Loû A, Delaunay V, et al. Impact of chloroquine resistance on malaria mortality. C R Acad Sci III. 1998;321:689–97. http://dx.doi.org/10.1016/S0764-4469(98)80009-7
5. Talisuna AO, Bloland P, D'Alessandro U. History, dynamics, and public health importance of malaria parasite resistance. Clin Microbiol Rev. 2004;17:235–54. http://dx.doi.org/10.1128/CMR.17.1.235-254.2004
6. Verdrager J, Riche A, Chheang CM. Treatment of "P. falciparum" malaria by delayed-action sulfonamides [in French]. Presse Med. 1967;75:2839–40.
7. Roper C, Pearce R, Nair S, Sharp B, Nosten F, Anderson T. Intercontinental spread of pyrimethamine-resistant malaria. Science. 2004;305:1124. http://dx.doi.org/10.1126/science.1098876
8. Maïga O, Djimdé AA, Hubert V, Renard E, Aubouy A, Kironde F, et al. A shared Asian origin of the triple-mutant *dhfr* allele in *Plasmodium falciparum* from sites across Africa. J Infect Dis. 2007;196:165–72. http://dx.doi.org/10.1086/518512
9. Plowe CV, Cortese JF, Djimde A, Nwanyanwu OC, Watkins WM, Winstanley PA, et al. Mutations in *Plasmodium falciparum dihydrofolate reductase* and *dihydropteroate synthase* and epidemiologic patterns of pyrimethamine–sulfadoxine use and resistance. J Infect Dis. 1997;176:1590–6. http://dx.doi.org/10.1086/514159
10. Pearce RJ, Pota H, Evehe MS, Bâ el-H, Mombo-Ngoma G, Malisa AL, et al. Multiple origins and regional dispersal of resistant dhps in African *Plasmodium falciparum* malaria. PLoS Med. 2009;6:e1000055. http://dx.doi.org/10.1371/journal.pmed.1000055
11. Gatton ML, Martin LB, Cheng Q. Evolution of resistance to sulfadoxine–pyrimethamine in *Plasmodium falciparum.* Antimicrob Agents Chemother. 2004;48:2116–23. http://dx.doi.org/10.1128/AAC.48.6.2116-2123.2004
12. Jelinek T, Ronn AM, Lemnge MM, Curtis J, Mhina J, Duraisingh MT, et al. Polymorphisms in the *dihydrofolate reductase* (*DHFR*) and *dihydropteroate synthetase* (*DHPS*) genes of *Plasmodium falciparum* and in vivo resistance to sulphadoxine/pyrimethamine in isolates from Tanzania. Trop Med Int Health. 1998;3:605–9. http://dx.doi.org/10.1046/j.1365-3156.1998.00280.x
13. World Health Organization. Guidelines for the treatment of malaria. 2nd ed. Geneva: The Organization; 2010 [cited 2014 May 23]. http://whqlibdoc.who.int/publications/2010/9789241547925_eng.pdf
14. World Health Organization. WHO policy recommendation on intermittent preventive treatment during infancy with sulphadoxine–pyrimethamine (SP-IPTi) for *Plasmodium falciparum* malaria control in Africa. Geneva: The Organization; 2010 [cited 2014 May 23]. http://www.who.int/malaria/news/WHO_policy_recommendation_IPTi_032010.pdf
15. World Health Organization. Draft recommendations on intermittent preventive treatment in pregnancy. Geneva: The Organization; 2013 [cited 2014 May 23]. http://www.who.int/malaria/mpac/mpac_sep13_erg_ipt_malaria_pregnancy_report.pdf
16. World Health Organization. WHO policy recommendation: seasonal malaria chemoprevention (SMC) for *Plasmodium falciparum* malaria control in highly seasonal transmission areas of the Sahel sub-region in Africa. Geneva: The Organization; 2012 [cited 2014 May 23]. http://www.who.int/malaria/publications/atoz/smc_policy_recommendation_en_032012.pdf
17. Triglia T, Menting JG, Wilson C, Cowman AF. Mutations in dihydropteroate synthase are responsible for sulfone and sulfonamide resistance in *Plasmodium falciparum.* Proc Natl Acad Sci U S A. 1997;94:13944–9. http://dx.doi.org/10.1073/pnas.94.25.13944
18. Cowman AF, Morry MJ, Biggs BA, Cross GA, Foote SJ. Amino acid changes linked to pyrimethamine resistance in the dihydrofolate reductase-thymidylate synthase gene of *Plasmodium falciparum.* Proc Natl Acad Sci U S A. 1988;85:9109–13. http://dx.doi.org/10.1073/pnas.85.23.9109
19. Brooks DR, Wang P, Read M, Watkins WM, Sims PF, Hyde JE. Sequence variation of the hydroxymethyldihydropterin pyrophosphokinase: dihydropteroate synthase gene in lines of the human malaria parasite, *Plasmodium falciparum*, with differing resistance to sulfadoxine. Eur J Biochem. 1994;224:397–405. http://dx.doi.org/10.1111/j.1432-1033.1994.00397.x
20. Gosling RD, Gesase S, Mosha JF, Carneiro I, Hashim R, Lemnge M, et al. Protective efficacy and safety of three antimalarial regimens for intermittent preventive treatment for malaria in infants: a randomised, double-blind, placebo-controlled trial. Lancet. 2009;374:1521–32. http://dx.doi.org/10.1016/S0140-6736(09)60997-1
21. Gesase S, Gosling RD, Hashim R, Ord R, Naidoo I, Madebe R, et al. High resistance of *Plasmodium falciparum* to sulphadoxine/pyrimethamine in northern Tanzania and the emergence of *dhps* resistance mutation at codon 581. PLoS ONE. 2009;4:e4569. http://dx.doi.org/10.1371/journal.pone.0004569
22. Naidoo I, Roper C. Mapping "partially resistant", "fully resistant", and "super resistant" malaria. Trends Parasitol. 2013;29:505–15. http://dx.doi.org/10.1016/j.pt.2013.08.002
23. Harrington WE, Mutabingwa TK, Muehlenbachs A, Sorensen B, Bolla MC, Fried M, et al. Competitive facilitation of drug-resistant *Plasmodium falciparum* malaria parasites in pregnant women who receive preventive treatment. Proc Natl Acad Sci U S A. 2009;106:9027–32. http://dx.doi.org/10.1073/pnas.0901415106
24. Harrington WE, Morrison R, Fried M, Duffy PE. Intermittent preventive treatment in pregnant women is associated with increased risk of severe malaria in their offspring. PLoS ONE. 2013;8:e56183. http://dx.doi.org/10.1371/journal.pone.0056183
25. Minja DT, Schmiegelow C, Mmbando B, Boström S, Oesterholt M, Magistrado P, et al. *Plasmodium falciparum* mutant haplotype infection during pregnancy associated with reduced birthweight, Tanzania. Emerg Infect Dis. 2013;19:1446–54. http://dx.doi.org/10.3201/eid1909.130133

26. Alifrangis M, Lusingu JP, Mmbando B, Dalgaard MB, Vestergaard LS, Ishengoma D, et al. Five-year surveillance of molecular markers of *Plasmodium falciparum* antimalarial drug resistance in Korogwe District, Tanzania: accumulation of the 581G mutation in the *P. falciparum* dihydropteroate synthase gene. Am J Trop Med Hyg. 2009;80:523–7 .
27. Lynch C, Pearce R, Pota H, Cox J, Abeku TA, Rwakimari J, et al. Emergence of a *dhfr* mutation conferring high-level drug resistance in *Plasmodium falciparum* populations from southwest Uganda. J Infect Dis. 2008;197:1598–604. http://dx.doi.org/10.1086/587845
28. Karema C, Imwong M, Fanello CI, Stepniewska K, Uwimana A, Nakeesathit S, et al. Molecular correlates of high-level antifolate resistance in Rwandan children with *Plasmodium falciparum* malaria. Antimicrob Agents Chemother. 2010;54:477–83. http://dx.doi.org/10.1128/AAC.00498-09
29. Alker AP, Kazadi WM, Kutelemeni AK, Bloland PB, Tshefu AK, Meshnick SR. *dhfr* and *dhps* genotype and sulfadoxine–pyrimethamine treatment failure in children with falciparum malaria in the Democratic Republic of Congo. Trop Med Int Health. 2008;13:1384–91. http://dx.doi.org/10.1111/j.1365-3156.2008.02150.x
30. Spalding MD, Eyase FL, Akala HM, Bedno SA, Prigge ST, Coldren RL, et al. Increased prevalence of the *pfdhfr*/*phdhps* quintuple mutant and rapid emergence of *pfdhps* resistance mutations at codons 581 and 613 in Kisumu, Kenya. Malar J. 2010;9:338. http://dx.doi.org/10.1186/1475-2875-9-338
31. Ishengoma DS, Mmbando BP, Segeja MD, Alifrangis M, Lemnge MM, Bygbjerg IC. Declining burden of malaria over two decades in a rural community of Muheza district, north-eastern Tanzania. Malar J. 2013;12:338. http://dx.doi.org/10.1186/1475-2875-12-338
32. Wooden J, Kyes S, Sibley CH. PCR and strain identification in *Plasmodium falciparum*. Parasitol Today. 1993;9:303–5. http://dx.doi.org/10.1016/0169-4758(93)90131-X
33. Alifrangis M, Enosse S, Pearce R, Drakeley C, Roper C, Khalil IF, et al. A simple, high-throughput method to detect Plasmodium falciparum single nucleotide polymorphisms in the *dihydrofolate reductase, dihydropteroate synthase*, and *P. falciparum chloroquine resistance transporter* genes using polymerase chain reaction- and enzyme-linked immunosorbent assay-based technology. Am J Trop Med Hyg. 2005;72:155–62 .
34. Roper C, Pearce R, Bredenkamp B, Gumede J, Drakeley C, Mosha F, et al. Antifolate antimalarial resistance in southeast Africa: a population-based analysis. Lancet. 2003;361:1174–81. http://dx.doi.org/10.1016/S0140-6736(03)12951-0
35. Zeile I, Gahutu JB, Shyirambere C, Steininger C, Musemakweri A, Sebahungu F, et al. Molecular markers of *Plasmodium falciparum* drug resistance in southern highland Rwanda. Acta Trop. 2012;121:50–4. http://dx.doi.org/10.1016/j.actatropica.2011.09.009
36. Gadalla NB, Abdallah TM, Atwal S, Sutherland CJ, Adam I. Selection of *pfdhfr*/*pfdhps* alleles and declining artesunate/sulphadoxine–pyrimethamine efficacy against *Plasmodium falciparum* eight years after deployment in eastern Sudan. Malar J. 2013;12:255. http://dx.doi.org/10.1186/1475-2875-12-255
37. Iriemenam NC, Shah M, Gatei W, van Eijk AM, Ayisi J, Kariuki S, et al. Temporal trends of sulphadoxine–pyrimethamine (SP) drug-resistance molecular markers in *Plasmodium falciparum* parasites from pregnant women in western Kenya. Malar J. 2012;11:134. http://dx.doi.org/10.1186/1475-2875-11-134
38. Al-Saai S, Kheir A, Abdel-Muhsin AM, Al-Ghazali A, Nwakanma D, Swedberg G, et al. Distinct haplotypes of *dhfr* and *dhps* among *Plasmodium falciparum* isolates in an area of high level of sulfadoxine–pyrimethamine (SP) resistance in eastern Sudan. Infect Genet Evol. 2009;9:778–83. http://dx.doi.org/10.1016/j.meegid.2009.04.010
39. Pindolia DK, Garcia AJ, Huang Z, Smith DL, Alegana VA, Noor AM, et al. The demographics of human and malaria movement and migration patterns in East Africa. Malar J. 2013;12:397. http://dx.doi.org/10.1186/1475-2875-12-397

Address for correspondence: Sidsel Nag, Center for Medical Parasitology, 7680 CSS Bldg 22, Bartholinsgade 2 1356 København K, Denmark; email: sidselnag@sund.ku.dk

Global and Local Persistence of Influenza A(H5N1) Virus

Xianbin Li, Zhong Zhang, Ailian Yu, Simon Y. W. Ho, Michael J. Carr, Weimin Zheng, Yanzhou Zhang, Chaodong Zhu,[1] Fumin Lei,[1] and Weifeng Shi[1]

An understanding of the global migration dynamics of highly pathogenic avian influenza A(H5N1) virus is helpful for surveillance and disease prevention. To characterize the migration network of this virus, we used genetic analysis, which supported a global persistence model in which each of 9 regions acts to some extent as a source. Siberia is the major hub for the dispersal of the virus. Southeast Asia and Africa are major sources of genetically and antigenically novel strains. We found evidence of local persistence of the virus in Southeast Asia and Africa, which is rare for human influenza A viruses. The differences in migration dynamics between avian and human influenza viruses might help with the design of region-specific surveillance efforts and the selection of vaccine candidates.

Highly pathogenic avian influenza (HPAI) A(H5N1) virus is an ineradicable zoonotic virus that continues to mutate and reassort in nature and poses a serious threat to avian and human health. As the natural hosts of avian influenza viruses, wild birds are the main reservoir for the HPAI (H5N1) pandemic; whether these birds contribute to the viruses' global circulation remains under debate (*1*–*3*).

Since their emergence in China in 1996 (*4*), HPAI (H5N1) viruses have spread to most Eurasian and African countries and have caused 650 laboratory-confirmed cases of human infection and 386 deaths (*5*). Understanding the migration dynamics of HPAI (H5N1) viruses is thus essential for surveillance and prevention of these infections in birds and humans and for policy decisions on vaccine development and/or implementation.

Numerous genetic studies have been conducted to determine the mechanisms underlying influenza A virus seasonality among humans; most results support a model of global migration (*6*–*11*). Rambaut et al. proposed a source–sink model for virus ecology (*7*), in which the tropics are the source regions and the subtropical and temperate zones of the Northern and Southern Hemispheres are the sink regions. Similarly, Russell et al. suggested that eastern and Southeast Asia comprise a regional circulation network that is the leading region for the evolution of human influenza viruses (*8*). However, Bedford et al. found that seasonal epidemics in the United States had seeded epidemics around the world in a pattern called global persistence (*9*). More recently, Bahl et al. found that the tropics (e.g., Southeast Asia and Hong Kong) did not maintain a source for annual epidemics of influenza A(H3N2) virus infection (*12*). Alternatively, each geographic region might act as a potential source, supporting the global persistence model.

Also extensively studied have been the migration mechanisms of avian influenza A(H5N1) virus (*13*–*15*). Despite the use of different methods, many studies reached the same conclusion: China is the source of multiple clusters of influenza A(H5N1) viruses identified from other countries in eastern and Southeast Asia (*13*–*18*). Liang et al. have also proposed that southern China and Southeast Asia might be the source of influenza A(H5N1) virus, seeding outbreaks elsewhere, and that eastern Siberia might be the source of influenza A(H5N1) virus cross-infection and genetic reassortment (*19*).

However, several questions remain with regard to the migration of HPAI (H5N1) viruses. For example, what are the features of their global migration network? Which region acts as the key node? Is southern China the only source of novel HPAI (H5N1) viruses? If not, what are the other sources? Are the sources stable, or do their contributions change with time?

To address these questions, we analyzed a large number of hemagglutinin gene sequences of influenza A(H5N1) viruses from avian hosts by using BEAST (*20*) and Migrate (*21*,*22*), which can estimate genetic diversity of each region and migration rates between regions. On the basis of these findings, we characterized the global migration network and studied the migratory mechanism of HPAI (H5N1) viruses.

Author affiliations: Chinese Academy of Sciences, Shenzhen, China (X. Li); Taishan Medical College, Taian, Shandong, China (Z. Zhang, A. Yu, W. Shi); Chinese Academy of Sciences, Beijing, China (X. Li, W. Zheng, Y. Zhang, C. Zhu, F. Lei); University of Sydney, Sydney, New South Wales, Australia (S.Y.W. Ho); and University College Dublin, Dublin, Ireland (M.J. Carr)

DOI: http://dx.doi.org/10.3201/eid2008.130910

[1]These authors contributed equally to this article.

Materials and Methods

Sequence Data and Genetic Diversity

All available sequences of the hemagglutinin gene of HPAI (H5N1) viruses isolated from avian hosts were obtained from Influenza Virus Resources at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/FLU/). These sequences were aligned by using MUSCLE (*23*). After short sequences (>60 bp shorter than the full-length hemagglutinin-1) were removed, the final dataset included 3,365 sequences from 9 geographic regions, which, to our knowledge, made it the largest influenza A(H5N1) virus dataset analyzed (Table 1; online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/20/8/13-0910-Techapp1.pdf). Therefore, we consider that the sequence data available in the database are informative and representative of the geographic distribution and global circulation of HPAI (H5N1) viruses, although they were not obtained through systematic global influenza virus sampling that was random in terms of time and space.

To evaluate whether the classification of 9 regions was appropriate, we used the same method for estimating nucleotide diversity of avian influenza A(H5N1) virus that had been used for influenza A(H3N2) virus (9). Within-region nucleotide diversity was estimated in terms of π_w:

$$\pi_w = \frac{1}{n}\sum_{i=1}^{n} \pi^{(i,i)}$$

where n is the number of regions and $\pi^{(ii)}$ refers to diversity estimates in which both samples in each pair are from region *i*.

The overall between-region diversity was estimated as π_b

$$\pi_b = \frac{2}{n(n-1)}\sum_{i=1}^{n}\sum_{j=i+1}^{n} \pi^{(i,j)}$$

where $\pi^{(i,j)}$ refers to diversity estimates in which 1 sample is from region *i* and the other sample is from region *j*.

Confidence intervals were estimated by taking 1,000 bootstrap replicates from the total pool of sequences. F_{ST} (genetic distance) was calculated as $(\pi_b\text{-}\pi_w)/\pi_b$, with $F_{ST} > 0$ indicating genetic isolation among regions (*24*) and supporting the geographic classification mentioned above.

Estimating Global Parameters and Testing Geographic Association at Tips

The program Migrate requires input of 2 parameters: the transition/transversion ratio (κ) and the rate of nucleotide substitution (μ). We estimated these parameters by using the Bayesian phylogenetic method implemented in BEAST version 1.7.2 (*20*). For all analyses, we used the uncorrelated lognormal relaxed molecular clock to accommodate rate variation among lineages (*25*). We used the HKY85 model (*26*) of nucleotide substitution to parameterize the mutational process; equilibrium nucleotide frequencies were derived from observed frequencies; equilibrium nucleotide frequency rates were homogeneous across sites. Posterior distributions of parameters were estimated by using Markov chain Monte Carlo (MCMC) sampling. Samples were drawn every 5,000 steps over a total of 4.0×10^7 steps, and 2.5×10^7 steps were removed as burn-in. The transition/transversion ratio (κ) was estimated to be 9.163 (95% CI 8.633–9.676). The rate of nucleotide substitution () was estimated to be 5.595×10^{-3} substitutions/site/year (95% CI 5.249×10^{-3} to 5.970×10^{-3} substitutions/site/year).

To estimate the extent of geographic structure (extent to which viruses from the same geographic region are more likely to cluster together in the phylogenetic tree than expected by chance) in the HPAI (H5N1) influenza virus populations, we performed a phylogenetic-trait association analysis on the posterior distribution of trees produced by BEAST. These geographic regions were coded onto the tips of the 3,000 trees sampled from the posterior, which were then analyzed by using the maximum monophyletic clade size statistic implemented in the Bayesian Analysis of Time Series program with 1,000 randomizations (*27*). For each of the 9 regions included in the analysis, the Bayesian Analysis of Time Series program was used to calculate a p value, which indicated whether the sequences from this region are more inclined to cluster together in the tree than expected by chance.

Estimating Migration Rates between Regions through Resampling

To estimate coalescent parameters for each geographic region, we used an MCMC technique implemented in Migrate version 3.3.0 (*21*,*22*). The prior distribution of Θ (mutation-scaled population size) and 2 Nm (migration rate) values was assumed to be exponential with a mean of 1, and mutational parameters were fixed in the analyses. To minimize the influence of potential sampling biases on our results, we performed independent analyses of 100 resampled replicates. For each replicate, we randomly sampled 50 sequences without replacement from each region (online Technical Appendix Table 2). For each of the 100 bootstrap replicates, 50 MCMC simulations were run for 6×10^6 steps each. The first 5×10^6 steps of each chain were removed as burn-in. Parameter values were sampled every 10^4 steps. Convergence was assessed visually and through comparison of chains by using the Gelman-Rubin convergence statistic (*28*). We combined the remaining samples from each chain to give a total of 5,000 samples for each of the resampled replicates. Estimates of migration rates varied little across the 100 replicates (online Technical Appendix Table 1); mean values are shown in Table 2.

Table 1. Statistical analysis of geographic structure for highly pathogenic avian influenza A(H5N1) viruses*

Region	Maximum monophyletic clade size				
	Observed mean	95% CI	Null mean	95% CI	Significance, p value
South Korea and Japan	50.44	49.0–68.0	1.66	1.19–2.04	0.001
Siberia	8.90	8.0–11.0	1.59	1.11–2.01	0.001
Southeast Asia	311.54	296.0–440.0	4.57	3.93–5.95	0.001
Africa	47.73	38.0–64.0	3.55	3.12–4.30	0.001
Hong Kong	17.00	17.0–17.0	1.99	1.65–2.24	0.001
China	32.88	28.0–42.0	3.74	3.23–4.65	0.001
Europe	80.08	80.0–80.0	2.21	2.01–3.00	0.001
Central and western Asia	5.23	3.0–8.0	1.32	1.00–2.00	0.001
Southern Asia	106.00	106.0–106.0	2.03	1.75–2.30	0.001

*Analyzed by using Bayesian Analysis of Time Series (*27*).

Genealogical Inference and Trunk Extraction

Most of the viruses we analyzed were isolated from domestic chickens and ducks. To infer the genealogy, we reduced the dataset so that a maximum of 30 sequences per year were sampled from chickens and ducks from each region. Combining this subset of sequences with those from the other hosts yielded a final dataset of 2,392 sequences; this dataset had notably fewer sequences from Africa, China, and Southeast Asia than did the original dataset (online Technical Appendix Table 2). We fixed Θ and 2Nm at the values estimated in the previous analysis.

We ran 4 MCMC chains for 2×10^8 steps each, of which the first 10^8 steps were removed as burn-in; genealogies were sampled every 10^5 steps. We combined the remaining samples of 4,000 genealogical trees and performed trunk reconstruction on them.

Trunk extraction and processing was performed by using the program PACT (http://www.trevorbedford.com/pact), which is able to estimate the mean and 95% credible interval for the proportion of the trunk assigned to each geographic region (*9*). The bigger the proportion, the more the corresponding region accounts for virus variation and evolution. By calculating the proportion of sampled genealogies for which the trunk is assigned to a particular region at different points in time, we could also assess the temporal dynamics, which illustrate the annual change of trunk proportion for each region.

Testing the Robustness of the Results

For many sequences, the time of isolation is known to the nearest year only, which limits the precision of estimates of relative genetic diversity through time. Therefore, we further analyzed a subsample in which we included only sequences isolated during 2006–2011 and for which detailed isolation times were available. This dataset included 1,173 samples from 6 geographic regions. We repeated the above analyses on this subset of the dataset. Our sampling strategies can be found in online Technical Appendix Table 3. MCMC simulations were run for 4×10^7 steps, and 2×10^7 steps were removed as burn-in.

Estimating Genetic Diversity of Each Region

To estimate the relative genetic diversity of each region through time, we extracted sequences of viruses from each region from the subsampled subdataset with 1,173 sequences from 2006 through 2011, which composed 5 new datasets: for Africa, Southeast Asia, China, southern Asia, and Europe (including Siberia). Each of these datasets was analyzed by using the Bayesian skyride method (*29*) in BEAST. Because the sizes of these subdatasets differed, we ran MCMC simulations for different steps for each and collected samples every 10^4 steps.

Results

Geographic Structure

Our dataset included 3,365 HPAI (H5N1) virus hemagglutinin gene sequences exclusively identified from nonmammalian hosts. On the basis of geography and sampling density, we classified these sequences into 9 regions: China (mainland China, n = 768), Hong Kong Special Administrative Region (n = 168), South Korea and Japan (n = 105), Siberia (n = 95), Southeast Asia (n = 1,024), southern Asia (n = 176), western and central Asia (n = 64), Europe (n = 261), and Africa (n = 704). This geographic classification is significantly supported by a phylogenetic trait–association test; all p values were <0.001, indicating that the sequences are more inclined to cluster together by geographic location than would be expected by chance (Table 1). Moreover, on average, genetic diversity between regions, $\pi_b = 42.64 \times 10^{-3}$ (95% CI 41.09×10^{-3}–48.81×10^{-3}), is greater than within regions, $\pi_w = 17.21 \times 10^{-3}$ (95% CI 16.33×10^{-3}–18.19×10^{-3}). F_{ST} was estimated to be 0.596 (95% CI 0.573-0.649), indicating genetic isolation among regions and also supporting the rationale for classifying the HPAI (H5N1) virus hemagglutinin sequences into 9 regions (*24*). Further information about regional genetic diversity is provided in online Technical Appendix Table 4.

Global Migration and Persistence Indicated by Migration Rates

We used the sampling strategy of Bedford et al. to study the global migration of human influenza A(H3N2)

Table 2. Means and 95% credible intervals of the total immigration and emigration rates for highly pathogenic avian influenza A(H5N1) viruses across resampled replicates and trunk proportion of each region, 1996–2012*

Region	Mean (95% credible interval)		
	Immigration	Emigration	Trunk proportion
Europe	1.24 (0.61–2.25)	0.89 (0.49–1.58)	0.01 (0.00–0.05)
Hong Kong	0.65 (0.34–1.13)	0.67 (0.41–1.05)	0.06 (0.04–0.12)
Africa	0.26 (0.18–0.38)	0.98 (0.62–1.45)	0.25 (0.23–0.29)
China	0.46 (0.25–0.81)	0.69 (0.41–1.08)	0.24 (0.17–0.31)
Central and western Asia	1.95 (1.18–2.95)	0.71 (0.40–1.24)	0.01 (0.00–0.03)
Southern Asia	0.40 (0.28–0.60)	0.56 (0.32–0.93)	0.02 (0.00–0.10)
Southeast Asia	0.25 (0.16–0.38)	0.37 (0.23–0.56)	0.38 (0.35–0.41)
South Korea and Japan	1.05 (0.52–2.72)	1.03 (0.36–2.60)	0.00 (0.00–0.02)
Siberia	1.45 (0.57–3.35)	1.81 (0.84–4.05)	0.04 (0.00–0.11)

*Migration rates are given in migration events per lineage per year.

virus (*9*). Our results support a global migration model (Figure 1; Table 2) in which all regions in the analysis are connected to form a global migration network; the migration rate between any 2 regions is >0 (online Technical Appendix Table 1). However, the geographic regions play different roles in the connectivity of the global migration network, as indicated by the varying circle sizes in Figure 1; all 9 regions act to some extent as sources. Specifically, migration rates between temperate regions >0 indicate that 1 temperate region could seed epidemics in other temperate regions. Therefore, these results support a model for the global persistence of HPAI (H5N1) virus (*9*,*12*).

In detail, this network contains 4 notable features (Figure 1; Table 2). First, Siberia is the most active node; the immigration rate is the second highest, and the emigration rate is the highest. High rates of migration are found for Siberia to South Korea and Japan, western and central Asia, and Europe. Second, for central and western Asia, the immigration rate is very high; the 3 major sources are Africa, Siberia, and Europe. Third, for Southeast Asia and Africa, immigration rates are relatively low (0.25 and 0.26, respectively), and for Africa and China, immigration rates are lower than emigration rates. Fourth, for South Korea and Japan, immigration and emigration rates are high. Pairwise immigration and emigration rates are given in online Technical Appendix Table 1.

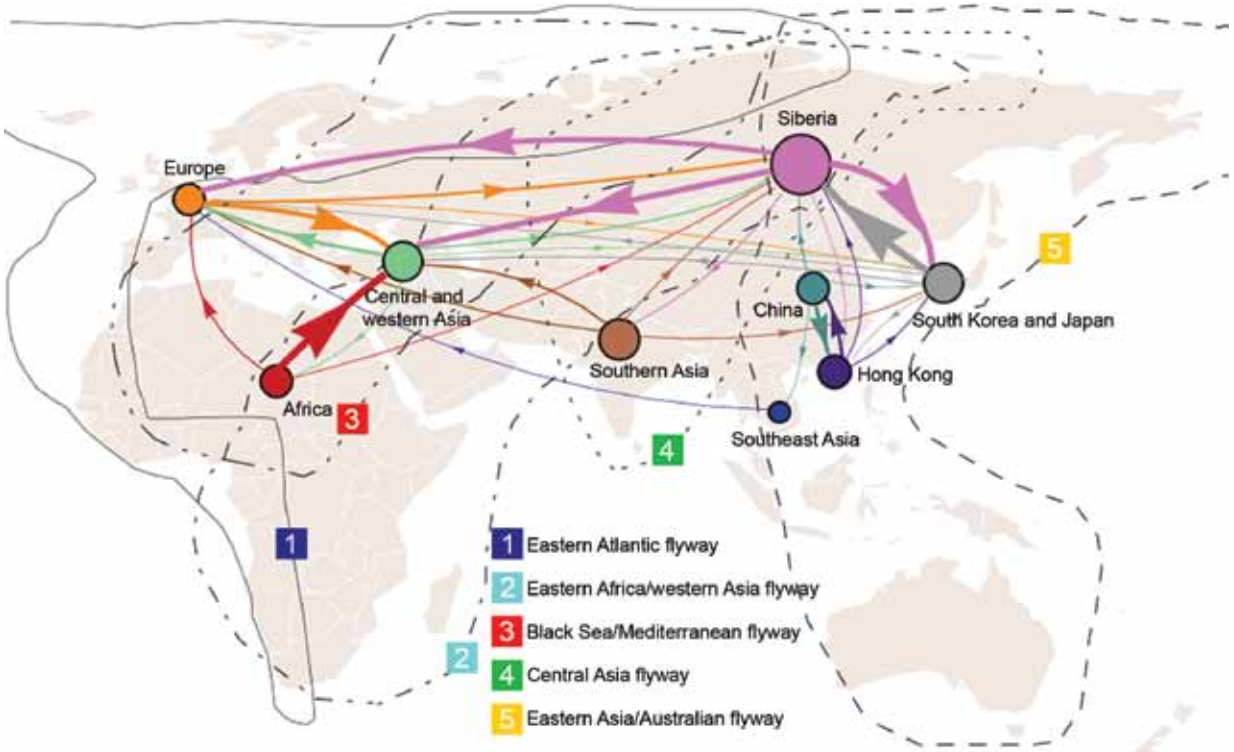


Figure 1. Global migration patterns of highly pathogenic avian influenza A(H5N1) viruses estimated from sequence data sampled during 1996–2012. Arrows represent direction of movement, and arrow width is proportional to the migration rate. Migration rates <0.07 migration events per lineage per year are not shown. The area of each circle is proportional to the region's eigenvector centrality; larger circles indicate crucial nodes in the migration network.

Genealogical History and Persistence of HPAI (H5N1) Viruses

We next inferred the genealogical history of the HPAI (H5N1) virus population. As described in previous studies, a human influenza virus tree can be characterized by a long trunk and short side branches (*9*,*30*). In brief, the trunk of a genealogical tree is composed of progenitor strains whose mutations are maintained (*9*). Therefore, strains located along the trunk account for a greater amount of virus evolution and genetic variation than do the strains on the side branches.

Consistent with our findings described above, the reconstructed genealogical history supports a global migration model because several lineages consist of virus strains from different geographic regions (e.g., g1–g4 in Figure 2). However, after viruses of these lineages emerge, they spread to other geographic regions, and generally these viruses persist over years through migration (e.g., g2–g4 in Figure 2). Therefore, the genealogy of HPAI (H5N1) viruses also supports a model of global persistence.

In contrast with human influenza A/H3N2 virus phylogenies, the HPAI (H5N1) influenza virus tree shows several long side branches (Figure 2), which supports a model of local persistence (lp) (*9*,*30*,*31*). These viruses on the side branches reside in Southeast Asia (lp3.1 to lp3.3), Africa (lp1.1 and lp1.2), and southern Asia (lp2). Of particular

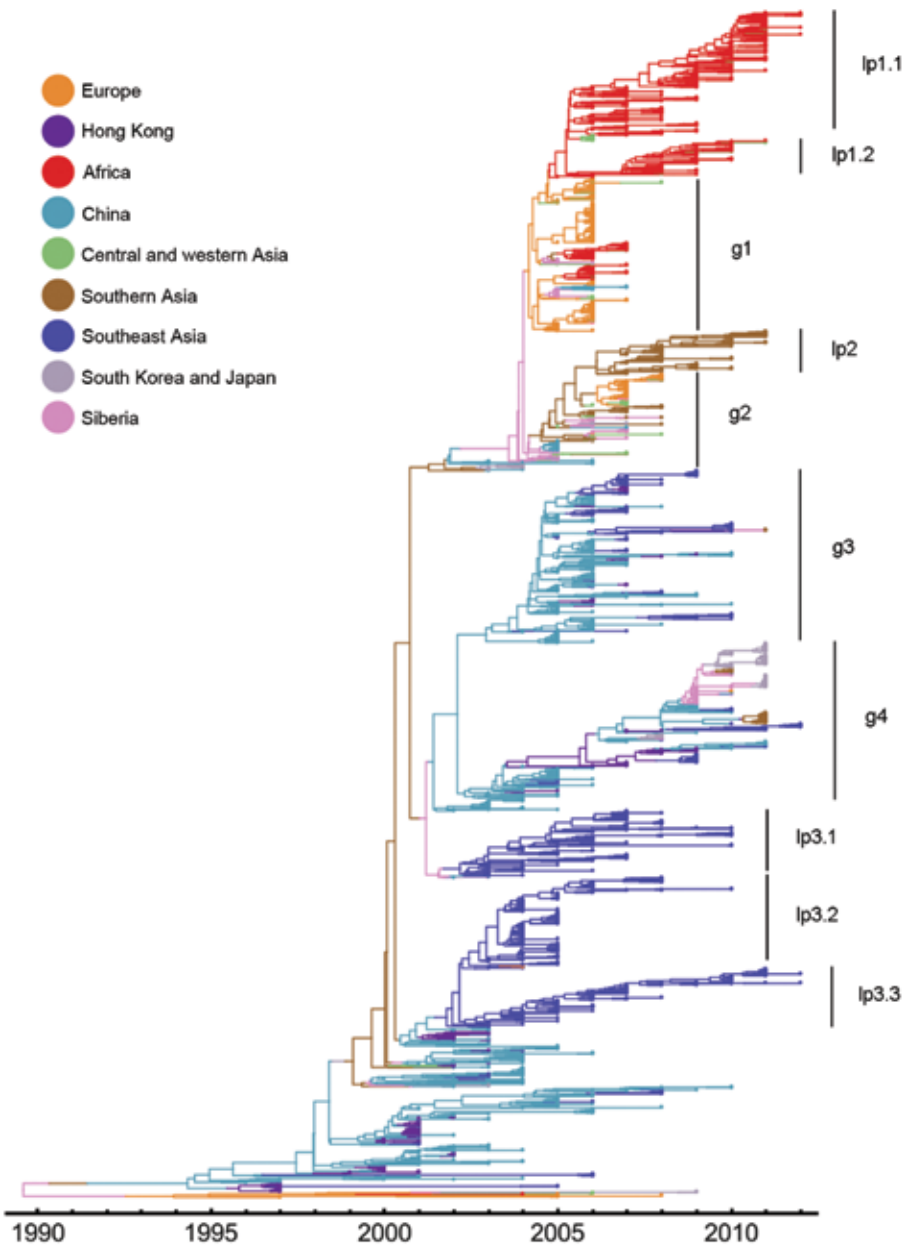


Figure 2. Estimated genealogy of 3,365 highly pathogenic avian influenza A(H5N1) viruses sampled during 1996–2012. The maximum a posteriori tree was estimated by using Migrate version 3.3.0 (*21*,*22*). Each tip represents a virus sequence. Colors indicate the sampling region, either actual (tips) or estimated (branches).

note, these lineages can persist over extended periods. For example, a lineage from Southeast Asia persisted from around 2002 to 2012 (lp3.3). Local persistence of HPAI (H5N1) virus has led to the co-circulation of different lineages in Southeast Asia during 2002–2010.

Trunk Proportion for Different Regions

Using a structured coalescent approach, we calculated the proportion of the trunk assigned to each geographic region (Table 2). Generally, a higher trunk proportion implies that the corresponding region accounts for more virus variation and evolution and that that region is more likely to be the source of the virus. In contrast, despite previous evidence that China was the region containing the influenza source population for HPAI (H5N1) viruses (*13–18*), only 24% (95% CI 17%–31%) of the trunk of the genealogical tree is assigned to China. Instead, Southeast Asia occupies the largest proportion of the trunk, 38% (95% CI 35%–41%), and the contribution of Africa to the trunk is 25% (95% CI 23%–29%). Although Hong Kong has a robust surveillance and reporting system for cases of HPAI virus infection, it accounts for a comparatively low level of the trunk of the influenza A(H5N1) virus genealogy, 6% (95% CI 4%–12%).

Further analysis of the trunk proportion reveals a dynamic change of regions over time (Figure 3), suggesting that the contribution of each geographic region to virus variation and evolution changes annually. Before 2004, China was the source for most of the evolution and variation of HPAI (H5N1) viruses. After 2001, however, Southeast Asia began to act as another primary source for novel strains. Since 2004, viruses on the trunk have resided less in China and more in Southeast Asian and African countries.

Genetic Diversity of HPAI (H5N1) Viruses from Different Regions

Because only regions with higher genetic diversity are most likely to be the virus source, we calculated the genetic diversity of 5 geographic regions by using sequences from 2006 through 2011 for which month of isolation was known. In this analysis, Europe and Siberia were combined because $F_{ST} = 0$, indicating a lack of genetic isolation between them (*24*). Although Africa, Southeast Asia, and China show influenza seasonality to different degrees, genetic diversities of HPAI (H5N1) viruses from these 3 regions are higher than those of the remaining regions throughout the year (Figure 4). Specifically, virus genetic diversity is highest in Africa and second highest in Southeast Asia; both regions maintain a relatively high level of diversity throughout the year. Furthermore, virus genetic diversity in Africa shows a clear seasonal pattern of change; peaks occur during the Northern Hemisphere winter. Activity of HPAI (H5N1) virus in Southeast Asia and China typically, but not always, peaks during the Northern Hemisphere winter (Figure 4).

Discussion

Our large-scale genetic analysis of HPAI (H5N1) viruses supports a global persistence model in which each region acts to some extent as a source. Siberia seems to play a vital role in this migration network, connecting Europe, central and western Asia, and South Korea and Japan. This finding is consistent with the fact that multiple bird migration flyways intersect in Siberia; the region is also one of the most commonly used breeding sites and in summer contains a high number of wild birds (*19*).

Although it has not been widely acknowledged that migratory birds act as vectors for the spread of HPAI (H5N1) viruses in Eurasia and Africa (*32–34*), it is intriguing that the migration network described here approximates the major flyways of migratory birds. This finding coincides with evidence obtained by comparing the spatiotemporal characteristics of wild bird migration and influenza A(H5N1) virus outbreaks, especially along the central Asia flyway (*19*,*35–37*).

Southern China has been regarded as the source of HPAI (H5N1) viruses (*13–18*). However, our results show

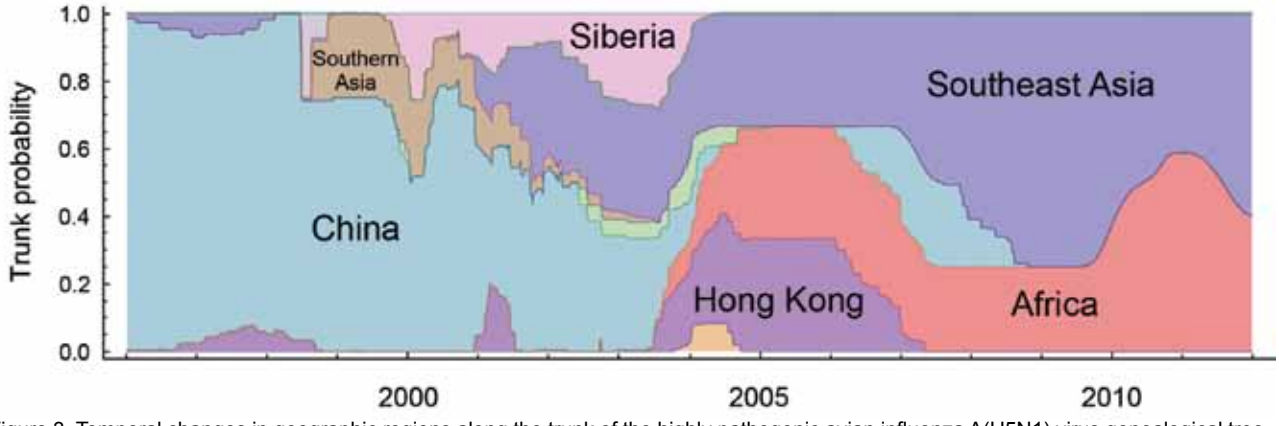


Figure 3. Temporal changes in geographic regions along the trunk of the highly pathogenic avian influenza A(H5N1) virus genealogical tree.

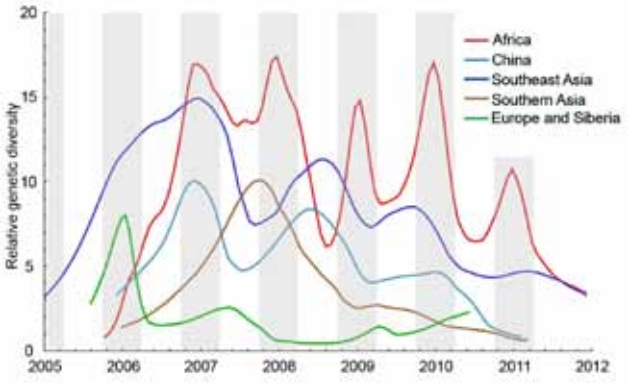


Figure 4. Bayesian skyride median of relative genetic diversity of highly pathogenic avian influenza (H5N1) virus in each region, 2006–2011. Shading represents winter (October–March) in the Northern Hemisphere.

that China is not the only source, although our data support the view that China was the major center for the evolution and variation of HPAI (H5N1) virus before 2004. The data also indicate that the tropics (Southeast Asia and Africa) have been the major sources since 2004. The role of these regions as genetic reservoirs for subtype H5N1 viruses is underscored by the higher genetic diversities of viruses in these regions compared with those of other regions. Therefore, increased sampling from Southeast Asia and Africa is vital for understanding the global dynamics of HPAI (H5N1) viruses.

With regard to emergence of novel virus variants, our results also support a source–sink model for HPAI (H5N1) virus, as described for human influenza A virus subtypes H1N1 and H3N2 (*7*), in which the tropics are the source regions and the Northern and Southern Hemispheres are the sink regions. This finding is not inconsistent with the global migration pattern of HPAI (H5N1) virus because China, as referred to here, is largely represented by tropical/subtropical southern China (*13*–*16*). In addition, most (93%) of the trunk of the genealogical tree is in the tropics and China (including Hong Kong), and the remaining regions, such as Siberia, play major roles in migration and genetic reassortment and are less responsible for emergence of variants with novel hemagglutinin proteins.

The relative genetic diversities of the viruses in Africa, Southeast Asia, and China change regularly over time, but peaks do not always appear concurrently among regions. Generally, peaks of influenza virus activity in Africa appear in winter, whereas those of Southeast Asia and China sometimes appear in other seasons. This finding can potentially be explained by the fact that influenza A(H5N1) virus has become endemic to Southeast Asia and China, where >70% of domestic ducks are raised. Domestic ducks can asymptomatically shed high titers of subtype H5N1 virus for several days (*38*).

The influenza A(H5N1) virus genealogical tree notably contains long side branches; some lineages (mostly comprising hemagglutinin sequences from Southeast Asia, Africa, and southern Asia) persist for years. These phylogenies support a model of local persistence of HPAI (H5N1) viruses. Specially, local persistence has led to co-circulation of multiple lineages and is likely to confound efforts to control the spread and selection of HPAI (H5N1) virus vaccine candidates. However, these patterns contrast with those described for seasonal human influenza A(H3N2) virus, for which global persistence plays a much larger role in the migration network (*9*,*12*).

Likewise, for human influenza A viruses, mutations on side branches have limited effects with regard to producing antigenically novel variants. This limitation is because mutations on side branches experience genetic bottlenecks and are quickly lost, such as at the end of peak influenza season (*9*,*30*). For HPAI (H5N1) viruses, however, mutations on side branches can have influential effects, because these mutations will be maintained for years because of local and/or global virus persistence.

The main study results were obtained by using the sampling year of the sequences. To evaluate effects of this potential bias on our results, we repeated the analyses by using a subsample of our dataset with sequences for which year and month of collection were known. Results from this subsample are broadly consistent with those from the whole dataset. Therefore, use of only the sampling year plays a limited role in the results. Detailed information about this subsample and the results of our additional analyses are provided in the online Technical Appendix Figures 2–5 and Tables 3 and 5–8.

In conclusion, we characterized the major features of the HPAI (H5N1) virus migration network and found evidence to support global and local persistence of this virus. We also drew attention to the role of Southeast Asia and Africa as genetic reservoirs in the origins of genetically and antigenically novel influenza A(H5N1) virus variants, which has, to our knowledge, been previously underestimated. Our results call for reassessment of the role of each geographic region in the migration network and in the genetic source of HPAI (H5N1) viruses and suggest that region-specific surveillance policies and vaccine candidate selection strategies should be considered.

Acknowledgments

We thank T. Bedford for his assistance with the program Migrate. Most of the computation in this study was performed on the clusters in the Institute of Zoology, Chinese Academy of Sciences. We also thank X. Y. Lang, X. N. Wang, and their colleagues in the Supercomputing Center, Computer Network Information Center of The Chinese Academy of Sciences, for help with computation on the Science Grid.

This study was supported by the Doctoral Starting up Foundation of Taishan Medical College (to W.S.) and by a grant from the International Development Research Centre (to W.S. and F.L.). F.L. was also supported by an intramural special grant for influenza virus research from the Chinese Academy of Sciences (KSZD-EW-A-002).

Mr Li is a master's degree candidate at the Shenzhen Institute of Advanced Technology, Chinese Academy of Sciences. His research interests involve computer science and bioinformatics.

References

1. Kilpatrick AM, Chmura AA, Gibbons DW, Fleischer RC, Marra PP, Daszak P. Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci U S A. 2006;103:19368–73. http://dx.doi.org/10.1073/pnas.0609227103
2. Gauthier-Clerc M, Lebarbenchon C, Thomas F. Recent expansion of highly pathogenic avian influenza H5N1: a critical review. Ibis. 2007;149:202–14. http://dx.doi.org/10.1111/j.1474-919X.2007.00699.x
3. Altizer S, Bartel R, Han BA. Animal migration and infectious disease risk. Science. 2011;331:296. http://dx.doi.org/10.1126/science.1194694
4. Xu X, Subbarao, Cox NJ, Guo Y. Genetic characterization of the pathogenic influenza A/goose/Guangdong/1/96 (H5N1) virus: similarity of its hemagglutinin gene to those of H5N1 viruses from the 1997 outbreaks in Hong Kong. Virology. 1999;261:15–9. http://dx.doi.org/10.1006/viro.1999.9820
5. World Health Organization. Cumulative number of confirmed human cases of avian influenza A(H5N1) reported to WHO, 2003–2013. 2013 June 4 [cited 2013 Jun 11]. http://www.who.int/influenza/human_animal_interface/EN_GIP_20130604CumulativeNumberH5N1cases.pdf
6. Nelson MI, Simonsen L, Viboud C, Miller MA, Holmes EC. Phylogenetic analysis reveals the global migration of seasonal influenza A viruses. PLoS Pathog. 2007;3:1220–8. http://dx.doi.org/10.1371/journal.ppat.0030131
7. Rambaut A, Pybus OG, Nelson MI, Viboud C, Taubenberger JK, Holmes EC. The genomic and epidemiological dynamics of human influenza A virus. Nature. 2008;453:615–9. http://dx.doi.org/10.1038/nature06945
8. Russell CA, Jones TC, Barr IG, Cox NJ, Garten RJ, Gregory V, et al. The global circulation of seasonal influenza A (H3N2) viruses. Science. 2008;320:340–6. http://dx.doi.org/10.1126/science.1154137
9. Bedford T, Cobey S, Beerli P, Pascual M. Global migration dynamics underlie evolution and persistence of human influenza A (H3N2). PLoS Pathog. 2010;6:e1000918. http://dx.doi.org/10.1371/journal.ppat.1000918
10. Chan J, Holmes A, Rabadan R. Network analysis of global influenza spread. PLOS Comput Biol. 2010;6:e1001005. http://dx.doi.org/10.1371/journal.pcbi.1001005
11. Le MQ, Lam HM, Cuong VD, Lam TT, Halpin RA, Wentworth DE, et al. Migration and persistence of human influenza A viruses, Vietnam, 2001–2008. Emerg Infect Dis. 2013;19:1756–65. http://dx.doi.org/10.3201/eid1911.130349
12. Bahl J, Nelson MI, Chan KH, Chen R, Vijaykrishna D, Halpin RA, et al. Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans. Proc Natl Acad Sci U S A. 2011;108:19359–64. http://dx.doi.org/10.1073/pnas.1109314108
13. Wu B, Wang C, Dong G, Guo Y, Nolte DL, Deliberto TJ, et al. New evidence suggests southern China as a common source of multiple clusters of highly pathogenic H5N1 avian influenza virus. J Infect Dis. 2010;202:452–8. http://dx.doi.org/10.1086/653709
14. Wallace RG, Hodac H, Lathrop RH, Fitch WM. A statistical phylogeography of influenza A H5N1. Proc Natl Acad Sci U S A. 2007;104:4473–8. http://dx.doi.org/10.1073/pnas.0700435104
15. Wallace RG, Fitch WM. Influenza A H5N1 immigration is filtered out at some international borders. PLoS ONE 2008;3(2):e1697.
16. Smith GJ, Fan XH, Wang J, Li KS, Qin K, Zhang JX, et al. Emergence and predominance of an H5N1 influenza variant in China. Proc Natl Acad Sci U S A. 2006;103:16936–41. http://dx.doi.org/10.1073/pnas.0608157103
17. Wang J, Vijaykrishna D, Duan L, Bahl J, Zhang JX, Webster RG, et al. Identification of the progenitors of Indonesian and Vietnamese avian influenza A (H5N1) viruses from southern China. J Virol. 2008;82:3405–14. http://dx.doi.org/10.1128/JVI.02468-07
18. Guan Y, Smith GJ, Webby R, Webster RG. Molecular epidemiology of H5N1 avian influenza. Rev Sci Tech. 2009;28:39–47.
19. Liang L, Xu B, Chen Y, Liu Y, Cao W, Fang L, et al. Combining spatial-temporal and phylogenetic analysis approaches for improved understanding on global H5N1 transmission. PLoS ONE. 2010;5:e13575. http://dx.doi.org/10.1371/journal.pone.0013575
20. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214. http://dx.doi.org/10.1186/1471-2148-7-214
21. Beerli P, Felsenstein J. Maximum likelihood estimation of a migration matrix and effective population sizes in *n* subpopulations by using a coalescent approach. Proc Natl Acad Sci U S A. 2001;98:4563–8. http://dx.doi.org/10.1073/pnas.081068098
22. Beerli P. Comparison of Bayesian and maximum-likelihood inference of population genetic parameters. Bioinformatics. 2006;22:341–5. http://dx.doi.org/10.1093/bioinformatics/bti803
23. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32:1792–7. http://dx.doi.org/10.1093/nar/gkh340
24. Hudson RR, Slatkin M, Maddison WP. Estimation of levels of gene flow from DNA sequence data. Genetics. 1992;132:583–9.
25. Drummond AJ, Ho SYW, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. PLoS Biol. 2006;4:e88. http://dx.doi.org/10.1371/journal.pbio.0040088
26. Hasegawa M, Kishino H, Yano T. Dating of the human-ape splitting by a molecular clock of mitochondrial DNA. J Mol Evol. 1985;22:160–74. http://dx.doi.org/10.1007/BF02101694
27. Parker J, Rambaut A, Pybus OG. Correlating viral phenotypes with phylogeny: accounting for phylogenetic uncertainty. Infect Genet Evol. 2008;8:239–46. http://dx.doi.org/10.1016/j.meegid.2007.08.001
28. Brooks SP, Gelman A. General methods for monitoring convergence of iterative simulations. J Comput Graph Stat. 1998;7:434–55. http://dx.doi.org/10.2307/1390675
29. Minin VN, Bloomquist EW, Suchard MA. Smooth skyride through a rough skyline: Bayesian coalescent-based inference of population dynamics. Mol Biol Evol. 2008;25:1459–71. http://dx.doi.org/10.1093/molbev/msn090
30. Nelson MI, Holmes EC. The evolution of epidemic influenza. Nat Rev Genet. 2007;8:196–205. http://dx.doi.org/10.1038/nrg2053
31. Tang JW, Ngai KLK, Lam WY, Chan PKS. Seasonality of influenza A (H3N2) virus: a Hong Kong perspective (1997–2006). PLoS ONE. 2008;3:e2768. http://dx.doi.org/10.1371/journal.pone.0002768
32. Liu J, Xiao H, Lei F, Zhu Q, Qin K, Zhang XW, et al. Highly pathogenic H5N1 influenza virus infection in migratory birds. Science. 2005;309:1206. http://dx.doi.org/10.1126/science.1115273
33. Takekawa JY, Prosser DJ, Newman SH, Muzaffar SB, Hill NJ, Yan B, et al. Victims and vectors: highly pathogenic avian influenza H5N1 and the ecology of wild birds. Avian Biology Research. 2010;3:51–73. http://dx.doi.org/10.3184/175815510X12737339356701
34. Si Y, Skidmore AK, Wang T, de Boer WF, Debba P. Spatiotemporal dynamics of global outbreaks match bird migration patterns. Geospat Health. 2009;4:65–78.

35. Prosser DJ, Cui P, Takekawa JY, Tang M, Hou Y, Collins BM, et al. Wild bird migration across the Qinghai-Tibetan Plateau: a potential transmission route for highly pathogenic H5N1. PLoS ONE. 2011;6:e17622. http://dx.doi.org/10.1371/journal.pone.0017622
36. Chen H, Li Y, Li Z, Shi J, Shinya K, Deng G, et al. Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China. J Virol. 2006;80:5976–83. http://dx.doi.org/10.1128/JVI.00110-06
37. Newman SH, Hill NJ, Spragens KA, Janies D, Voronkin IO, Prosser DJ, et al. Eco-virological approach for assessing the role of wild birds in the spread of avian influenza H5N1 along the Central Asian flyway. PLoS ONE. 2012;7:e30636. http://dx.doi.org/10.1371/journal.pone.0030636
38. Guan Y, Webby R, Capua I, Waldenström J. H5N1: how to track a flu virus. Nature. 2012;483:535–6. http://dx.doi.org/10.1038/483535a

Address for correspondence: Weifeng Shi, Institute of Pathogen Biology, School of Basic Medical Sciences, Taishan Medical College, Yingshengdonglu 2, Taian 271000, Shandong, China; email: wfshi@tsmc.edu.cn

Human Exposure to Live Poultry and Psychological and Behavioral Responses to Influenza A(H7N9), China

Liping Wang,[1] Benjamin J. Cowling,[1] Peng Wu,[1] Jianxing Yu,[1] Fu Li, Lingjia Zeng, Joseph T. Wu, Zhongjie Li, Gabriel M. Leung, and Hongjie Yu

To investigate human exposure to live poultry and changes in risk perception and behavior after the April 2013 influenza A(H7N9) outbreak in China, we surveyed 2,504 urban residents in 5 cities and 1,227 rural residents in 4 provinces and found that perceived risk for influenza A(H7N9) was low. The highest rate of exposure to live poultry was reported in Guangzhou, where 47% of those surveyed reported visiting a live poultry market ≥1 times in the previous year. Most (77%) urban respondents reported that they visited live markets less often after influenza A(H7N9) cases were first identified in China in March 2013, but only 30% supported permanent closure of the markets to control the epidemic. In rural areas, 48% of respondents reported that they raised backyard poultry. Exposure to live commercial and private poultry is common in urban and rural China and remains a potential risk factor for human infection with novel influenza viruses.

The novel influenza A(H7N9) virus was identified in early 2013; as of March 31, 2014, a total of 404 laboratory-confirmed cases of human infection had been reported. These cases included 394 in mainland China, 2 in Taiwan, 7 in Hong Kong, and 1 in Malaysia (*1*,*2*). Only 2 laboratory-confirmed cases were identified in the summer months (June–September 2013), but beginning in early October 2013, the virus reemerged and caused many new human infections (*3*,*4*).

Author affiliations: Division of Infectious Disease, Key Laboratory of Surveillance and Early-warning on Infectious Disease, Chinese Center for Disease Control and Prevention, Beijing, China (L. Wang, J. Yu, F. Li, L. Zeng, Z. Li, H. Yu); and Division of Epidemiology and Biostatistics, School of Public Health, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong Special Administrative Region, China (B.J. Cowling, P. Wu, J.T. Wu, G.M. Leung)

DOI: http://dx.doi.org/10.3201/eid2008.131821

Previously published studies have reported that most human infections appear to have occurred as a result of exposure to live poultry, particularly through visits to live poultry markets (LPMs) in urban areas (*3*,*5*–*8*). No published reports have detailed population exposure to live poultry and LPMs in influenza A(H7N9) virus–affected areas in China, and few data on live poultry exposure have been previously reported in areas in which the virus has not been detected (*4*,*9*,*10*). In addition, little information has been reported on how the population of China responded to the outbreak and the control measures that were implemented. To clarify responses to the influenza A(H7N9) outbreak in China, we investigated patterns in human exposure to live poultry in LPMs and at home, examined risk perception and behavioral responses in the population, and compared these parameters between urban and rural areas in China that were affected or unaffected by the virus.

Methods

Study Design

We collected information on human exposure to poultry, risk perception and psychological responses to the outbreak, preventive behaviors, and attitudes toward control measures, including closure of LPMs. We used 2 approaches to collect these data. In urban areas, we conducted telephone surveys because access to mobile telephones is high, making the approach feasible. In rural areas, where telephone accessibility is lower, we conducted door-to-door surveys.

We selected 5 large cities for our study to represent diverse levels of socioeconomic development and geographic location: Chengdu, Guangzhou, Shanghai, Shenyang, and Wuhan (Figure 1). Before our study, no laboratory-

[1]These authors contributed equally to this article.

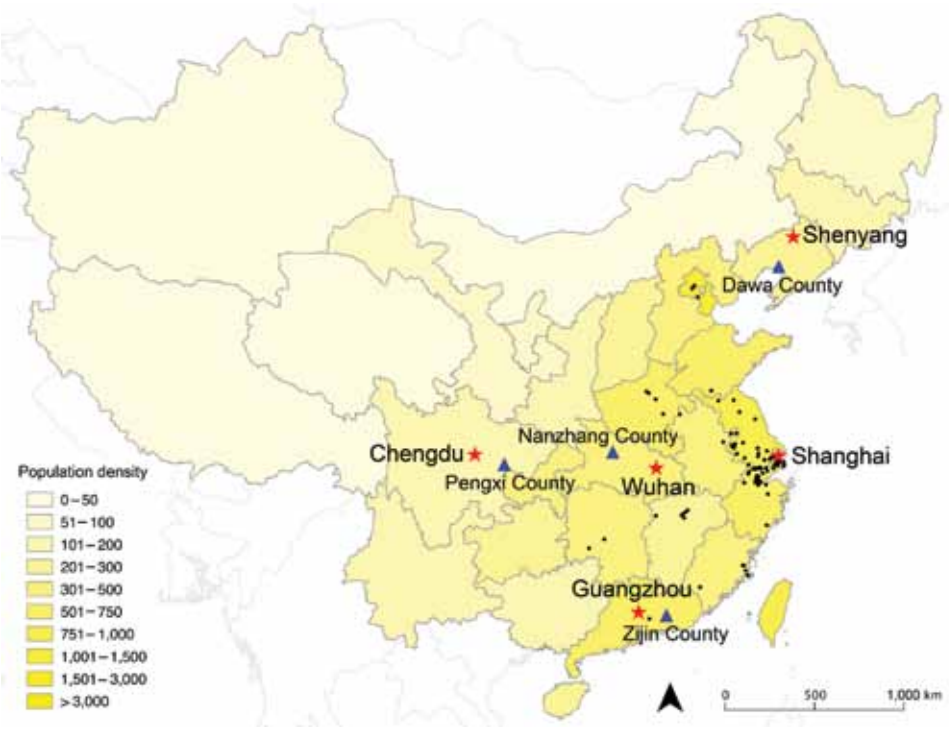


Figure 1. Geographic distribution of urban locations (red stars) and rural locations (blue triangles) selected for population survey to determine human exposure to live poultry and attitudes and behavior toward influenza A(H7N9) in China, 2013. Black dots indicate geographic locations of laboratory-confirmed cases of H7N9 through October 31, 2013. Shading indicates population density (persons per square kilometer). The 5 selected urban locations were Chengdu, capital of Sichuan Province in western China, population 10 million; Guangzhou, capital of Guangdong Province in southern China, population 13 million; Shanghai, a municipality in eastern China, population 23 million; Shenyang, capital of Liaoning Province in northeastern China, population 8 million; and Wuhan, capital of Hubei Province in central China, population 10 million. The 4 rural areas were Dawa County (Panjin city, Liaoning Province), Zijin County (Heyuan city, Guangdong Province), Nanzhang County (Xiangfan city, Hubei Province), and Pengxi County (Suining city, Sichuan Province).

confirmed human cases of influenza A(H7N9) had been reported in these cities except Shanghai; 1 environmental sample had tested positive for the virus in Guangzhou (*11*). In each city, we aimed to interview ≥500 adult residents (≥18 years of age) who had been living there for ≥1 year. The telephone surveys were conducted by using a computer-assisted interviewing system, which enabled random generation of mobile telephone numbers and systematic data collection across each city. On each call, after the study was explained and verbal consent obtained, the respondent would be recruited into the study and asked to complete the survey. If a respondent were busy, a call would be made later, when the respondent was available to finish the questionnaire. Unanswered numbers were given 4 follow-up calls, made at different hours and on different days of the week, before being classified as invalid. The online Technical Appendix shows the survey used in English and Chinese (http://wwwnc.cdc.gov/EID/article/20/8/13-1821-Techapp1.pdf).

Although we had planned to use the same telephone survey approach in rural areas, a pilot study revealed it was not feasible because the survey would occur during the busy farming season, when residents would not be readily available by telephone. Instead, in rural areas we conducted door-to-door surveys. In mainland China, some cities/counties that are administrated as rural regions actually include semiurban areas, such as towns in a county, and rural areas, such as villages in a town/county. The living conditions and lifestyle of residents in semiurban areas are similar to those of urban residents, whereas residents in rural areas live in a different environment, with low population density and a more self-sustainable life, mainly dependent on farming. We used convenience sampling to choose 4 counties from rural rather than semiurban areas. Rural sites were selected on the basis of the level of economic development (measured by gross domestic product per capita) and the overall incidence of infectious diseases in 2012. Given the tiers of administration levels in mainland China, including province, city, county, town, and village, we selected a city from each of the 4 provinces with mid-level gross domestic product per capita compared with other cities in the province and with an incidence of notifiable infectious diseases above the provincial average. Within each province, we then selected a rural county from each of the 4 cities areas. As a result, we chose Dawa County (Panjin city, Liaoning Province), Zijin County (Heyuan city, Guangdong Province), Nanzhang County (Xiangfan city, Hubei Province), and Pengxi County (Suining city, Sichuan Province) for the study (Figure 1). At time of the survey, none of these counties had laboratory-confirmed human infections with avian influenza A(H7N9) virus.

After the initial selections, all towns within a county were stratified into high, middle, and low levels of socioeconomic status on the basis of census data (*12*–*15*), and 1 town was selected at random within each strata. Then, 2 villages were selected at random within each town, a convenience sample of 50 households was recruited in each village, and 1 adult in each household (≥18 years of age and resident in the village for ≥1 year) was interviewed. To improve cooperation, each rural interviewee received a small gift worth ≈10 Chinese renminbi (6.1 renminbi = $1 US), such as a towel or a bottle of shampoo, after the survey was completed. All selected participants in the rural areas consented to be interviewed during the survey. The time taken to complete the survey was 16 minutes on average for each participant.

The urban surveys were conducted in May and June 2013 and the rural surveys in July and August 2013. Ethical approval was obtained from the Institutional Review Board of the Chinese Center for Disease Control and Prevention before the survey was conducted.

Survey Instrument

All surveys in urban and rural areas were conducted by using the same questionnaire, which was based on an instrument used during the outbreaks of severe acute respiratory syndrome (SARS) in 2003 (*16*,*17*) and influenza A(H1N1)pdm09 in 2009 (*18*). The survey instrument was pretested for face and content validity, length, and comprehensibility. Most answers were ranked on ordinal Likert scales. We used the State Trait Anxiety Inventory to measure the general level of anxiety in the population (*16*–*18*).

We investigated exposure to live poultry in backyards and in LPMs, which are defined as markets where the public can buy live chickens, ducks, pigeons, and other birds. Because LPMs are rare in rural areas and rural residents seldom visit LPMs, we did not ask rural respondents about exposures to live poultry in LPMs, only about backyard poultry exposure. In urban areas, we asked respondents about frequency of visits to LPMs and behaviors in LPMs (i.e., frequency of purchases, practice of picking up birds before purchasing, location where purchased live poultry were slaughtered). We asked all respondents about perception of risk for influenza A(H7N9) infection and perceived severity of such an infection, preventive practices in general and specifically in response to influenza A(H7N9), and attitudes toward influenza A(H7N9) and closure of LPMs.

Statistical Analysis

Statistical analyses were conducted in R version 2.13.0 (R Foundation for Statistical Computing, Vienna, Austria). We performed descriptive analyses of responses in each location and compared responses between urban areas with and without laboratory-confirmed cases of influenza A(H7N9) by using χ^2 tests. For the subset of respondents who reported purchasing live poultry in LPMs during the previous year, we used a multivariate logistic regression model to estimate the associations of age, sex, educational level, and geographic location with attitudes toward closure of LPMs and changes in habits of buying live poultry after public health authorities announced the first human influenza A(H7N9) case on March 31, 2013 (*19*).The sample size of 500 respondents in each city and 300 respondents in each rural county was chosen to ensure precision of answers to within ±4% and ±6%, respectively, and to ensure reasonable statistical power to identify differences in responses of 5%–10% or more between locations.

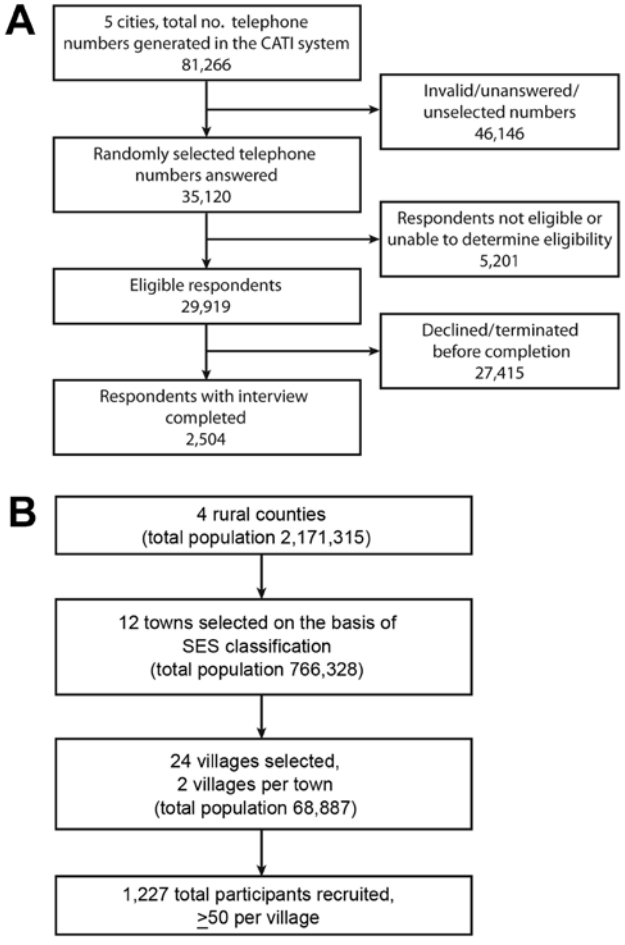


Figure 2. Flow charts for recruitment of participants for telephone surveys and face-to-face interviews to determine human exposure to live poultry and attitudes and behavior toward influenza A(H7N9) in China, 2013. A) Flowchart for telephone surveys conducted in 5 urban areas: Chengdu (capital of Sichuan Province), Guangzhou (capital of Guangdong Province), Shanghai municipality, Shenyang (capital of Liaoning Province), and Wuhan (capital of Hubei Province). B) Flowchart for face-to-face interviews conducted in 3 rural areas: Dawa county (Panjin city, Liaoning Province), Zijin county (Heyuan city, Guangdong Province), Nanzhang county (Xiangfan city, Hubei Province), and Pengxi county (Suining city, Sichuan Province). CATI, computer-assisted telephone interview; SES, socioeconomic status.

Results

In the 5 urban areas, 81,266 unique telephone numbers were dialed, and the overall response rate was 8% (number of participants [2,504] divided by number of calls with eligible respondents [29,919]) (Figure 2, panel A). The selection of 1,227 participants in 4 rural sites is illustrated in Figure 2, panel B. The surveys were conducted from May 23 through August 24. During this period, the influenza A(H7N9) epidemic had passed its peak, and few cases occurred. Guangdong Province notified its first human influenza A(H7N9) case on August 9, after the completion of the survey in Guangzhou on June 26.

Respondents in urban areas tended to have white-collar jobs or were unemployed, were younger, had more education and higher income, and were less likely to be married than those in rural areas (Table 1). However, because the surveys were conducted in different forms in urban versus rural areas and the general characteristics of participants were different, including the risk for becoming infected with influenza A(H7N9) virus and the types of potential exposure to avian influenza viruses, we did not make any further direct quantitative comparisons between urban and rural respondents. For comparisons among urban areas, respondents were generally similar, but reported incomes were higher for Shanghai and Guangzhou than for the other 3 cities (data not shown).

We assessed exposures to live poultry and visits to LPMs in the 5 cities. In total, 33% of respondents reported visiting LPMs during the preceding year, the highest proportion in Guangzhou; notable differences were found between cities (Table 2). By imputing midpoints of reported purchasing rates, we estimated that the mean number of live poultry purchased per year varied between cities: 6.8 for Shenyang, 19 for Shanghai, 20 for Wuhan, 28 for Chengdu, and 47 for Guangzhou. Age-specific patterns in exposure to live poultry were generally similar for men and women within each city, with some exceptions. In Guangzhou, women 35–54 years of age purchased poultry in LPMs much more frequently than did men of the same age, but the reverse was true for those ≥65 years of age (Figure 3). We found no evidence of a substantial difference in poultry exposures by sex in Shanghai (Figure 3).

We further analyzed exposures in LPMs among urban residents on the basis of responses from the 829 (33%) of

Table 1.Sociodemographic characteristics of participants recruited for urban and rural surveys of influenza A(H7N9) awareness, China, 2013*

Characteristic	No. (%) persons	
	Urban, n = 2,504	Rural, n = 1,227
Male sex	1,288 (51.4)	626 (51.0)
Age group, y		
18–44	1,938 (77.5)	685 (55.8)
45–64	415 (16.6)	405 (33.0)
≥65	147 (5.9)	137 (11.2)
Educational attainment		
No formal education	38 (1.5)	86 (7.0)
Primary school	191 (7.6)	259 (21.1)
Middle school	391 (15.6)	464 (37.9)
High school	593 (23.7)	268 (21.9)
College and above	1,291 (51.6)	148 (12.1)
Occupation		
Service workers and shop sales workers	601 (24.0)	164 (13.4)
Professionals	504 (20.1)	66 (5.4)
Retired	293 (11.7)	61 (5.0)
Unemployed	678 (27.1)	195 (15.9)
Full-time students	232 (9.3)	111 (9.0)
Homemakers	96 (3.8)	86 (7.0)
Agricultural and fishery workers	100 (4.0)	544 (44.3)
Marital status		
Single	941 (38.1)	269 (22.0)
Married	1,458 (59.0)	923 (75.4)
Divorced/separated	35 (1.4)	12 (1.0)
Widowed	36 (1.5)	20 (1.6)
Average household income, in renminbi*		
No income	65 (3.0)	83 (6.8)
<3,000	368 (17.0)	748 (61.2)
3,001–6,000	627 (28.9)	264 (21.6)
6,001–10,000	408 (18.8)	80 (6.5)
10,001–50,000	396 (18.2)	28 (2.3)
Not sure	307 (14.1)	20 (1.6)
Recent history of travel away from home		
Yes	479 (19.1)	117 (9.6)

*6.1 Chinese renminbi = $1 US.

Table 2. Exposure to live poultry and attitudes toward closure of LPMs among participants recruited in urban areas for surveys related to influenza A(H7N9) awareness, by area, China, 2013*

Exposure	No. (%) persons					p value
	Chengdu, n = 500	Guangzhou, n = 500	Shanghai, n = 500	Shenyang, n = 504	Wuhan, n = 500	
Frequency of LPM visits in the previous year						<0.001
≥1	183 (36.6)	237 (47.4)	161 (32.2)	97 (19.2)	151 (30.2)	
No. live poultry bought in the previous year†						<0.001
1–2/y	33 (18.0)	32 (13.5)	25 (15.5)	35 (36.1)	25 (16.6)	
3–5/y	31 (16.9)	27 (11.4)	30 (18.6)	23 (23.7)	28 (18.5)	
6–11/y	27 (14.8)	25 (10.5)	23 (14.3)	4 (4.1)	23 (15.2)	
1–3/mo	33 (18.0)	56 (23.6)	32 (19.9)	10 (10.3)	29 (19.2)	
1–2/wk	19 (10.4)	49 (20.7)	20 (12.4)	2 (2.1)	19 (12.6)	
3–5/wk	2 (1.1)	8 (3.4)	2 (1.2)	0	2 (1.3)	
Almost every day	2 (1.1)	4 (1.7)	2 (1.2)	0	2 (1.3)	
Almost none	36 (19.7)	36 (15.2)	27 (16.8)	23 (23.7)	23 (15.2)	
Pick up live poultry before buying‡						<0.001
Yes	120 (81.6)	136 (67.7)	94 (69.6)	38 (51.4)	97 (75.8)	
Where did you slaughter the live poultry?§						0.601
In LPM	123 (83.7)	175 (87.1)	119 (88.1)	66 (89.2)	113 (88.3)	
In household	22 (15.0)	23 (11.4)	15 (11.1)	6 (8.1)	13 (10.2)	
Other places	2 (1.4)	3 (1.5)	1 (0.7)	2 (2.7)	2 (1.6)	
Not buying or buying less since March 2013¶						<0.001
Yes	101 (68.7)	139 (69.2)	123 (91.1)	59 (79.7)	104 (81.3)	
Views toward closure of LPMs#						0.06
Agree	37 (25.2)	54 (26.9)	53 (39.3)	25 (33.8)	35 (27.3)	
Closure caused any inconvenience**						
More inconvenient	NA	NA	45 (31.5)	NA	NA	
Distance of nearest LPM from home, km						<0.001
≤0.50	12 (13.3)	39 (31.0)	21 (18.9)	5 (13.5)	6 (15.0)	
0.51–1.00	23 (25.6)	42 (33.3)	32 (28.8)	4 (10.8)	10 (25.0)	
1.01–2.00	16 (17.8)	20 (15.9)	16 (14.4)	6 (16.2)	7 (17.5)	
>2.00	39 (43.3)	25 (19.8)	42 (37.8)	22 (59.5)	17 (42.5)	
Backyard poultry exposure	73 (14.6)	76 (15.2)	34 (6.8)	37 (7.3)	54 (10.8)	<0.001

*LPM, live poultry market; NA, not applicable.
†Respondents who bought live poultry ≥1/year were further asked about the number of live poultry bought in the previous year, picking up poultry or not before buying, locations where poultry was slaughtered, and changes in poultry purchase behavior since influenza A(H7N9) outbreak.
‡Respondents who answered always/usually to the question "Did you pick up poultry for examination before deciding to buy it?" were categorized as "Yes."
§Respondents who stated that they always/usually have live poultry slaughtered in LPMs were categorized as "In LPM," whereas those who answered always/usually in household were categorized as "in household."
¶Respondents who answered not buying since then/still buying but less than before to the question "Has your habit of buying live poultry changed since H7N9 was identified in China in March 2013?" were categorized as "Yes."
#Respondents who answered strongly agree/agree to the question "Would you agree to permanent closure of live poultry markets in order to control avian influenza epidemics?" were categorized as "Agree."
**Respondents who reported that market closure caused great/some inconvenience were categorized as "More inconvenient." This question was only asked of respondents in Shanghai because Shanghai was the only area where LPMs were closed at the time of the survey.

2,504 participants who visited LPMs ≥1 time in the preceding year. Overall, 69% of these respondents reported that they always visited the nearest LPM; median distance from home to the nearest LPM was 1 km. Most respondents reported that they "usually" or "always" picked up poultry for examination before deciding to buy, with the highest proportion in Chengdu and lowest in Shenyang; 87% of respondents always arranged for slaughter of purchased poultry in the LPM, with no notable differences between cities.

During the study period, the general anxiety level among urban respondents (measured by the State Trait Anxiety Inventory) was low to moderate, but levels varied substantially between cities; the lowest mean scores were seen in Wuhan and Shenyang (Table 3). Perceived risk for influenza A(H7N9) in the following month (absolute susceptibility) and relative to others (relative susceptibility) were generally low in all cities, but highest in Shanghai. Respondents in Shanghai and Guangzhou were more likely to respond that they would be more worried than usual if they experienced an influenza-like illness (ILI). Twelve percent of respondents reported that they had worried about becoming ill with influenza A(H7N9) during the previous week; levels varied among cities, with a greater frequency of worry in Shanghai and Guangzhou (Table 3). Respondents in Shenyang reported the highest perceived severity of influenza A(H7N9) compared with seasonal influenza and avian influenza A(H5N1); respondents in Guangzhou reported the highest perceived severity of influenza A(H7N9) compared with that of SARS (Table 3).

In rural areas, as in urban areas, the mean State Trait Anxiety Inventory was low to moderate (Table 4). A total of 48% of respondents reported that they raised ≥1 type of

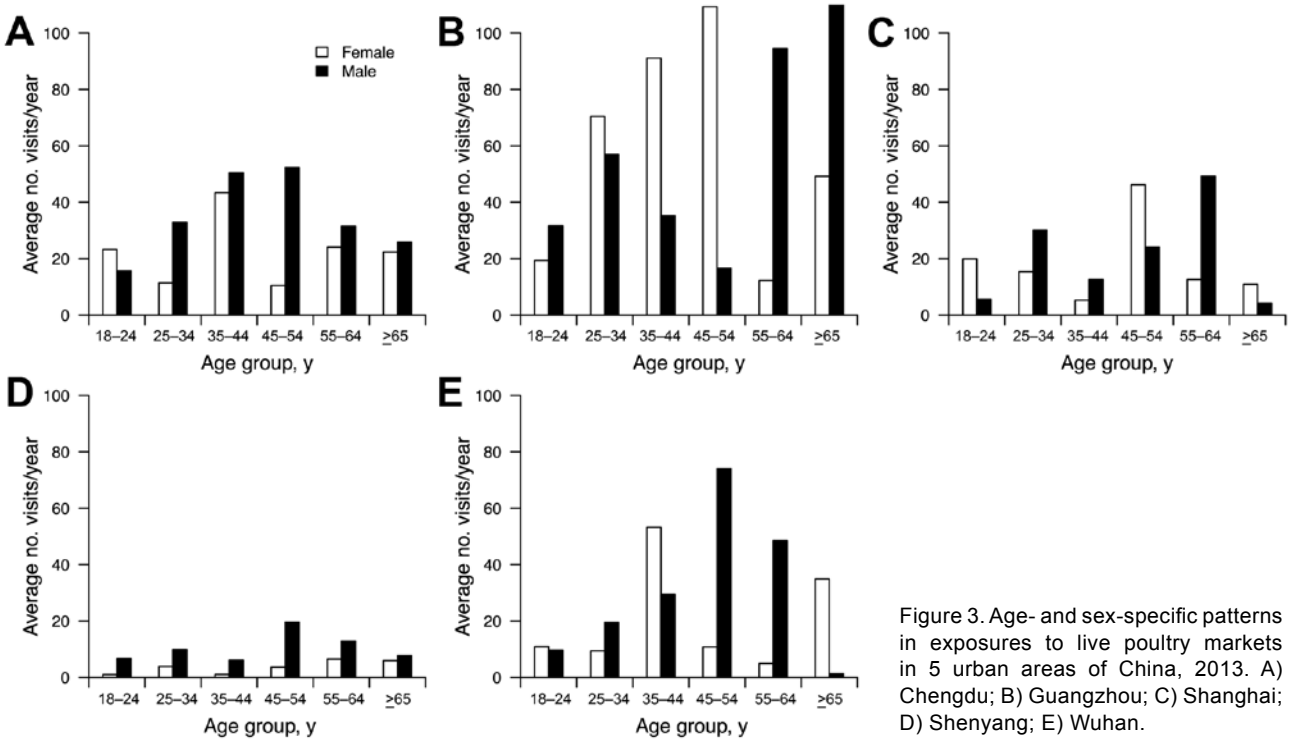


Figure 3. Age- and sex-specific patterns in exposures to live poultry markets in 5 urban areas of China, 2013. A) Chengdu; B) Guangzhou; C) Shanghai; D) Shenyang; E) Wuhan.

poultry at home. Overall, 47% reported raising chickens, 15% raised ducks, and 8% raised geese; these proportions varied between counties (Table 4). In rural areas, levels of perceived absolute and relative susceptibility and concern about ILI or confirmed influenza A(H7N9) infection were generally low; some differences were seen between the 4 rural areas. Respondents in Nanzhang and Zijin were more likely to respond that they would be more worried than usual if they had an ILI; 24% of respondents in Zijin reported that they had worried about becoming ill with influenza A(H7N9) in the previous week, and the average level of worry in Zijin was higher than that for other counties (Table 4). Most respondents in each area perceived influenza A(H7N9) to be more severe than seasonal influenza but less severe than influenza A(H5N1) and SARS.

Among respondents in urban areas who visited LPMs ≥1 time in the preceding year, 77% reported that they had stopped buying or bought lower amounts of live poultry since March 2013; this proportion was highest (91%) for Shanghai (Table 2). We examined factors affecting the likelihood of changing habits of buying live poultry and found greater changes among women, those with higher educational attainment, and those residing in Shanghai and Wuhan rather than in Chengdu. We found no statistically significant differences by age group (Table 5).

On average, across the 5 cities, 30% of respondents reported that they would support the closure of LPMs to control the epidemic; the proportion in support of closures was highest in Shanghai (39%) and lowest in Guangzhou (27%) and Chengdu (25%) (Table 2). We examined factors affecting the likelihood of supporting the closure of LPMs and found greater support among persons 55–64 years of age (odds ratio [OR] 3.28, 95% CI 1.71–6.29) and ≥65 years of age (OR 2.36, 95% CI 1.04–5.32). We also found greater support for closure of LPMs in Shanghai (OR 1.77, 95% CI 1.05–2.99) than in Chengdu but no significant differences by sex or educational attainment (Table 5). However, 32% of respondents in Shanghai reported that the closure of LPMs had caused them inconvenience.

Discussion

We have reported empirical information on human exposures to live poultry, perception of risk for influenza A(H7N9), and behavioral responses to the 2013 influenza A(H7N9) outbreak in China. We found that exposure to LPMs in urban areas is common: 20%–50% of urban residents report ≥1 visit to an LPM in the preceding year (Table 2). Most respondents who purchased poultry in LPMs reported close contact with live poultry before slaughter. It is likely that the number of laboratory-confirmed cases of influenza A(H7N9) virus infection is lower than the actual number of human infections to date (*8*), and our results show that a broad cross-section of urban residents could be exposed to influenza A(H7N9) virus if it were prevalent among poultry in LPMs. In the spring 2013 outbreak, some evidence pointed to high prevalence of influenza A(H7N9)

Table 3.Risk perception related to influenza A(H7N9) among participants recruited for surveys in urban areas, by area, China, 2013*

Characteristic	Chengdu, n = 500	Guangzhou, n = 500	Shanghai, n = 500	Shenyang, n = 504	Wuhan, n = 500	p value
Mean STAI scores (95% CI)	1.89 (1.85–1.94)	1.80 (1.75–1.84)	1.82 (1.78–1.86)	1.73 (1.69–1.77)	1.74 (1.71–1.78)	<0.001†
Self-perceived susceptibility to influenza A(H7N9)‡						<0.001
High	13 (2.6)	9 (1.8)	14 (2.8)	1 (0.2)	5 (1.0)	
Even	61 (12.2)	98 (19.6)	61 (12.2)	54 (10.7)	90 (18.0)	
Low	426 (85.2)	393 (78.6)	425 (85.0)	449 (89.1)	405 (81.0)	
Perceived susceptibility to influenza A(H7N9) compared with others§						0.431
High	5 (1.0)	5 (1.0)	9 (1.8)	4 (0.8)	7 (1.4)	
Even	40 (8.0)	52 (10.4)	39 (7.8)	32 (6.3)	50 (10.0)	
Low	455 (91.0)	443 (88.6)	452 (90.4)	468 (92.9)	443 (88.6)	
ILI symptoms induced worry¶						<0.001
More	105 (21.0)	151 (30.2)	140 (28.0)	113 (22.4)	107 (21.4)	
Same as usual	197 (39.4)	198 (39.6)	192 (38.4)	165 (32.7)	233 (46.6)	
Less	198 (39.6)	151 (30.2)	168 (33.6)	226 (44.8)	160 (32.0)	
Infection with influenza A(H7N9) in next week#						0.004
Worry	64 (12.8)	68 (13.6)	68 (13.6)	49 (9.7)	53 (10.6)	
Think about it but no worry	77 (15.4)	57 (11.4)	104 (20.8)	92 (18.3)	78 (15.6)	
Never think about it	359 (71.8)	375 (75.0)	328 (65.6)	363 (72.0)	369 (73.8)	
Relative severity of influenza A(H7N9) compared with**						
Seasonal influenza	313 (62.6)	319 (63.8)	290 (58.0)	361 (71.6)	312 (62.4)	<0.001
Avian influenza A(H5N1)	159 (31.8)	163 (32.6)	170 (34.0)	203 (40.3)	156 (31.2)	0.028
SARS	52 (10.4)	57 (11.4)	54 (10.8)	45 (8.9)	51 (10.2)	0.779
Distance, km††	804	383	–	601	233	

*Values are no. (%) persons except as indicated. STAI, State Trait Anxiety Inventory; ILI, influenza-like illness; SARS, severe acute respiratory syndrome.
†Differences between groups was examined with the Kruskal Wallis Test (assuming nonhomogeneous variances).
‡Respondents who answered certain/very likely/likely to the question "How likely do you think it is that you will contract H7N9 avian flu over the next 1 month?" were categorized as "High"; those who answered never/very unlikely/unlikely were categorized as "Low."
§Respondents who answered certain/much more /more to the question "What do you think is your chance of getting infected with H7N9 avian flu over the next 1 month compared to other people outside your family of a similar age?" were categorized as "High"; those who answered not at all/much less/less were categorized as "Low."
¶Respondents who answered extremely concerned/concerned much more than normal/concerned more than normal to the question "If you were to develop ILI symptoms tomorrow, would you be…?" were categorized as "More"; those who answered not at all concerned/much less concerned than normal/ concerned less than normal were categorized as "Less."
#Respondents who answered worried about it all the time/worried a lot/worried a bit to the question "Did you worry about H7N9 in the past week?" were categorized as "Worry."
**Respondents who answered much higher/a little higher regarding the severity of influenza A(H7N9) compared with seasonal influenza, avian influenza A(H5N1), and SARS.
††Distance between the survey location and the nearest area in which influenza A(H7N9) case(s) were reported.

virus in certain LPMs (*6*), whereas official surveillance data from the Ministry of Agriculture identified the virus in only a small proportion of samples collected from across the country (of 4,488 samples tested, 0.9% were positive for the virus) (*11*). The absolute risk for human infection after close contact with poultry infected with the influenza A(H7N9) virus remains unclear.

We found that men in the 55–64-year age group had more exposures to live poultry than women in that age group, but no difference by sex among the small number of respondents ≥65 years of age in Shanghai (Figure 3). We had previously hypothesized that exposure to poultry in LPMs might be higher for older men than for older women (*3*). Our findings suggest that the higher risk for laboratory-confirmed influenza A(H7N9) virus infection among men during the spring 2013 outbreak in the Yangtze River Delta might not be explained by sex differences in exposure but rather by increased susceptibility to serious disease after infection among men (e.g., because of greater prevalence of co-existing conditions) or by increased access to health care and laboratory testing for men. However, our sample size was relatively small, particularly for respondents ≥65 years of age. As in a previous report of live poultry exposures in the southern China cities of Guangzhou in 2006 and Shenzhen in 2007 (*9*), we did not identify major differences in exposures among middle-aged adults compared with exposures among the elderly. However, most laboratory-confirmed influenza A(H7N9) cases have been in persons ≥60 years of age (*3*), consistent with our hypothesis that exposures in middle-aged adults may have led to milder disease that was less likely to result in laboratory testing (*3*,*9*).

A minority of respondents reported willingness to accept LPM closures in the event of future outbreaks of influenza A(H7N9). During the winter 2013–14 influenza season, in some areas where human cases of influenza A(H7N9) had been reported, local governments implemented short-term LPM closures; other administrations, including that of Shanghai, closed LPMs for longer periods. However, such interventions can have serious economic consequences. Given the lack of public support for LPM closure and the related economic concerns, whether

Table 4. Risk perception related to influenza A(H7N9) and backyard poultry exposure among participants recruited for surveys in rural areas, by area, China, 2013*

Characteristic	Dawa, n = 310	Zijin, n = 308	Nanzhang, n = 308	Pengxi, n = 301	p value
Mean STAI scores (95% CI)	1.52 (1.47–1.57)	1.85 (1.80–1.90)	1.66 (1.62–1.70)	1.54 (1.48–1.61)	<0.001†
Self-perceived susceptibility to influenza A(H7N9)‡					<0.001
Higher	2 (0.6)	1 (0.3)	1 (0.3)	9 (3.0)	
Even	29 (9.4)	41 (13.3)	21 (6.8)	31 (10.3)	
Lower	279 (90.0)	266 (86.4)	286 (92.9)	261 (86.7)	
Perceived susceptibility to influenza A(H7N9) compared with others§					<0.001
Higher	0	1 (0.3)	2 (0.6)	8 (2.7)	
Even	10 (3.2)	25 (8.1)	3 (1.0)	36 (12.0)	
Lower	300 (96.8)	282 (91.6)	303 (98.4)	257 (85.4)	
Worry induced by ILI symptoms¶					<0.001
More	69 (22.3)	79 (25.6)	118 (38.4)	49 (16.3)	
Same as usual	73 (23.5)	113 (36.7)	118 (38.4)	113 (37.5)	
Less	168 (54.2)	116 (37.7)	71 (23.1)	139 (46.2)	
Infection with influenza A(H7N9) in next week#					<0.001
Worry	32 (10.3)	75 (24.4)	71 (23.1)	51 (16.9)	
Think about it but no worry	51 (16.5)	42 (13.7)	20 (6.5)	33 (11.0)	
Never think about it	227 (73.2)	190 (61.9)	217 (70.5)	217 (72.1)	
Severity of influenza A(H7N9) compared with**					
Seasonal influenza	201 (64.8)	181 (58.8)	224 (72.7)	182 (60.5)	0.001
Avian influenza A(H5N1)	105 (33.9)	112 (36.4)	67 (21.8)	92 (30.6)	<0.001
SARS	51 (16.5)	63 (20.5)	30 (9.7)	44 (14.6)	0.003
Distance, km††	482	2448	351	665	
Raising backyard poultry	141 (45.5)	135 (43.8)	166 (53.9)	168 (49.7)	0.067
Type of backyard poultry raised					
Chicken	120 (38.7)	134 (43.5)	162 (52.6)	161 (53.5)	<0.001
Ducks	49 (15.8)	45 (14.6)	20 (6.5)	65 (21.6)	<0.001
Geese	34 (11.0)	17 (5.5)	2 (0.6)	43 (14.3)	<0.001
Median no. live poultry raised	6	20	13	12	<0.001†

*Values are no. (%) persons except as indicated. STAI, State Trait Anxiety Inventory; ILI, influenza-like illness; SARS, severe acute respiratory syndrome.
†Differences between groups were examined with the Kruskal-Wallis Test (assuming nonhomogeneous variances).
‡Respondents who answered certain/very likely/likely to the question "How likely do you think it is that you will contract H7N9 avian flu over the next 1 month?" were categorized as "High"; those who answered never/very unlikely/unlikely were categorized as "Low."
§Respondents who answered certain/much more /more to the question "What do you think is your chance of getting infected with H7N9 avian flu over the next 1 month compared to other people outside your family of a similar age?" were categorized as "High"; those who answered not at all/much less/less were categorized as "Low."
¶Respondents who answered extremely concerned/concerned much more than normal/concerned more than normal to the question "If you were to develop ILI symptoms tomorrow, would you be…?" were categorized as "More"; those who answered not at all concerned/much less concerned than normal/ concerned less than normal were categorized as "Less."
#Respondents who answered worried about it all the time/worried a lot/worried a bit to the question "Did you worry about H7N9 in the past week?" were categorized as "Worry."
**Respondents who answered much higher/a little higher regarding the severity of influenza A(H7N9) compared with seasonal influenza, avian influenza A(H5N1), and SARS.
††Distance between the survey location and the nearest area in which influenza A(H7N9) case(s) were reported.

to make additional closures should be considered carefully. Regular rest days (i.e., days on which live poultry are not sold and stalls must be disinfected and left empty of live birds) and bans on overnight retention of live poultry in markets have been successful in controlling the transmission of avian influenza viruses in LPMs in Hong Kong (*20*,*21*) and have been proposed in some areas of China (*8*).

Although almost all cases of influenza A(H7N9) cases have been identified in areas within or surrounding large cities, about half of the laboratory-confirmed avian influenza A(H5N1) cases in China were identified in rural residents, which indicates that avian influenza viruses can reach backyard poultry flocks and pose a risk to human health (*3*). Influenza A(H7N9) virus does not appear to have spread to backyard flocks at this time, however. Most confirmed human cases have occurred in urban areas among persons who have reported recent exposure to live poultry in LPMs, although a smaller number of cases occurred in persons who have reported recent exposure to backyard poultry (*3*). However, if the circulation of influenza A(H7N9) virus in backyard poultry were to increase, the number of potential exposures could be substantial because almost half of rural residents report raising backyard poultry. The risk for influenza A(H5N1) virus infection among rural residents has been reduced through better education about the danger of close contact with, or consumption of, sick or dead backyard poultry (*22*,*23*). Unfortunately, this approach would not be effective for controlling spread of influenza A(H7N9) virus because infected chickens do not show signs of illness.

Perception of risk for influenza A(H7N9) infection by respondents to our surveys was generally low, as might be expected given the small number of laboratory-confirmed cases in China. However, low perception of risk could pose

Table 5. Factors associated with attitudes and behavior toward influenza A(H7N9) among survey respondents from urban areas who had visited a live poultry market during the previous year, China, 2013*

Characteristic	Odds ratio (95% CI)	
	Support closure of LPMs	Change purchase behavior
Sex		
F	1.19 (0.84–1.68)	**2.42 (1.61–3.63)**
M	Referent	Referent
Age group, y		
18–24	0.73 (0.37–1.45)	0.70 (0.36–1.36)
25–34	1.36 (0.85–2.17)	0.81 (0.49–1.34)
35–44	Referent	Referent
45–54	1.43 (0.72–2.83)	0.62 (0.3–1.26)
55–64	**3.28 (1.71–6.29)**	0.86 (0.39–1.9)
≥65	**2.36 (1.04–5.32)**	1.42 (0.51–3.97)
Educational attainment		
Primary or below	Referent	Referent
Secondary	1.80 (0.92–3.50)	**1.95 (1.01–3.76)**
Tertiary or above	1.78 (0.90–3.53)	1.79 (0.91–3.51)
Urban sites		
Chengdu	Referent	Referent
Guangzhou	1.13 (0.69–1.85)	0.99 (0.62–1.60)
Shanghai	**1.77 (1.05–2.99)**	**4.89 (2.42–9.89)**
Shenyang	1.40 (0.74–2.64)	1.95 (0.97–3.95)
Wuhan	1.07 (0.62–1.86)	**2.05 (1.15–3.65)**

*Odds ratios were estimated by adjustment for all variables shown. Boldface indicates significance (p<0.05).

difficulties for policy measures such as closure of LPMs. Indeed, we found generally low levels of public support for long-term closure of LPMs (Table 2), particularly in cities that had not been affected by influenza A(H7N9). Respondents in Guangzhou and Shanghai reported higher likelihood than residents of other cities that they would be worried if they showed signs and symptoms of ILI. This finding is unsurprising for Shanghai, but there had been no confirmed influenza A(H7N9) cases in Guangzhou at the time of our survey.

Our study has several limitations. First, the cross-sectional study design did not enable us to identify changes over time in risk perception or preventive behaviors. Having access to data on live poultry exposures before the identification of influenza A(H7N9) virus infections would have been helpful because the epidemic may have led to changes in exposure patterns by the time our survey was conducted. Second, because the survey was conducted by telephone in urban areas and face-to-face in rural areas, our results may have been affected by selection bias. We did attempt multiple calls to unanswered telephone numbers in an attempt to mitigate this bias, but the overall response rate for the telephone survey was low. Also, because the respondents self-reported their behaviors, the results might be affected by response biases (e.g., if respondents had incomplete recollection of past visits to LPMs). In particular, results could have been affected by social desirability bias if respondents felt uncomfortable reporting true patterns of poultry exposure or attitudes toward government interventions and preferred to report what they perceived to be ideal or most acceptable.

Third, our analyses did not explore in depth the social or psychological factors underlying behavioral responses to influenza A(H7N9), such as the effect of perceived risk or severity. This area might be productive for further investigation. Fourth, similar to other cross-sectional knowledge–attitude–behavior studies, our survey could only provide descriptive data on live poultry exposure, risk perception, and behavioral changes. Inferences on the associations between different psychobehavioral factors will require further study. Furthermore, we did not investigate seasonal variation in poultry-purchasing behaviors, which could also be studied in longitudinal surveys.

In conclusion, exposures to live poultry are common in many areas of China. If influenza A(H7N9) virus were to become more prevalent among poultry, the number of human exposures could be substantial in the absence of control measures. Our findings highlight possible problems in the structure of the live poultry trade in China and the potential for improved protection of human and animal health (*8*,*24*).

Acknowledgments

We thank Hang Zhou and Zhibin Peng for questionnaire preparation and Qiaohong Liao, Hui Jiang, Xiang Ren, Vicky Fang, Michael Ni, and Hoi Wa Wong for technical assistance.

This study was funded by the US National Institutes of Health (Comprehensive International Program for Research on AIDS grant U19 AI51915); the China–US Collaborative Program on Emerging and Re-emerging Infectious Diseases; and grants from the Ministry of Science and Technology, China (2012 ZX10004-201); the Harvard Center for Communicable Disease Dynamics from the National Institute of General Medical Sciences (grant no. U54 GM088558); the Research Fund for the Control of Infectious Disease, Food and Health Bureau, Government of the Hong Kong Special Administrative Region (grant no. HKU-13-06-01); and the Area of Excellence Scheme of the Hong Kong University Grants Committee (grant no. AoE/M-12/06). The funding bodies had no role in study design, data collection and analysis, preparation of the manuscript, or the decision to publish.

L.W., B.J.C., P.W., G.M.L., and H.Y. designed the study; L.W., J.Y., F.L., and L.Z. collected data; and L.W., B.J.C., P.W., and J.Y. analyzed data. L.W. and B.J.C. wrote the first draft of this article, and all authors contributed to review and revision of the report.

Dr Wang is a director of the Branch of General Affairs on Infectious Disease, Division of Infectious Disease, Key Laboratory of Surveillance and Early-warning on Infectious Disease, Chinese Center for Disease Control and Prevention. Her research interests include notifiable infectious diseases surveillance and related policy research.

References

1. World Health Organization. Human infection with avian influenza A(H7N9) virus—update [cited 2014 Apr 1]. http://www.who.int/csr/don/2013_11_06/en/index.html
2. China Information System for Disease Control and Prevention. Beijing: Chinese Center for Disease Control and Prevention [cited 2014 Apr 1]. http://1.202.129.170/
3. Cowling BJ, Jin L, Lau EH, Liao Q, Wu P, Jiang H, et al. Comparative epidemiology of human infections with avian influenza A H7N9 and H5N1 viruses in China: a population-based study of laboratory-confirmed cases. Lancet. 2013;382:129–37. http://dx.doi.org/10.1016/S0140-6736(13)61171-X
4. Chen E, Chen Y, Fu L, Chen Z, Gong Z, Mao H, et al. Human infection with avian influenza A(H7N9) virus re-emerges in China in winter 2013. Euro Surveill. 2013;18:20616.
5. Li Q, Zhou L, Zhou M, Chen Z, Li F, Wu H, et al. Epidemiology of human infections with avian influenza A(H7N9) virus in China. N Engl J Med. 2014;370:520–32. http://dx.doi.org/10.1056/NEJMoa1304617
6. Chen Y, Liang W, Yang S, Wu N, Gao H, Sheng J, et al. Human infections with the emerging avian influenza A H7N9 virus from wet market poultry: clinical analysis and characterisation of viral genome. Lancet. 2013;381:1916–25. http://dx.doi.org/10.1016/S0140-6736(13)60903-4
7. Bao CJ, Cui LB, Zhou MH, Hong L, Gao GF, Wang H. Live-animal markets and influenza A(H7N9) virus infection. N Engl J Med. 2013;368:2337–9. http://dx.doi.org/10.1056/NEJMc1306100
8. Yu H, Wu JT, Cowling BJ, Liao Q, Fang VJ, Zhou S, et al. Effect of closure of live poultry markets on poultry-to-person transmission of avian influenza A H7N9 virus: an ecological study. Lancet. 2014;383:541–8. http://dx.doi.org/10.1016/S0140-6736(13)61904-2
9. Cowling BJ, Freeman G, Wong JY, Wu P, Liao Q, Lau EH, et al. Preliminary inferences on the age-specific seriousness of human disease caused by avian influenza A(H7N9) infections in China, March to April 2013. Euro Surveill. 2013;18:20475.
10. Liao Q, Lam WT, Leung GM, Jiang C, Fielding R. Live poultry exposure, Guangzhou, China, 2006. Epidemics. 2009;1:207–12. http://dx.doi.org/10.1016/j.epidem.2009.09.002
11. Epidemic release of the Ministry of Agriculture of the People's Republic of China [cited 2014 Apr 1]. http://www.moa.gov.cn/zwllm/yjgl/yqfb/
12. Dawa county, Liaoning province, North-eastern China [Internet] [updated 2014 Jan 23; cited 2014 Feb 1]. http://baike.baidu.com/view/119258.htm
13. Zijin county, Guangdong province, Southern China [Internet] [updated 2014 Feb 1; cited 2014 Feb 1]. http://baike.baidu.com/view/204129.htm
14. Nanzhang county, Hubei province, Mid China. [Internet] [updated 2013 Dec 19; cited 2014 Feb 1]. http://baike.baidu.com/link?url=hC6vMe6VQP8rq3kHC3GI0SK2NIVlmbkgM6TI2OBUy0GQl9Twvjk1eOqLb_MzD4ER
15. Pengxi county, Sichuan province, Western China. [Internet] [updated 2012 Mar 20; cited 2014 Feb 1]. http://wenku.baidu.com/view/e1c74214f18583d0496459f3.html
16. Leung GM, Ho LM, Chan SK, Ho SY, Bacon-Shone J, Choy RY, et al. Longitudinal assessment of community psychobehavioral responses during and after the 2003 outbreak of severe acute respiratory syndrome in Hong Kong. Clin Infect Dis. 2005;40:1713–20. http://dx.doi.org/10.1086/429923
17. Leung GM, Lam TH, Ho LM, Ho SY, Chan BH, Wong IO, et al. The impact of community psychological responses on outbreak control for severe acute respiratory syndrome in Hong Kong. J Epidemiol Community Health. 2003;57:857–63. http://dx.doi.org/10.1136/jech.57.11.857
18. Cowling BJ, Ng DM, Ip DK, Liao Q, Lam WW, Wu JT, et al. Community psychological and behavioral responses through the first wave of the 2009 influenza A(H1N1) pandemic in Hong Kong. J Infect Dis. 2010;202:867–76. http://dx.doi.org/10.1086/655811
19. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A(H7N9) virus. N Engl J Med. 2013;368:1888–97. http://dx.doi.org/10.1056/NEJMoa1304459
20. Fournié G, Guitian FJ, Mangtani P, Ghani AC. Impact of the implementation of rest days in live bird markets on the dynamics of H5N1 highly pathogenic avian influenza. J R Soc Interface. 2011;8:1079–89. http://dx.doi.org/10.1098/rsif.2010.0510
21. Leung YH, Lau EH, Zhang LJ, Guan Y, Cowling BJ, Peiris JS. Avian influenza and ban on overnight poultry storage in live poultry markets, Hong Kong. Emerg Infect Dis. 2012;18:1339–41. http://dx.doi.org/10.3201/eid1808.111879
22. Di Giuseppe G, Abbate R, Albano L, Marinelli P, Angelillo IF. A survey of knowledge, attitudes and practices towards avian influenza in an adult population of Italy. BMC Infect Dis. 2008;8:36. http://dx.doi.org/10.1186/1471-2334-8-36
23. Leslie T, Billaud J, Mofleh J, Mustafa L, Yingst S. Knowledge, attitudes, and practices regarding avian influenza (H5N1), Afghanistan. Emerg Infect Dis. 2008;14:1459–61. http://dx.doi.org/10.3201/eid1409.071382
24. Fournié G, Pfeiffer DU. Can closure of live poultry markets halt the spread of H7N9? Lancet. 2014;383:496–7. http://dx.doi.org/10.1016/S0140-6736(13)62109-1

Address for correspondence: Hongjie Yu, Division of Infectious Disease, Key Laboratory of Surveillance and Early-warning on Infectious Disease, Chinese Center for Disease Control and Prevention, No. 155, Changbai Road, Changping District, Beijing, 102206, China; email: yuhj@chinacdc.cn

Rapid Whole-Genome Sequencing for Surveillance of *Salmonella enterica* Serovar Enteritidis

Henk C. den Bakker, Marc W. Allard, Dianna Bopp, Eric W. Brown, John Fontana, Zamin Iqbal, Aristea Kinney, Ronald Limberger, Kimberlee A. Musser, Matthew Shudt, Errol Strain, Martin Wiedmann, and William J. Wolfgang

For *Salmonella enterica* serovar Enteritidis, 85% of isolates can be classified into 5 pulsed-field gel electrophoresis (PFGE) types. However, PFGE has limited discriminatory power for outbreak detection. Although whole-genome sequencing has been found to improve discrimination of outbreak clusters, whether this procedure can be used in real-time in a public health laboratory is not known. Therefore, we conducted a retrospective and prospective analysis. The retrospective study investigated isolates from 1 confirmed outbreak. Additional cases could be attributed to the outbreak strain on the basis of whole-genome data. The prospective study included 58 isolates obtained in 2012, including isolates from 1 epidemiologically defined outbreak. Whole-genome sequencing identified additional isolates that could be attributed to the outbreak, but which differed from the outbreak-associated PFGE type. Additional putative outbreak clusters were detected in the retrospective and prospective analyses. This study demonstrates the practicality of implementing this approach for outbreak surveillance in a state public health laboratory.

For genetically monomorphic bacteria, current typing methods often prove inadequate for outbreak detection, trace back, and identification of transmission routes. Some of these bacteria, such as *Salmonella enterica* serovar Enteritidis, *S. enterica* serovar Montevideo, *Staphylococcus aureus*, *Clostridium difficile*, *Klebsiella pneumoniae*, and *Mycobacterium tuberculosis*, cause diseases that have major public health effects. For these pathogens, retrospective studies have unambiguously demonstrated that phylogenetic analysis based on whole-genome–derived single nucleotide polymorphisms (SNPs) improves cluster resolution and would be an invaluable tool in epidemiologic investigations (*1*–*8*). We refer to this approach as whole-genome cluster analysis.

Introduction of small, affordable, and rapid benchtop whole-genome sequencers, such as the Illumina MiSeq (Illumina, San Diego, CA, USA) MiSeq and the Ion Torrent PGM (Life Technologies, Carlsbad, CA, USA), has made it possible for clinical and public health laboratories to contemplate adding genome sequencing as a rapid typing tool. Eyre et al. (*9*) showed the utility of Illumina MiSeq in the detection of nosocomial outbreaks of *S. aureus* and *C. difficile* infections. Although Eyre et al. (*9*) showed the utility of this approach in improving typing of these monomorphic pathogens, the utility of these sequencers in a larger public health setting, in which capacity and turn-around times are critical parameters, has not been demonstrated.

The standard typing method for *Salmonella* species, which is used by PulseNet laboratories, is pulsed-field gel electrophoresis (PFGE) (*10*). However, PFGE has limited discriminatory power for *S. enterica* serovar Enteritidis strains and clusters. At the New York State Department of Health (NYSDOH) Wadsworth Laboratories (Albany, NY, USA), ≈50% of the 350–500 *S. enterica* serovar Enteritidis isolates received each year are PFGE type JEGX01.0004. Multilocus variable-number tandem-repeat analysis (MLVA) of these isolates improves discrimination of disease clusters for this pathogen, but even this tool assigns 30% of isolates to a single MLVA type. Because genomic homogeneity of *S. enterica* serovar Enteritidis is

Author affiliations: Cornell University, Ithaca, New York, USA (H. den Bakker, M. Wiedmann); US Food and Drug Administration, College Park, Maryland, USA (M.W. Allard, E.W. Brown, E. Strain); New York State Department of Health/Wadsworth Center, Albany, New York, USA (D. Bopp, R. Limberger, M. Shudt, K.A. Musser, W.J. Wolfgang); Connecticut Department of Public Health, Rocky Hill, Connecticut, USA (J. Fontana, A. Kinney); and Wellcome Trust Centre for Human Genetics, Oxford, UK (Z. Iqbal)

DOI: http://dx.doi.org/10.3201/eid2008.131399

also observed on a national and international level (*11*), a clear need exists for improved typing methods for *S. enterica* serovar Enteritidis in the public health laboratory.

To determine if whole-genome cluster analysis can improve subtype discrimination and cluster detection in the public health laboratory, we sequenced 93 *S. enterica* serovar Enteritidis isolates received during routine surveillance activities at the NYSDOH by using the Ion Torrent PGM located in the core sequencing facility. The sequence data were used to create SNP-based phylogenetic trees. This study consisted of 2 parts. First, we conducted a retrospective analysis that focused on an epidemiologically defined outbreak of *S. enterica* serovar Enteritidis JEGX01.0004 within a long-term care facility (LTCF). Second, we conducted a prospective study in which nearly all *S. enterica* serovar Enteritidis PFGE patterns JEGX01.0004 and JEGX01.0021 were sequenced during a 4-month period during the summer of 2012. In addition, we retrospectively sequenced JEGX01.0009 *S. enterica* serovar Enteritidis isolates that had been associated with contaminated ground beef early in the summer of 2012.

The retrospective part of the study serves as a proof of principle and clearly demonstrates increased resolution of whole-genome cluster analysis for typing of common PFGE pattern types and subsequent outbreak detection. In the prospective study, we show the feasibility of detecting outbreaks in near real time, as well as improved resolution, of the method that enables detection of numerous potential outbreak clusters that would likely go undetected by PFGE.

Materials and Methods

Ninety-three *S. enterica* serovar Enteritidis isolates received at the Wadsworth Center were selected from our routine surveillance (online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/20/8/13-1399-Techapp1.pdf). Serotype, PFGE PulseNet pattern and NYS MLVA designation were determined by using standard methods (*10*,*12*–*14*) before sequencing. Retrospective study isolates had been collected during August 10, 2010–October 22, 2011. For this period, we sequenced all isolates with the outbreak–associated PFGE pattern JEGX01.0004 and NYS-MLVA pattern W (JEGX01.0004/NYS-W) (n = 28), selected isolates with the most common pattern JEGX01.0004/NYS-B (n = 6), and 1 isolate with pattern JEGX01.0004/NYS-AE, which was initially believed to be part of the outbreak. These isolates represent 5% of all *S. enterica* serovar Enteritidis isolates and 10% of all pattern JEGX01.0004 isolates received by the Wadsworth Center during this period.

Prospective study isolates were obtained during April 17, 2012–August 16, 2012. Isolates sequenced from this period included all JEGX01.0004/NYS-B (n = 22) except 2, all JEGX01.0004/NYS-W except 1 (n = 3), all JEGX01.0021/NYS-B (n = 22), selected JEGX01.0009/NYS-CR (n = 8), and 1 isolate each of JEGX01.0843/NYS-CR, JEGX01.0968/NYS-CR, and JEGX01.0034/NYS-B. These isolates represented 9% of all *Salmonella* species isolates received by our laboratory over the 4-month period. In addition, sequence data from earlier studies of *S. enterica* serovar Enteritidis (*11*,*15*) were included in the data analysis.

Enzymatic shearing of genomic DNA samples and generation of barcoded libraries were carried out by using the Ion Xpress Plus Fragment Library Kit and Ion Xpress Barcode Adapters Kit (Life Technologies). Libraries were size-selected by using a 2-step AMPure XP isolation method that was optimized for use in Ion 200 bp Template kits (*16*). Templates were prepared by using the Ion OneTouch 200 Template Kit version 2 and the Ion OneTouch system. Sequencing was conducted by using the Ion PGM with a 316 chip and the Ion PGM 200 Sequencing Kit. The Torrent Suite versions 2.0.1–2.2 (https://olex-secure.openlogic.com/packages/ts-iontorrent/2.2) was used for base calling. Isolates were sequenced with an average coverage of 16–135 times (median 53 times).

Two SNP detection methods were used in this study: a traditional reference-based method that used VarScan (*17*) for SNP detection, and a de novo genomic variant detection method as implemented in Cortex Variation Assembler (*18*). The traditional reference-based pipeline relied on BWA 0.6.1 (*19*) to map reads against a reference genome, and VarScan was used for SNP detection. Only SNPs that were in agreement with the following parameters were used in the analysis: minimal coverage of 8, minimal variant coverage of 8, minimum variant frequency of 90%, and a p value ≤0.01. A consensus sequence was created by using vcftools (http://vcftools.sourceforge.net/), and sites with coverage <8 were hard-masked in the consensus sequence.

The consensus sequences were used as input for BRATNextGen (*20*), a homologous recombination detection software. Recombinogenic regions detected by BRATNextGen and rRNA-encoding regions were excluded from further analyses. The publicly available genome sequence of *S. enterica* serovar Enteritidis P125109 (GenBank accession no. NC_011294) and a de novo–assembled draft genome of *S. enterica* serovar Enteritidis 10_34587 (GenBank accession no, AWOI00000000) were used as references in the analysis. The de novo assembly of *S. enterica* serovar Enteritidis 10_34587 was created by using MIRA version 3.2.1 (*21*).

Cortex_var version v1.0.5.14 (http://cortexassembler.sourceforge.net/index_cortex_var.html) was used for de novo variant detection. The run calls script was used to call variants by using the independent work flow (*18*) by using the bubble caller. Read filter parameters were

adjusted so that the maximum possible read length could be used without use of an excessive amount of random access memory. For the Ion Torrent, reads used in this study bases with a Phred score <15 were clipped and reads were split when homopolymers longer than 5 bp were encountered. SNPs and indels were used for further analysis if they passed the Cortex population filter/site classifier by using default parameters.

Population-level phylogenetic analysis was performed only on variable sites. Only sites that were correctly called in ≥95% of the isolates were included in the analysis. Maximum-likelihood–based phylogenetic inference and nucleotide substitution model selection were performed in MEGA 5.1 (*22*). A bootstrap analysis based on 150 bootstrap replicates was performed to assess robustness of individual clades.

Results

Identification of SNPs

Reference mapping against the publicly available genome sequence of *S. enterica* serovar Enteritidis P125109 yielded 1,240 SNPs among the 93 newly sequenced isolates from New York, and the de novo pipeline yielded 903 SNPs for the same dataset; 714 SNPs were called by both pipelines. Phylogenetic analysis showed the same population structure based on the reference-derived dataset, the de novo–derived dataset, and a dataset consisting of SNPs called by both pipelines. Results of the reference-based pipeline are reported in the remainder of this study. Addition of 41 previously sequenced isolates (*11*,*15*) to the reference mapping–based pipeline increased the number of SNPs to 4,510. After exclusion of regions that were putatively affected by homologous recombination and sites that were called in <95% of the isolates, 2,031 SNPs were used for further analysis.

Retrospective Study

To determine if whole-genome cluster analysis could improve the resolution of outbreak clusters for *S. enterica* serovar Enteritidis, we selected a retrospective cohort from an epidemiologically defined outbreak that occurred in Connecticut and New York during September 1, 2010–September 30, 2010. This outbreak was associated with an LTCF. The study cohort (isolates obtained during August 10, 2010–October 22, 2011) contained 7 JEGX01.0004/NYS-W (combined PulseNet PFGE type and NYS-MLVA type) isolates that were epidemiologically linked to the outbreak and 21 JEGX01.0004/NYS-W, 6 JEGX01.0004/NYS-B, and 1 JEGX01.0004/NYS-AE that were considered to be from sporadic outbreaks (Table).

Maximum-likelihood analysis of the SNP matrix placed the epidemiologically defined outbreak isolates in a well-supported clade with an average pairwise SNP difference of <1.0 (Figure 1). The clade is 78 SNPs distant from the nearest neighbor in the cohort. Whole-genome cluster analysis identified 9 additional isolates as part of this outbreak cluster (Table; Figure 1). These additional isolates were obtained during the time of the outbreak in the same regions of New York and Connecticut and showed pattern JEGX01.0004/NYS-W, but were not epidemiologically linked to the LTCF at the time of the outbreak (Table). No attempt was made to link these isolates to the LTCF outbreak. On the basis of whole-genome cluster analysis, 12 JEGX01.0004/NYS-W, 6 JEGX01.0004/NYS-B, and 1 JEGX01.0004/NYS-AE were unambiguously excluded from the outbreak. Excluded isolates were characterized by an MLVA type other than NYS-W or were obtained at distant sites or at times outside the outbreak period. Among these excluded isolates, we detected 2 additional clusters (clusters A and B) not associated with any known outbreak (Table; Figure 1).

Prospective Study

To further evaluate the application of whole-genome cluster analysis to *S. enterica* serovar Enteritidis typing and cluster detection, we sequenced isolates with 2 of the most common combined PFGE/MLVA patterns (JEGX01.0004/NYS-B and JEGX01.0021/ NYS-B) as they were obtained during April 17, 2012–August 16, 2012. All JEGX01.0004/NYS-W isolates, the type associated with the LTCF outbreak, were sequenced to determine if this clone persisted. In addition, we conducted a retrospective analysis of isolates from another outbreak (online Technical Appendix Table) to test the performance of whole-genome cluster analysis in a second bona fide outbreak. Trees were constructed in an ad hoc manner as data were acquired, which was not real time because of slow turnaround times.

Whole-genome sequence data for these 58 isolates, as well as for 41 isolates that were sequenced as part of a large study of *S. enterica* serovar Enteritidis infections that were associated with eggs (*11*,*15*), were combined with the whole-genome sequence data from the retrospective study described above. Four well-supported (bootstrap values 100) clades were apparent (Figure 2). Clade 1 contained 62 isolates with PulseNet PFGE type JEGX01.0004 and 1 isolate with PFGE type JEGX01.0034. Clade 2 contained 23 isolates with PFGE type JEGX01.0021. Clade 3 contained 16 isolates associated with the 2010 LTCF outbreak described above. Clade 4 contained 10 isolates associated with a 2012 outbreak linked to consumption of contaminated ground beef (http://www.cdc.gov/salmonella/enteritidis-07-12/) and represented PFGE types JEGX01.0009 (n = 8), JEGX01.0843 (n = 1), or JEGX01.0968 (n = 1). Clades 1–3 all belong to *S. enterica* serovar Enteritidis lineage V, a clade that is the prevalent lineage in the United States

Table. Retrospective cohort of *Salmonella enterica* serovar Enteritidis isolates analyzed by whole-genome cluster analysis*

ID no.	Collection date	State	PFGE-MLVA combined†	Cluster detected by epidemiology‡	Cluster detected by WGCA‡
10_28670	2010 Aug 8	NY	JEGX01.0004-B	–	–
10_29153	2010 Aug 10	NY	JEGX01.0004-W	–	–
10_29949	2010 Aug 16	NY	JEGX01.0004-B	–	–
10_30147	2010 Aug 22	NY	JEGX01.0004-W	–	Cluster B
10_31528	2010 Aug 26	NY	JEGX01.0004-W	–	–
10_33213	2010 Sep 10	NY	JEGX01.0004-W	–	LTCF
10_33369	2010 Sep 10	NY	JEGX01.0004-W	–	LTCF
10_33371	2010 Sep 11	NY	JEGX01.0004-W	–	LTCF
10_35179	2010 Sep 12	CT	JEGX01.0004-W	LTCF	LTCF
10_35180	2010 Sep 12	NY	JEGX01.0004-W	LTCF	LTCF
10_35182	2010 Sep 12	NY	JEGX01.0004-W	LTCF	LTCF
10_35178	2010 Sep 13	NY	JEGX01.0004-W	LTCF	LTCF
10_35181	2010 Sep 13	NY	JEGX01.0004-W	LTCF	LTCF
10_34601	2010 Sep 13	NY	JEGX01.0004-W	–	LTCF
10_34213	2010 Sep 13	NY	JEGX01.0004-B	–	–
10_33603	2010 Sep 14	NY	JEGX01.0004-B	–	–
10_34599	2010 Sep 15	NY	JEGX01.0004-W	–	Cluster A
10_35183	2010 Sep 16	CT	JEGX01.0004-W	LTCF	LTCF
10_35184	2010 Sep 16	NY	JEGX01.0004-AE	–	–
10_36119	2010 Sep 17	NY	JEGX01.0004-W	LTCF	LTCF
10_34587	2010 Sep 20	NY	JEGX01.0004-W	–	LTCF
10_35417	2010 Sep 22	NY	JEGX01.0004-W	–	LTCF
10_36319	2010 Sep 28	NY	JEGX01.0004-W	–	LTCF
10_37723	2010 Oct 4	NY	JEGX01.0004-B	–	–
10_36979	2010 Oct 8	NY	JEGX01.0004-W	–	LTCF
10_39087	2010 Oct 27	NY	JEGX01.0004-B	–	–
10_38792	2010 Oct 29	NY	JEGX01.0004-W	–	LTCF
11_03844	2011 Feb 1	NY	JEGX01.0004-W	–	Cluster B
11_06235	2011 Feb 21	NY	JEGX01.0004-W	–	Cluster A
11_21079	2011 Jul 13	NY	JEGX01.0004-W	–	Cluster A
11_22186	2011 Jul 22	NY	JEGX01.0004-W	–	Cluster B
11_27690	2011 Sep 6	NY	JEGX01.0004-W	–	Cluster B
11_31312	2011 Oct 5	NY	JEGX01.0004-W	–	Cluster B
11_30508	2011 Oct 9	NY	JEGX01.0004-W	–	Cluster B
11_32014	2011 Oct 22	NY	JEGX01.0004-W	–	Cluster B

*ID, identification; PFGE, pulsed-field gel electrophoresis; MLVA, multilocus variable-number tandem-repeat analysis; WGCA, whole-genome cluster analysis; –, sporadic outbreaks; LTCF, isolates assigned to long-term care facility outbreak.
†The letter designating the New York MLVA type follows PulseNet PFGE pattern designation.
‡Cluster designations are indicated by boxes in Figure 1.

and is predominately associated with poultry products, such as shelled eggs and broilers (K. Deng et al., unpub. data). Clades 1 and 2 also contained isolates from clade C2 associated with the shelled egg outbreak in 2010 (*11*,*15*). Clade 4 belongs to *S. enterica* serovar Enteritidis lineage II, which is rare among *S. enterica* serovar Enteritidis isolates from the United States, but has been isolated from mammalian hosts (K. Deng et al., unpub. data).

Clades 3 and 4 are composed of isolates from epidemiologically defined outbreaks that are divergent from other *S. enterica* serovar Enteritidis isolates in this study. Within-clade variability is limited for both clades (clade 3: 0–2 pairwise SNP differences, average pairwise SNP difference <1 SNP; clade 4: 0–3 pairwise SNP differences, average pairwise SNP difference 1.2 SNP), which, together with the short time span from the first isolate to the last isolate, points toward a point source outbreak.

Clade 4 consists of 10 isolates (9 from humans and 1 from contaminated hamburger [12_19824]) from a multistate outbreak in 2012 associated with beef. A total of 46 cases in 9 states were associated with this outbreak (http://www.cdc.gov/salmonella/enteritidis-07-12/). This outbreak was unusual because it was associated with contaminated beef, and *S. enterica* serovar Enteritidis is more commonly associated with poultry products (*23*). Eight of the isolates sequenced were PFGE type JEGX01.0009, and 2 isolates (12_18137 and 12_21314) were PFGE types JEGX01.0968 and JEGX01.0843, respectively (these differ by only 1–2 bands from JEGX01.0009).

Isolates with PFGE type JEGX01.0968 or JEGX01.0843 have the same SNP profile as 2 PFGE type JEGX01.0009 isolates (12_18775 and 12_19824), which suggests that the difference in 3 PFGE types is not associated with differences in the genomic backbone of these isolates. De novo assembly of sequence data for isolates 12_18137 and 12_21314 by using MIRA version 3.2.1 corroborated this finding and showed that each isolate

contains ≥1 large plasmids. To assess the distribution of these plasmids among the isolates from the outbreak, we used Cortex_var for a plasmid presence/absence analysis. This analysis showed that these plasmids are absent from all other isolates in this outbreak.

Most isolates obtained by NYSDOH and sequenced in this study belonged to clades 1 and 2. Pairwise SNP differences within the clades are similar: 0–53 pairwise SNP differences (average 29.5) between isolates in clade 1, and 0–42 pairwise SNP differences (average 25.1) between isolates in clade 2. Indicative of a highly structured population, clades 1 and 2 can be further subdivided into 6 and 5 well-supported (bootstrap value >97%) subclades, respectively (Figure 2). These subclades most likely represent strains that persist in the environment (i.e., in poultry) and consequently caused multiple human cases. Evidence for persistence is particularly strong for clade 1, in which 5 of 6 clusters contain isolates obtained during the summer or fall of 2010–summer of 2012. Further research and epidemiologic data are needed to determine if these strains are widely distributed or represent exposure to a specific source.

To assess the distribution of plasmids and prophages, we queried de novo assemblies of representative isolates from each clade and each PFGE type by using Cortex_var. This analysis showed that the *S. enterica* serovar Enteritidis virulence plasmid pSLA5 (GenBank accession no. NC_019002.1) was present in all isolates sequenced, with the exception of 12_23426 from clade 1. This analysis also confirmed the presence of the unique plasmids found by

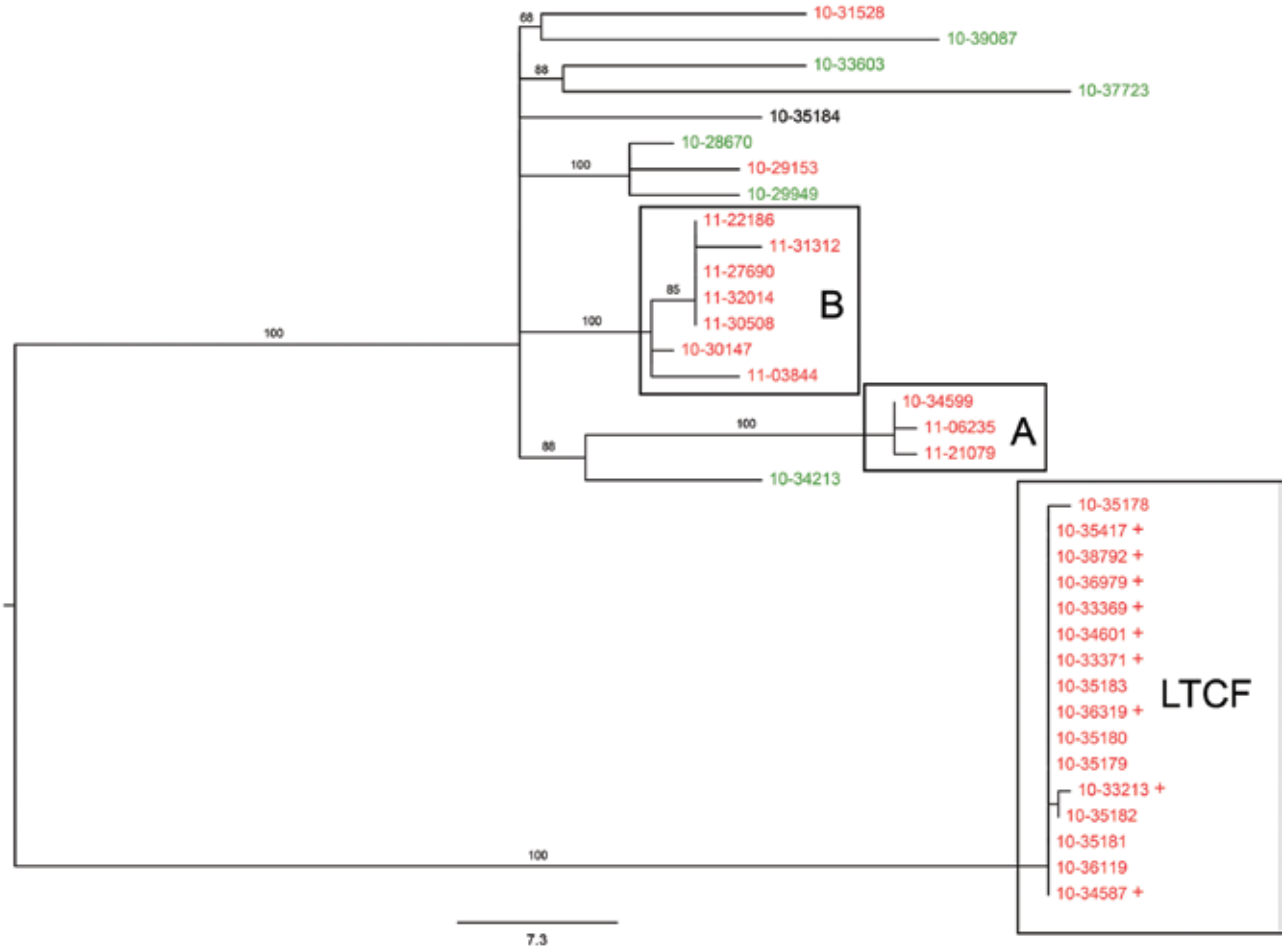


Figure 1. Maximum-likelihood tree of population structure of *Salmonella enterica* serovar Enteritidis isolates obtained in New York and neighboring states, USA. The tree was inferred by using a general time-reversible model with a gamma distribution and was inferred to be the best fit model by the maximum-likelihood method implemented in MEGA 5.1 (*22*). Values on branches are bootstrap values based on 150 bootstrap replicates. Note the well supported and distant cluster associated with the long-term care facility (LTCF), as well as additional clusters A and B. Labels of isolates are colored according to their New York State Department of Health Wadsworth Laboratories multilocus variable-number tandem-repeat analysis (MLVA) subtype designation. Green, MLVA subtype B; red, MLVA subtype W; black, MLVA subtype AE. + indicates isolates in the LTCF cluster that were detected only by whole-genome analysis and were not detected epidemiologically. Scale bar indicates single-nucleotide polymorphisms per site.

de novo assembly for isolates 12_18137 and 12_21314 described above. In clades 1, 2, and 3, prophage distribution is highly conserved, and all genomes sequenced in this study contained an ELPhiS-like (*24*) prophage. Although the ELPhis-like prophage is absent from all clade 4 isolates, these isolates contain a unique prophage region of ≈49 kb, which was not found in other clades studied here or in currently published *Salmonella* genomes.

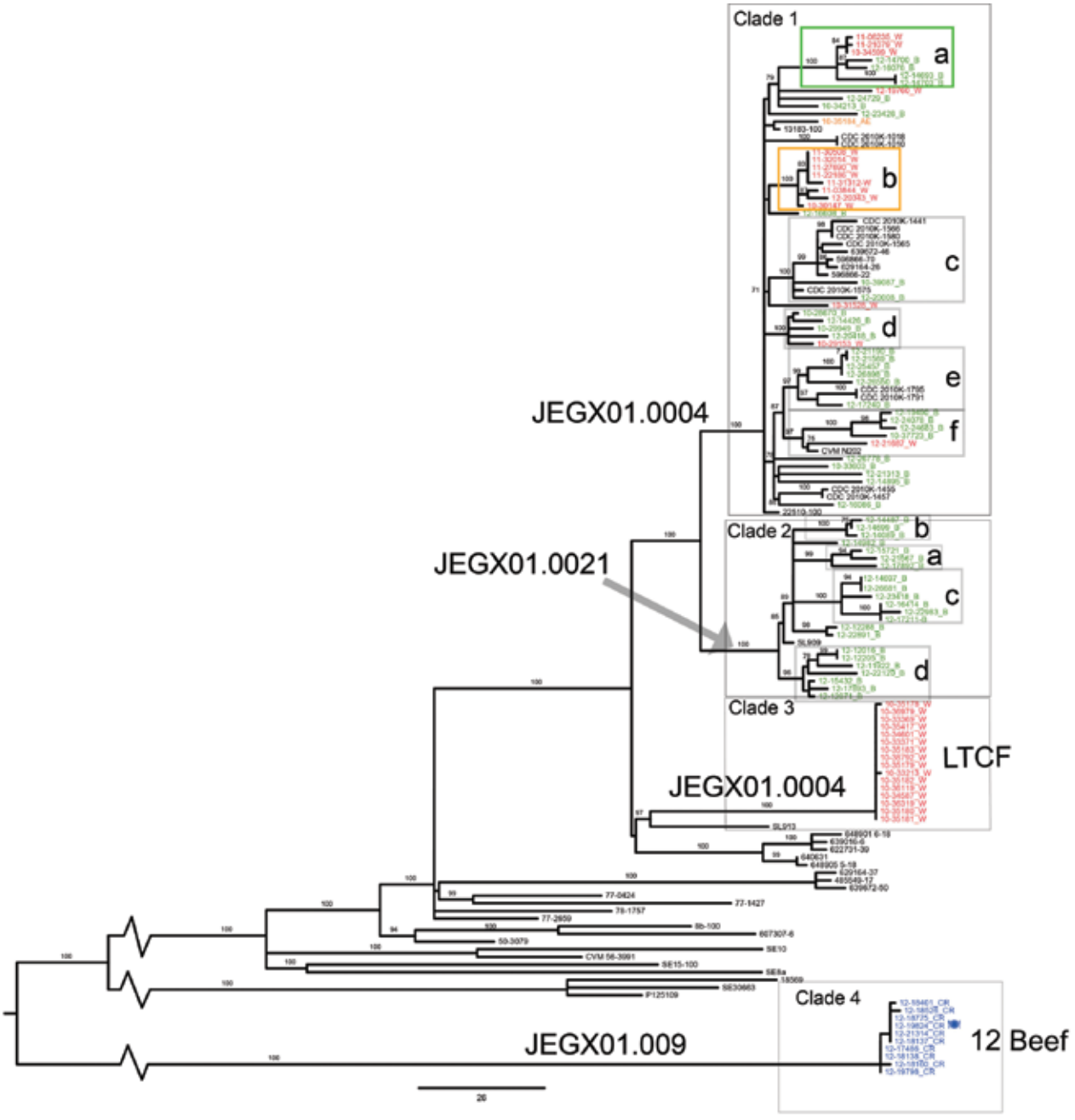


Figure 2. Maximum-likelihood tree of population structure of *Salmonella enterica* serovar Enteritidis isolates obtained in New York and neighboring states, USA. The tree was inferred by using a general time-reversible model with a gamma distribution, which was inferred to be the best fit model by the maximum-likelihood method implemented in MEGA 5.1 (*22*). Values on branches are bootstrap values based on 150 bootstrap replicates. Pulsed-field gel electrophoresis (PFGE) types are indicated on branches. Labels of isolates are colored according to their New York State Department of Health Wadsworth Laboratories multilocus variable-number tandem-repeat subtype designation. Green, MLVA subtype B; red, MLVA subtype W; orange, MLVA subtype AE; blue, MLVA subtype CR; black, MLVA subtype data missing and isolates from Allard et al. (*11*). Rectangles indicate well-supported clusters of at least 3 isolates, letters within the rectangles correspond to the cluster designation in the Table. LTCF, long-term care facility. Scale bar indicates single-nucleotide polymorphisms per site.

Discussion

In this study, we demonstrated that whole-genome cluster analysis of *S. enterica* serovar Enteritidis results in vastly improved detection of clusters of common PFGE types and outbreak resolution than PFGE, the current standard. Analysis of a retrospective dataset showed that all isolates associated with an LTCF outbreak in 2010 belonged to a well-supported clade with an average <1.0 SNP distance between all the isolates in the clade. Furthermore, this clade is 78 SNPs distant from the nearest neighboring sporadic isolates. Additional clinical isolates obtained during the time of the outbreak from patients in surrounding communities not previously associated with the outbreak also belonged to the clade, which expanded the number of possible outbreak cases from 7 to 16. Identification of these additional 9 matching isolates suggests a common contaminated source outside the LTCF. Knowledge of these cases at the time of the outbreak might have improved the chances of finding the outbreak source, which was never identified. Furthermore, whole-genome cluster analysis showed that the LTCF outbreak belonged to the same monophyletic lineage as isolates in 2 clades associated with the 2010 shelled egg outbreak, suggesting that shelled eggs are a common source of infection (*11*,*15*). For the LTCF outbreak, MLVA data showed concordance with whole-genome sequencing data. In contrast, PFGE analysis of all isolates (i.e., from the LTCF and the shelled eggs outbreak) resulted in a single type (JEGX01.0004), which yielded no useful molecular clustering information.

When we combined retrospective and prospective datasets, no additional isolates clustered with those from the LTCF outbreak (these datasets included 3 JEGX01.0004/NYS-W isolates). However, several other clusters were detected. One well-supported and distant cluster associated with an outbreak linked to contaminated beef contains PFGE patterns JEGX 01.009, JEGX01.0968, and JEGX01.0843. These PFGE types are rarely seen in the United States. Other smaller well-supported clusters were observed that contained isolates obtained during a 2.5-year period, which suggested persistence of point sources in the environment.

During the outbreak associated with contaminated beef, 2 isolates (12_18137 and 12_21314), which have distinct PFGE types (JEGX 01.0968 and JEGX 01.0843, respectively), had not been included in the outbreak. However, whole-genome cluster analysis placed these isolates in the outbreak cluster. De novo assembly of the sequences of these 2 strains showed the presence of plasmids that are not found in other isolates in this clade. This observation is consistent with observations of Zhou et al. (*25*), who found that differences in PFGE types in *S. enterica* serovar Agona could be attributed mainly to differences in the content of mobile elements (e.g., prophages and plasmids), and not to SNP-related differences in the genomic backbone. Similar to our observations, Gilmour et al. (*26*) also linked *Listeria monocytogenes* isolates with PFGE types that differed by <3 bands to an outbreak, on the basis of whole-genome sequencing data, which indicated that PFGE pattern diversification was caused by mobile elements.

Other retrospective studies of *S. aureus*, *K. pneumoniae*, *C. difficile*, and *M. tuberculosis* have also demonstrated improved resolution of whole-genome cluster analysis (*2*–*8*,*15*). Whether this approach can be translated to the public health laboratory setting is still unclear. These laboratories currently support the bulk of outbreak investigations through various formal and informal networks. Interpretation of whole-genome sequencing data are relatively straightforward and no more challenging than the interpretation of PFGE or MLVA data. During our prospective study, we were able to sequence 12 isolates at the NYSDOH and analyze the output at the Cornell Food Safety Laboratories within 8 days. Recently, our throughput has increased to 32 isolates in the same period, and analysis can be conducted in house. At this rate, all *Salmonella* isolates received for surveillance can be sequenced in a timeframe that is useful to epidemiologists. In addition, once whole-genome cluster analysis is fully implemented for all *Salmonella* isolates, serovar (*27*) and multilocus sequence typing (*28*) information could be inferred solely from the genome data to link sequenced isolates to historical data, which would shorten the turnaround time by 2 days. Thus, it is reasonable to expect that public health laboratories can serve as centers for these new technologies and will be able to detect clusters in a meaningful time frame.

For national surveillance, whole-genome cluster analysis could use public health laboratories and standardized protocols and procedures to analyze data locally and upload raw sequence data to centralized sites for analysis. This approach builds upon the PulseNet model (*10*) and would enable a rapid local response and centralized control. One model for centralized data is being tested in a collaboration between the US Food and Drug Administration, the National Center for Biotechnology Information, and selected state public health laboratories (http://www.fda.gov/Food/FoodScienceResearch/WholeGenomeSequencingProgramWGS/default.htm). Surveillance laboratories upload raw sequence reads that are processed and added to a single tree that harbors all sequenced *S. enterica* isolates and associated metadata (date of isolation, isolation source, location, unique identifier). As clusters appear, they would be reported to the surveillance laboratories, which would communicate the information to epidemiologists. The uploading, analysis, and reporting could be highly automated.

Many challenges need to be addressed before a whole-genome sequence–based surveillance system can be implemented. In addition to standardization of protocols and

analyses, several questions still need to be resolved. What constitutes an epidemiologically meaningful phylogenetic cluster? Do circulating persistent clones confound this analysis? How will this information be reported to epidemiologists? Pilot studies at the Centers for Disease Control and Prevention (Atlanta, GA, USA) and NYSDOH to implement real-time whole-genome–based surveillance for *L. monocytogenes* and *S. enterica* serovar Enteritidis, respectively, will begin to address these questions.

Improving surveillance and tracking of pathogens is a high priority goal for federal and state agencies charged with protecting public health. Affordable and rapid next-generation sequencing technologies and associated bioinformatics will be potent tools in achieving these goals. This study demonstrates the practical feasibility and benefits of deploying these technologies in public health laboratories.

Acknowledgments

We thank the Wadsworth Center Applied Genomic Technologies Core for library preparation and whole-genome sequencing of isolates.

The study was supported by the Wadsworth Center Public Health Genomics Pilot Program (W.J.W.), the US Food and Drug Administration (M.W.A.), and US Department of Agriculture–Agriculture and Food Research Initiative special research grants 2010-34459-20756 and 2009-34459-19750 (M.W.).

Dr den Bakker is a research associate at Cornell University, Ithaca, New York. His research focuses on genomics and population genetics of foodborne pathogens.

References

1. Bakker HC, Switt AI, Cummings CA, Hoelzer K, Degoricija L, Rodriguez-Rivera LD, et al. A whole genome SNP based approach to trace and identify outbreaks linked to a common *Salmonella enterica* subsp. *enterica* serovar Montevideo pulsed-field gel electrophoresis type. Appl Environ Microbiol. 2011;77:8648–55. http://dx.doi.org/10.1128/AEM.06538-11
2. Lienau EK, Strain E, Wang C, Zheng J, Ottesen AR, Keys CE, et al. Identification of a salmonellosis outbreak by means of molecular sequencing. N Engl J Med. 2011;364:981–2. http://dx.doi.org/10.1056/NEJMc1100443
3. Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, et al. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med. 2012;366:2267–75. http://dx.doi.org/10.1056/NEJMoa1109910
4. Snitkin ES, Zelazny AM, Thomas PJ, Stock F; NISC Comparative Sequencing Program Group, Henderson DK, et al. Tracking a hospital outbreak of carbapenem-resistant *Klebsiella pneumoniae* with whole-genome sequencing. Sci Transl Med. 2012;2:148ra116. http://dx.doi.org/10.1126/scitranslmed.3004129.
5. Eyre DW, Cule ML, Griffiths D, Crook DW, Peto TE, Walker AS, et al. Detection of mixed infection from bacterial whole genome sequence data allows assessment of its role in *Clostridium difficile* transmission. PLOS Comput Biol. 2013;9:e1003059. http://dx.doi.org/10.1371/journal.pcbi.1003059
6. Roetzer A, Diel R, Kohl TA, Rückert C, Nübel U, Blom J, et al. Whole genome sequencing versus traditional genotyping for investigation of a *Mycobacterium tuberculosis* outbreak: a longitudinal molecular epidemiological study. PLoS Med. 2013;10:e1001387. http://dx.doi.org/10.1371/journal.pmed.1001387
7. Török ME, Reuter S, Bryant J, Köser CU, Stinchcombe SV, Nazareth B, et al. Rapid whole-genome sequencing for investigation of a suspected tuberculosis outbreak. J Clin Microbiol. 2013;51:611–4. http://dx.doi.org/10.1128/JCM.02279-12
8. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al. Whole-genome sequencing to delineate *Mycobacterium tuberculosis* outbreaks: a retrospective observational study. Lancet Infect Dis. 2013;13:137–46. http://dx.doi.org/10.1016/S1473-3099(12)70277-3
9. Eyre DW, Golubchik T, Gordon NC, Bowden R, Piazza P, Batty EM, et al. A pilot study of rapid benchtop sequencing of *Staphylococcus aureus* and *Clostridium difficile* for outbreak detection and surveillance. BMJ Open. 2012;2:pii: e0011240. http://dx.doi.org/10.1136/bmjopen-2012-001124.
10. Ribot EM, Fair MA, Gautom R, Cameron DN, Hunter SB, Swaminathan B, et al. Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. Foodborne Pathog Dis. 2006;3:59–67. http://dx.doi.org/10.1089/fpd.2006.3.59
11. Allard MW, Luo Y, Strain E, Pettengill J, Timme R, Wang C, et al. On the evolutionary history, population genetics and diversity among isolates of *Salmonella* Enteritidis PFGE pattern JEGX01.0004. PLoS ONE. 2013;8:e55254. http://dx.doi.org/10.1371/journal.pone.0055254
12. Cho S, Boxrud DJ, Bartkus JM, Whittam TS, Saeed M. Multiple-locus variable-number tandem repeat analysis of *Salmonella* Enteritidis isolates from human and non-human sources using a single multiplex PCR. FEMS Microbiol Lett. 2007;275:16–23. http://dx.doi.org/10.1111/j.1574-6968.2007.00875.x
13. Boxrud D, Pederson-Gulrud K, Wotton J, Medus C, Lyszkowicz E, Besser J, et al. Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis, and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. J Clin Microbiol. 2007;45:536–43. http://dx.doi.org/10.1128/JCM.01595-06
14. Brenner FW, McWhorter-Murlin AC. Identification and serotyping of *Salmonella*. Atlanta: Centers for Disease Control and Prevention; 1998.
15. Timme RE, Allard MW, Luo Y, Strain E, Pettengill J, Wang C, et al. Draft genome sequences of 21 *Salmonella enterica* serovar Enteritidis strains. J Bacteriol. 2012;194:5994–5. http://dx.doi.org/10.1128/JB.01289-12
16. Rodrigue S, Materna AC, Timberlake SC, Blackburn MC, Malmstrom RR, Alm EJ, et al. Unlocking short read sequencing for metagenomics. PLoS ONE. 2010;5:e11840. http://dx.doi.org/10.1371/journal.pone.0011840
17. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, et al. VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. Genome Res. 2012;22:568–76. http://dx.doi.org/10.1101/gr.129684.111
18. Iqbal Z, Turner I, McVean G. High-throughput microbial population genomics using the Cortex variation assembler. Bioinformatics. 2013;29:275–6. http://dx.doi.org/10.1093/bioinformatics/bts673
19. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics. 2009;25:1754–60. http://dx.doi.org/10.1093/bioinformatics/btp324
20. Marttinen P, Hanage WP, Croucher NJ, Connor TR, Harris SR, Bentley SD, et al. Detection of recombination events in bacterial genomes from large population samples. Nucleic Acids Res. 2012;40:e6. http://dx.doi.org/10.1093/nar/gkr928

21. Chevreux B, Wetter T, Suhai S. Genome sequence assembly using trace signals and additional sequence information. Presented at: Computer Science and Biology. Proceedings of the German Conference on Bioinformatics, GCB'99; 1999 Oct 4–6; Hannover, Germany. p. 45–56.
22. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121
23. Shah DH, Casavant C, Hawley Q, Addwebi T, Call DR, Guard J. *Salmonella* Enteritidis strains from poultry exhibit differential responses to acid stress, oxidative stress, and survival in the egg albumen. Foodborne Pathog Dis. 2012;9:258–64. http://dx.doi.org/10.1089/fpd.2011.1009
24. Hanna LF, Matthews TD, Dinsdale EA, Hasty D, Edwards RA. Characterization of the ELPhiS prophage from *Salmonella enterica* serovar Enteritidis strain LK5. Appl Environ Microbiol. 2012;78:1785–93. http://dx.doi.org/10.1128/AEM.07241-11
25. Zhou Z, McCann A, Litrup E, Murphy R, Cormican M, Fanning S, et al. Neutral genomic microevolution of a recently emerged pathogen, *Salmonella enterica* serovar Agona. PLoS Genet. 2013;9:e1003471. http://dx.doi.org/10.1371/journal.pgen.1003471
26. Gilmour MW, Graham M, van Domselaar G, Tyler S, Kent H, Trout-Yakel KM, et al. High-throughput genome sequencing of two *Listeria monocytogenes* clinical isolates during a large foodborne outbreak. BMC Genomics. 2010;11:120. http://dx.doi.org/10.1186/1471-2164-11-120
27. Ranieri ML, Shi C, Switt AIM, Bakker Den HC, Wiedmann M. Comparison of typing methods with a new procedure based on sequence characterization for *Salmonella* serovar prediction. J Clin Microbiol. 2013;51:1786–97. http://dx.doi.org/10.1128/JCM.03201-12
28. Achtman M, Wain J, Weill F-X, Nair S, Zhou Z, Sangal V, et al. Multilocus sequence typing as a replacement for serotyping in *Salmonella enterica.* PLoS Pathog. 2012;8:e1002776. http://dx.doi.org/10.1371/journal.ppat.1002776

Address for correspondence: William J. Wolfgang, Bacteriology Laboratory, Wadsworth Center, New York State Department of Health, Albany, NY 12208, USA; email: wwolfgan@wadsworth.org

Novel Reassortant Influenza A(H5N8) Viruses in Domestic Ducks, Eastern China

Haibo Wu, Xiaorong Peng, Lihua Xu, Changzhong Jin, Linfang Cheng, Xiangyun Lu, Tiansheng Xie, Hangping Yao, and Nanping Wu

Domestic ducks are natural reservoirs of avian influenza viruses and serve as reassortant hosts for new virus subtypes. We isolated 2 novel influenza A(H5N8) viruses from domestic ducks in eastern China, sequenced their genomes, and tested their pathogenicity in chickens and mice. Circulation of these viruses may pose health risks for humans.

Avian influenza viruses are members of the family *Orthomyxoviridae* and contain 8 segments of single-stranded RNA with negative polarity (*1*). These viruses are classified into subtypes on the basis of their envelope proteins hemagglutinin (HA) and neuraminidase (NA). Aquatic birds, including domestic ducks, have been considered the natural reservoir of these viruses (*2*). Although domestic ducks do not usually display symptoms when they are infected with these viruses, they provide an environment for the reassortment of low pathogenicity avian influenza viruses, which can serve as progenitors of highly pathogenic avian influenza viruses (*3*).

Because live poultry markets are considered a major source of avian influenza virus dissemination and sites for potential influenza virus reassortment, as well as interspecies transfer (*3*,*4*), we participated in active surveillance of these virus in live poultry markets. We sequenced genes from 2 novel influenza A(H5N8) viruses isolated from domestic ducks in eastern China and evaluated their pathogenicity in chickens and mice.

The Study

During surveillance of poultry for avian influenza viruses in live poultry markets in Zhejiang Province in eastern China in 2013, we isolated 2 influenza A(H5N8) viruses, A/duck/Zhejiang/W24/2013(H5N8) (W24) and A/duck/Zhejiang/6D18/2013(H5N8) (6D18), from domestic ducks. To better understand genetic relatedness between these viruses, we sequenced all gene segments of these 2 viruses and compared them with influenza virus sequences in GenBank. We also determined the virulence of the 2 isolates in chickens and mice.

For virus isolation, cloacal swab specimens from domestic ducks were inoculated into embryonated chicken eggs as described (*5*). All experiments with viruses were performed in a Biosafety Level 3 laboratory.

RNA was extracted by using the Viral RNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. All gene segments were amplified with primers, and fragments were sequenced and analyzed as described (*6*–*8*). Sequence data obtained were submitted to GenBank under accession nos. KJ476663–KJ476678.

Sequence analysis showed that all sequences of 8 genes (HA, NA, basic polymerase 1, basic polymerase 2 [PB2], acidic polymerase, nucleoprotein, matrix protein, and nonstructural protein [NS]) of viruses W24 and 6D18 showed 99.9%–100% sequence similarity (Figure 1, panel A, http://wwwnc.cdc.gov/EID/article/20/8/14-0339-F1.htm). Results show that the HA gene of W24 was closely related to those HA genes of H5N8 subtype viruses circulating in South Korea in 2014 (*9*); W24 belongs to clade 2.3.4.

Sequence analysis suggested that these H5N8 subtype viruses were most closely related to isolates from poultry in countries in eastern Asia. Previous studies have shown that H5 subtype viruses within clade 2.3.2 have been circulating widely in poultry and wild birds in China since 2007 (*7*,*10*). Our results indicated that the 2 novel H5 subytpe viruses belong to clade 2.3.4, the prevalent lineage in southern China since 2005 (*11*); thus showing their presence in eastern China. NA gene phylogeny indicated that a novel influenza A(H10N8) virus, which infected humans, had different ancestors for this gene (Figure 1, panel B).Gene phylogenies for 6 other genes indicated that H9N2 subtype viruses circulating in China were not donors of these genes for W24.

Lee et al. (*9*) recently reported that that HA and NA genes of 3 H5N8 subtype viruses isolated in South Korea in 2014 had high nucleotide identities with A/duck/Jiangsu/k1203/2010(H5N8). All 8 genes of W24 were closely related to those of H5N8 subtype viruses, such as A/breeder duck/Korea/Gochang1/2014(H5N8), which are circulating in South Korea. Basic polymerase 1, acidic polymerase, HA, nucleoprotein, NA, and matrix genes of W24 were also closely related to those of A/duck/Jiangsu/k1203/2010(H5N8). PB2 and NS genes of W24 were most closely related to those of

Author affiliations: Zhejiang University, Hangzhou, China (H. Wu, X. Peng, C. Jin, L. Cheng, X. Lu, T. Xie, H. Yao, N. Wu); Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou (H. Wu, X. Peng, C. Jin, L. Cheng, X. Lu, T. Xie, H. Yao, N. Wu); and Zhejiang Academy of Agricultural Science, Hangzhou (L. Xu)

DOI: http://dx.doi.org/10.3201/eid2008.140339

Table 1. Sequence homology of whole genome of influenza A(H5N8) A/duck/Zhejiang/W24/2013 virus isolated from domestic ducks, eastern China, 2013, compared with nucleotide sequences available in GenBank*

Gene	Virus with the highest percentage of nucleotide identity	GenBank accession no.	Homology, %
PB2	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413831	99
	A/environment/Jiangxi/28/2009 (H11N9)	KC881295	98
PB1	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413832	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973692	99
PA	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413833	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973693	99
HA	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413834	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973694	99
NP	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413835	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973695	99
NA	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413836	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973696	99
M	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413837	99
	A/duck/Jiangsu/k1203/2010 (H5N8)	JQ973697	99
NS	A/breeder duck/Korea/Gochang1/2014 (H5N8)	KJ413838	99
	A/duck/Hunan/8–19/2009 (H4N2)	HQ285890	99

*PB2, basic polymerase 2; PB1, basic polymerase 1; PA, acidic polymerase; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix; NS, nonstructural protein.

A/environment/Jiangxi/28/2009(H11N9) and A/duck/Hunan/8–19/2009(H4N2), respectively (Table 1; Figure 2). On the basis of analysis of phylogenetic relationships, we found that W24 was a reassortant virus that derived its genes from a virus of a different subtype from poultry in China. We also found that H5N8 subtype viruses had been present in eastern China for several years and these viruses might have been spread to other countries by wild birds in recent years.

On the basis of deduced amino acid sequences of HA genes, we found that the HA cleavage site pattern (PLREKRRKR) of the 2 novel H5N8 subtype viruses indicated that these viruses were highly pathogenic. In this study, amino acid sequences of these H5N8 subtype viruses at positions 236–241 and 146–150 were NGQRGR and GVSAA, respectively. Receptor-binding sites (Gln226 and Gly228) of H5N8 subtype viruses were similar to those of the 2 novel H5N8 subtype viruses, which suggested that these 2 viruses would preferentially bind to avian-like receptors (*7*). The PB2 protein Lys627Glu mutation has been reported to influence the host range and confer increased virulence for H5N1 subtype viruses in animal models (*12*). This mutation was not observed in PB2 of the 2 novel H5N8 subtype viruses analyzed in this study, which indicated that these 2 viruses had low levels of pathogenicity for mice.

Deletion of several amino acids (position 80–84) in NS1 proteins had been observed more frequently in H5N1 subtype viruses, which indicated possible adaptation of these viruses to avian species (*13*). This deletion was not observed in the 2 novel H5N8 subtype viruses. These 2 viruses contained the NS1 Pro42Ser mutation, which is associated with increased virulence in mice (*14*).

To evaluate pathogenicity of W24 and 6D18 in chickens, we inoculated groups of ten 6-week-old specific pathogen–free chickens intravenously with a 10^6 median egg infective dose of each virus in a 0.2-mL volume of phosphate-buffered saline; deaths were observed over a 10-day period (*7*). Animal studies were conducted according to the recommendation of the World Organisation for Animal Health (Paris, France). Characteristics of W24 and 6D18 viruses are shown in Table 2. Results showed that these viruses were highly pathogenic to chickens.

To determine the pathogenicity of these viruses in a mammalian host, we inoculated BALB/c mice intranasally with a 10^6 median egg infective dose for each virus. Over a 14-day period, we observed virus replication in various

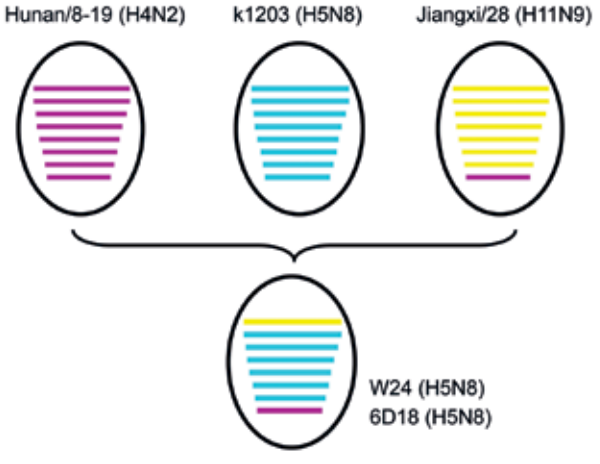


Figure 2. Putative genomic compositions of novel influenza A(H5N8) viruses isolated from poultry, eastern China, 2013, and their 3 possible parent viruses. The 8 gene segments (from top to bottom) in each virus are basic polymerase 2, basic polymerase 1, acidic polymerase, hemagglutinin, nucleoprotein, neuramindase, matrix, and nonstructural protein. Each color represents a separate virus background: purple indicates Hunan/8-19 (H4N2); A/duck/Hunan/8-19/2009(H4N2); blue indicates k1203 (H5N8), A/duck/Jiangsu/k1203/2010(H5N8); and yellow indicates Jiangxi/28 (H11N9), A/environment/Jiangxi/28/2009(H11N9). The simplified schematic illustration is based on nucleotide-distance comparison and phylogenetic analysis.

Table 2. Characteristics of 2 novel influenza A(H5N8) viruses isolated from domestic ducks, eastern China, 2013 *

Virus	Characteristic			Virus replication in experimentally infected mice, virus titers in organs of mice (log_{10} EID_{50}/mL)†			
	IVPI	EID_{50}	$TCID_{50}$	Tissue	3 d	6 d	9 d
W24	3.0	$10^{8.5}$	$10^{8.4}$	Lung	2.5 ± 0.5	3.0 ± 0.5	5.0 ± 0.5
	NA	NA	NA	Brain	–	–	–
	NA	NA	NA		–	–	–
	NA	NA	NA	Liver	–	–	–
6D18	3.0	$10^{8.5}$	$10^{9.6}$	Lung	2.0 ± 0.5	2.5 ± 0.5	3.0 ± 0.5
	NA	NA	NA	Brain	–	–	–
	NA	NA	NA	Heart	–	–	–
	NA	NA	NA	Liver	–	–	2.0 ± 0.5

*Groups of 14 adult female BALB/c mice were intranasally inoculated with a 10^6 median egg infective dose (EID_{50}) of each virus in a 0.05-mL volume of phosphate-buffered saline. Five mice per group were observed for survival within 14 d. The remaining 9 mice from each experimental group were exsanguinated on days 3, 6, and 9, and their lungs, brains, hearts and livers were collected for virus titration in embryonated chicken eggs. IVPI, intravenous pathogenicity index; $TCID_{50}$, median tissue culture infective dose; NA, not applicable.
†Values are mean ± SD. –, virus was not detected.

organs and deaths. After intranasal administration of W24 and 6D18, we observed 5 mice per group for survival over a 14-day period. On day 9 postinfection, high titers of virus was detected in lung and liver but were not detected in brain and heart. Mice had signs of illness but had survival rates of 80% (4/5) and 100% (5/5), respectively, for each virus during 14 days postinfection (Table 2). These results suggested that the 2 novel H5N8 subtype viruses did not kill mice but that they could replicate in the lung. Results of our study are consistent with those of Zhao et al. (*15*), who reported that H5N8 subtype virus from chickens did not cause deaths in mice.

Conclusions

We isolated 2 influenza A(H5N8) viruses were isolated from domestic ducks in eastern China in 2013. Results of phylogenetic analysis showed that these viruses were reassortant viruses that derived their genes from different virus subtypes. These reassortant H5N8 subtype viruses and their 3 possible parent viruses, A/duck/Jiangsu/k1203/2010 (H5N8), A/environment/Jiangxi/28/2009 (H11N9), and A/duck/Hunan/8-19/2009 (H4N2), were isolated in China. Both H5N8 subtype isolates were highly pathogenic for chickens and showed moderate pathogenicity for mice. Domestic ducks are considered the natural reservoir of avian influenza viruses and serve as reassortant hosts for creation of new virus subtypes. Continued circulation of these viruses may pose health threats for birds and humans.

This study was supported by grants from the National Key Technologies Research and Development Program for the 12th Five-Year Plan of China (2012ZX1000-004-005) and the State Key Laboratory of independent task (No.2010ZZ04).

Dr Haibo Wu is a faculty member at the State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University. His primary research interest is the epidemiology and pathogenic mechanism of avian influenza viruses.

References

1. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. Microbiol Rev. 1992;56:152–79.
2. Kawaoka Y, Chambers TM, Sladen WL, Webster RG. Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology. 1988;163:247–50. http://dx.doi.org/10.1016/0042-6822(88)90260-7
3. Liu M, He S, Walker D, Zhou N, Perez DR, Mo B, et al. The influenza virus gene pool in a poultry market in South central china. Virology. 2003;305:267–75. http://dx.doi.org/10.1006/viro.2002.1762
4. Cardona C, Yee K, Carpenter T. Are live bird markets reservoirs of avian influenza? Poult Sci. 2009;88:856–9. http://dx.doi.org/10.3382/ps.2008-00338
5. Wu HB, Guo CT, Lu RF, Xu LH, Wo EK, You JB, et al. Genetic characterization of subtype H1 avian influenza viruses isolated from live poultry markets in Zhejiang Province, China, in 2011. Virus Genes. 2012;44:441–9. http://dx.doi.org/10.1007/s11262-012-0716-y
6. Hoffmann E, Stech J, Guan Y, Webster RG, Perez DR. Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol. 2001;146:2275–89. http://dx.doi.org/10.1007/s007050170002
7. Hai-bo W, Chao-tan G, Ru-feng L, Li-hua X, En-kang W, Jin-biao Y, et al. Characterization of a highly pathogenic H5N1 avian influenza virus isolated from ducks in Eastern China in 2011. Arch Virol. 2012;157:1131–6. http://dx.doi.org/10.1007/s00705-012-1259-1
8. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121
9. Lee Y-J, Kang H-M, Lee E-K, Song B-M, Jeong J, Kwon Y-K, et al. Novel reassortant influenza A(H5N8) viruses, South Korea, 2014. Emerg Infect Dis. 2014;20:1086–9. http://dx.doi.org/10.3201/eid2006.140233
10. Hu X, Liu D, Wang M, Yang L, Zhu Q, Li L, et al. Clade 2.3.2 avian influenza virus (H5N1), Qinghai Lake region, China, 2009–2010. Emerg Infect Dis. 2011;17:560–2. http://dx.doi.org/10.3201/eid1703.100948
11. Smith GJ, Fan XH, Wang J, Li KS, Qin K, Zhang JX, et al. Emergence and predominance of an H5N1 influenza variant in China. Proc Natl Acad Sci U S A. 2006;103:16936–41. http://dx.doi.org/10.1073/pnas.0608157103
12. Shinya K, Hamm S, Hatta M, Ito H, Ito T, Kawaoka Y. PB2 amino acid at position 627 affects replicative efficiency, but not cell tropism, of Hong Kong H5N1 influenza A viruses in mice. Virology. 2004;320:258–66. http://dx.doi.org/10.1016/j.virol.2003.11.030

13. Zhu Q, Yang H, Chen W, Cao W, Zhong G, Jiao P, et al. A naturally occurring deletion in its NS gene contributes to the attenuation of an H5N1 swine influenza virus in chickens. J Virol. 2008;82:220–8. http://dx.doi.org/10.1128/JVI.00978-07
14. Jiao P, Tian G, Li Y, Deng G, Jiang Y, Liu C, et al. A single-amino-acid substitution in the NS1 protein changes the pathogenicity of H5N1 avian influenza viruses in mice. J Virol. 2008;82:1146–54. http://dx.doi.org/10.1128/JVI.01698-07
15. Zhao K, Gu M, Zhong L, Duan Z, Zhang Y, Zhu Y, et al. Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China. Vet Microbiol. 2013;163:351–7. http://dx.doi.org/10.1016/j.vetmic.2012.12.025

Address for correspondence: Nanping Wu, State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, 79 Qingchun Rd, Hangzhou, Zhejiang 310003, China; email: flwnp2013@163.com

Antibodies against MERS Coronavirus in Dromedary Camels, Kenya, 1992–2013

Victor M. Corman,[1] Joerg Jores,[1] Benjamin Meyer,[1] Mario Younan, Anne Liljander, Mohammed Y. Said, Ilona Gluecks, Erik Lattwein, Berend-Jan Bosch, Jan Felix Drexler, Set Bornstein, Christian Drosten, and Marcel A. Müller

Dromedary camels are a putative source for human infections with Middle East respiratory syndrome coronavirus. We showed that camels sampled in different regions in Kenya during 1992–2013 have antibodies against this virus. High densities of camel populations correlated with increased seropositivity and might be a factor in predicting long-term virus maintenance.

Middle East respiratory syndrome coronavirus (MERS-CoV) was discovered in a patient from Saudi Arabia in 2012 and has since caused ≥250 human infections and 93 deaths (*1*). The evolutionary origins of MERS-CoV and related viral species belonging to the genus *Betacoronavirus* clade C were attributed to insectivorous bats in Europe and Africa (*2–4*). Seroprevalence studies of livestock from diverse species showed that dromedary camels from Oman, Saudi Arabia, the United Arab Emirates, Jordan, Qatar, Spain, and Egypt harbored antibodies against MERS-CoV antigens (*5–8*). Direct evidence for MERS-CoV infection in camels has been found in Qatar, Saudi Arabia, and Egypt. Close similarity of camel-associated and human-associated MERS-CoV sequences suggests that camels are sources of infection for humans and might constitute a zoonotic animal reservoir (*5,9,10*). Where and when the putative introduction of MERS-CoV into camel populations took place and how the virus is maintained in camel populations remains obscure.

Author affiliations: University of Bonn Medical Centre, Bonn, Germany (V.M. Corman, B. Meyer, J.F. Drexler, C. Drosten, M.A. Müller); International Livestock Research Institute, Nairobi, Kenya (J. Jores, A. Liljander, M.Y. Said); Vétérinaires Sans Frontières Germany, Nairobi (M. Younan); Vétérinaires Sans Frontières Suisse, Nairobi (I. Gluecks); EUROIMMUN AG, Lübeck, Germany (E. Lattwein); Utrecht University, Utrecht, the Netherlands (B.-J. Bosch); and National Veterinary Institute, Uppsala, Sweden (S. Bornstein)

DOI: http://dx.doi.org/10.3201/eid2008.140596

Most livestock camels slaughtered in the Arabian Peninsula and in Egypt are imported from the Greater Horn of Africa, in particular Ethiopia, Somalia, Sudan, and Kenya (*11,12*). We investigated MERS-CoV antibody levels and distribution patterns in farmed and nomadic camels from Kenya.

The Study

Samples were obtained from 774 dromedary camels in 3 regions in Kenya (Northeastern, Eastern, and Rift Valley [former administrative provinces]) and 7 counties (Mandera, Wajir, Isiolo, Marsabit, Laikipia, Turkana, and Baringo) during 1992–2013 (Figure). Blood samples were obtained from farmed or nomadic camels by jugular vein puncture. Serum samples originated from the archives of the International Livestock and Research Institute (ILRI) (Nairobi, Kenya). Ethical clearance for collection was part of the agreement between the Government of Kenya and ILRI, which provided ILRI with approval to broadly investigate livestock disease in Kenya.

All serum samples were tested for MERS-CoV antibodies by using a recombinant MERS-CoV spike protein subunit 1–based ELISA (rELISA) as described (*13*). Serum samples were used at a 1:100 dilution, which had been shown to be optimal for screening (*13*). A positive serum sample from recent studies (*6,13*) was used as a reference in all experiments. We used the assay-specific cutoff (optical density ratio 0.3) that had been validated in a previous study of camel serum samples (*13*). A total of 228 (29.5%) of 774 dromedary camels were rated MERS-CoV positive by the rELISA (Table 1). All 228 rELISA-positive serum samples from these 228 camels were subsequently tested at a 1:40 dilution by using an established recombinant immunofluorescence assay and Vero cells expressing MERS-CoV spike protein (*6*). This confirmatory assay showed that 213 (93.4%) of 228 rELISA-positive serum samples had MERS-CoV antibodies (Table 1).

As a final step, antibody specificity was confirmed by using a highly specific MERS-CoV microneutralization assay as described (*6*). All 228 rELISA-positive serum samples were tested at a starting dilution of 1:80 and an ending dilution of 1:800 to identify animals with high neutralization titers. A total of 119 (52.2%) 228 rELISA-positive serum samples had MERS-CoV neutralizing antibody titers (range 1:80–1:800) and 14 (6.1%) of 228 had high (>1:800) titers. The highly reactive camel serum samples originated from 3 counties (Wajir, Mandera, and Marsabit) in 2 regions (Northeastern and Eastern). The highest determined endpoint titer was 1:5,120.

Dromedary camels that had MERS-CoV antibodies were present at all sampling sites and during the 20-year

[1]These authors contributed equally to this article.

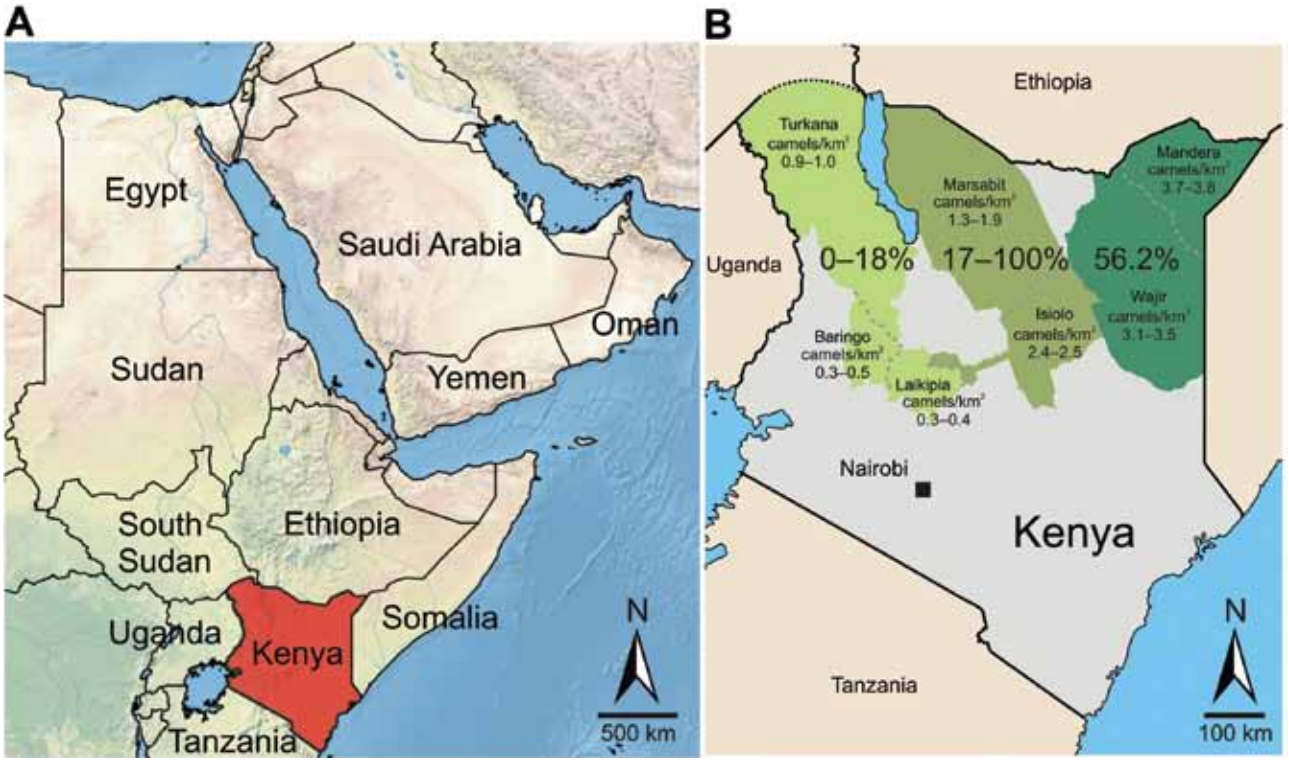


Figure. Greater Horn of Africa and Kenya. A) Arabian Peninsula and neighboring countries in the Greater Horn of Africa. B) Detailed map of Kenya showing sampling sites in 7 counties (Turkana, Baringo, Laikipia, Marsabit, Isiolo, Mandera, and Wajir) for Middle East respiratory syndrome coronavirus (MERS-CoV). Counties were assigned to 3 regions named after the former administrative provinces of Rift Valley, Eastern, and Northeastern (left to right). The 3 sampling regions are indicated in shades of green and other counties are indicated in gray. Percentages of camels positive for antibodies against MERS-CoV are shown with the density of camels (individuals/km^2) for the analyzed regions during 2 periods (1991–2000 and 2000–2013). Serosurveys were performed during 1992–2013. Camel population numbers were determined for 1991–2000 and 2000–2013. Maps were created by using data from http://www.naturalearthdata.com.

sampling period (Table 1; Figure). With the exception of 1 county, seroprevalence was generally higher in the Northeastern and Eastern regions (range 53.4%–100%) than in the northern Rift Valley region (range 0%–17.5%).

Serum samples from 28 dromedary camels from Wajir County that had been held at a research center in isolation conditions since 1998 were negative for MERV-CoV antibodies. To further confirm the observed seropositivity gradient, we compared those 129 camel serum samples with those that were obtained in the same year (2000) but at 2 locations (Eastern and northwestern Rift Valley regions). Antibody levels of nomadic dromedary camels from the Eastern region were significantly higher than those for farmed animals from the Rift Valley (corrected χ^2 34.1, $p<0.005$) (Table 2). Adult animals in both regions had a 7%–10% higher seroprevalence than juvenile animals, which is consistent with results of a previous study (*6*).

Because virus transmission might be influenced by population density, we attempted to correlate seroprevalence with dromedary camel population density across different regions. Data for dromedary camel density (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/14-0596-Techapp1.pdf) were calculated on the basis of livestock counts conducted by the Department of Resource Surveys and Remote Sensing as part of an ongoing Kenya-wide rangeland monitoring program (*14*). Increased seroprevalence showed a significant correlation (Spearman rank correlation coefficient 0.715, $p<0.005$) with higher densities of dromedary camel populations in the Northeastern region and the northern part of the Eastern region (range 0.73–2.9 animals/km^2) than in the Rift Valley region (0.58–0.6 animals/km^2) (Figure; online Technical Appendix).

Conclusions

The present study showed that dromedary camels from Kenya have antibodies against MERS-CoV, which complements the current finding that MERS-CoV is a common pathogen in dromedary camel populations (*5,6,8,9,13*). Our finding of MERS-CoV antibodies in dromedary camels as early as 1992 is consistent with findings of a recent report from Saudi Arabia, which suggested that MERS-CoV has been circulating in dromedary camels for ≥20 years (*5*).

Table 1. Analysis for MERS-CoV in serum samples of dromedary camel from 3 regions in Kenya, 1992–2013*

Region	County†	Husbandry/ management	Year	No. samples	No. rELISA positive samples (%)	No. rIFA positive samples/rELISA positive samples (%)
Northeastern	Mandera/Wajir‡	Nomadic§	2008	162	91 (56.2)	86/91 (94.5)
Eastern	Isiolo	Nomadic§	1998	12	2 (16.7)	1/2 (50.0)
	Marsabit	Nomadic§	1999	41	32 (78.0)	28/32 (87.5)
	Variable	Nomadic§	2000	73	39 (53.4)	38/39 (97.4)
	Marsabit	Nomadic§	2008	21	12 (57.1)	12/12 (100.0)
	Marsabit	Nomadic§	2013	7	7 (100.0)	7/7 (100.0)
Rift Valley	Laikipia¶	Ranch#	1992	22	1 (4.5)	0/1 (0.0)
	Laikipia	Ranch#	1996	37	2 (5.4)	2/2 (100.0)
	Laikipia	Ranch#	1998	50	0 (0.0)	ND
	Laikipia	Ranch#	1999	175	32 (18.3)	30/32 (93.8)
	Turkana	Nomadic**	1999	50	7 (14.0)	6/7 (85.7)
	Laikipia	Ranch#	2000	56	2 (3.6)	2/2 (100.0)
	Baringo	Research center††	2007	28	0 (0.0)	ND
	Laikipia	Ranch	2013	40	1 (2.5)	1/1 (100.0)
Total				774	228 (29.5)	213/228 (93.4)

*MERS-CoV, Middle East respiratory syndrome coronavirus; rELISA, recombinant ELISA for MERS-CoV subunit 1 spike protein (serum samples were tested at a dilution of 1:100); rIFA, recombinant immunofluorescence assay for MERS-CoV spike protein expression in Vero cells (serum samples were tested at a dilution of 1:40; ND, not done.
†Designated county refers to place of sampling or location in which camels were primarily located.
‡Data was merged because both counties had comparable antibody levels.
§High density of camels and regular contact between herds, including exchange of animals between herds in relation to lactation and reproduction status.
¶Formerly from Pakistan.
#Low density of camels and only sporadic contact between herds, with introduction of new animals only by purchase or livestock raiding, or restocking of camels.
**Low density of camels but more frequent contact between herds than on ranches This includes encounters at waterholes and night enclosures, as well as sharing of pastures with daily to weekly contact between herds.
††Isolated herd that originated in Wajir but was kept under quarantine-like isolation conditions for experimental work since 1998.

To project and potentially control virus spread, the public health community must understand factors determining virus maintenance. Our group and others have demonstrated that young dromedary camels have lower seroprevalences and are more likely to carry infectious virus (*5,6*). Similar observations have been made for coronaviruses in their original chiropteran hosts wherein strong virus amplification occurred soon after the time of parturition (*15*). Young, immunologically naive animals may thus facilitate virus amplification in dromedary camel populations.

We also demonstrated that dromedary camel population density shows a positive correlation with MERS-CoV seropositivity, which suggests efficient MERS-CoV maintenance or spread if herd density is high. Different types of animal husbandry in the Northeastern and Eastern regions of Kenya might be better predictors of virus transmission among camels. Dromedary camels in this area are often nomadic following rainfall patterns, and are taken across borders into neighboring countries, such as Ethiopia, for trade purposes (*13*). The observed increase in seropositivity from the Western region to the Northeastern and Eastern regions could be attributed to increased animal-to-animal contact in cross-border dromedary camel metapopulations.

Conversely, dromedary camels that originated in the Northeastern region but had been held in isolation since 1998 were negative for MERS-CoV antibodies, which is consistent with absence of antibodies in dromedary camels bred in isolation in Dubai (*6*). The combination of nomadic husbandry for a large population and presence of young virus-susceptible animals might facilitate virus maintenance. However, our retrospective study with archived samples could not assess hypotheses for each of the individual variables to determine their relative and absolute degrees of influence on virus circulation.

Because exportation of dromedary camels is largely unidirectional from eastern Africa into the Arabian Peninsula

Table 2. Antibodies against MERS-CoV in dromedary camels in 2 regions of Kenya, 2000*

Region	County†	Husbandry	Sex	Age	No. samples positive by rELISA/no. tested (%)
Eastern	Marsabit	Nomadic‡	F/M	A	24/42 (57.1)
			F/M	J	15/31 (48.4)
Subtotal					39/73 (53.4)
Rift Valley	Laikipia	Ranch§	F/M	A	2/28 (7.1)
			F/M	J	0/28 (0)
Subtotal					2/56 (3.6)
Total					41/129 (31.8)

*MERS-CoV, Middle East respiratory syndrome coronavirus; rELISA, recombinant ELISA; A, adult; J, juvenile.
†Designated county refers to place of sampling or location in which camels were primarily located.
‡Frequent herd contacts.
§Sporadic herd contacts.

(*11*), our findings might facilitate the search for more ancestral MERS-CoV variants to clarify the natural history of acquisition of MERS-CoV by dromedary camels and its putative transmission to humans. Our recent finding of a MERS-CoV ancestor in bats from South Africa (*3*) highlights the need for wider investigations of viral reservoirs. The fact that no human MERS cases have been observed in eastern Africa could indicate less transmissibility of viruses in regional lineages or lack of detection and reporting of cases. Serosurveys of persons handling dromedary camels in this region could help to determine whether silent or unrecognized infections are being maintained in humans.

Acknowledgments

We thank Stephan Kallies, Monika Eschbach-Bludau, Sebastian Brünink, Tobias Bleicker, and Andrea Sieberg for providing excellent technical assistance; Chris Field, the late Jasper Evans, Martin Evans, Gilfrid Powys, and all camel owners and herdsmen for providing help during field work in Kenya; the Kenyan Department of Veterinary Services for providing support during field work; and the Director of the Department of Resource Surveys and Remote Sensing for providing data on camel populations in Kenya.

This study was supported by the European Commission (FP7-EMPERIE no. 223498 and FP7-ANTIGONE no. 278976) and the German Research Foundation (DFG grant DR772/3-1 to C.D.) and the Consultative Group for International Agricultural Research Program on Agriculture for Nutrition and Health. A.L. was supported by the Centrum of International Migration. C.D. received infrastructural support from the German Center for Infection Research.

Dr Corman is a physician and virologist at the Institute of Virology in Bonn, Germany. His research interests include characterization of novel human and zoonotic viruses and development of molecular diagnostic assays.

References

1. World Health Organization. Middle East respiratory syndrome coronavirus—update. April 20, 2014 [cited 2014 Apr 26]. http://www.who.int/csr/don/2014_04_20_mers/en/
2. Reusken CB, Lina PH, Pielaat A, de Vries A, Dam-Deisz C, Adema J, et al. Circulation of group 2 coronaviruses in a bat species common to urban areas in western Europe. Vector Borne Zoonotic Dis. 2010;10:785–91. http://dx.doi.org/10.1089/vbz.2009.0173
3. Ithete NL, Stoffberg S, Corman VM, Cottontail VM, Richards LR, Schoeman MC, et al. Close relative of human middle East respiratory syndrome coronavirus in bat, South Africa. Emerg Infect Dis. 2013;19:1697–9. http://dx.doi.org/10.3201/eid1910.130946
4. Annan A, Baldwin HJ, Corman VM, Klose SM, Owusu M, Nkrumah EE, et al. Human betacoronavirus 2c EMC/2012-related viruses in bats, Ghana and Europe. Emerg Infect Dis. 2013;19:456–9. http://dx.doi.org/10.3201/eid1903.121503
5. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, de Wit E, et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. MBio. 2014;5:e00884–14. http://dx.doi.org/10.1128/mBio.01002-14
6. Meyer B, Muller MA, Corman VM, Reusken CB, Ritz D, Godecke GJ, et al. Antibodies against MERS coronavirus in dromedaries, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014;20:552–9. http://dx.doi.org/10.3201/eid2004.131746
7. Perera RA, Wang P, Gomaa M, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 2013;18:pii:20574 .
8. Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis. 2013;13:859–66. http://dx.doi.org/10.1016/S1473-3099(13)70164-6
9. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infect Dis. 2014;14:140–5. http://dx.doi.org/10.1016/S1473-3099(13)70690-X
10. Chu DK, Poon LM, Gomaa MM, Shehata MM, Perera RA, Abu Zeid D, et al. MERS coronaviruses in dromedary camels, Egypt. Emerg Infect Dis. 2014;20:1050–4.
11. Aklilu Y, Catley A. Livestock exports from the Horn of Africa: an analysis of benefits by pastoralist wealth group and policy implications. Medford (MA): Feinstein International Center, Tufts University; 2009.
12. Mahmoud HA. Camel marketing in the northern Kenya/southern Ethiopia borderlands. FAC Research Update 005 DFID, 2010 [cited 2014 Apr 11]. http://www.future-agricultures.org
13. Memish ZA, Cotten M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, et al. Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. Emerg Infect Dis. 2014;20:1016–9.
14. Ottichilo W, Grunblatt J, Said M, Wargute P. Wildlife and livestock population trends in the Kenya rangeland. In: Prins HT, Grootenhuis J, Dolan T, editors. Wildlife conservation by sustainable use. Amsterdam: Springer; 2000. p. 203–18.
15. Drexler JF, Corman VM, Wegner T, Tateno AF, Zerbinati RM, Gloza-Rausch F, et al. Amplification of emerging viruses in a bat colony. Emerg Infect Dis. 2011;17:449–56. http://dx.doi.org/10.3201/eid1703.100526

Address for correspondence: Christian Drosten, Institute of Virology, University of Bonn Medical Centre, Sigmund Freud Strasse 25, 53105 Bonn, Germany; email: drosten@virology-bonn.de

Shelter Dogs as Sentinels for *Trypanosoma cruzi* Transmission across Texas, USA

Trevor D. Tenney, Rachel Curtis-Robles, Karen F. Snowden, and Sarah A. Hamer

Chagas disease, an infection with the parasite *Trypanosoma cruzi*, is increasingly diagnosed among humans in the southern United States. We assessed exposure of shelter dogs in Texas to *T. cruzi*; seroprevalence across diverse ecoregions was 8.8%. Canine serosurveillance is a useful tool for public health risk assessment.

The protozoan parasite *Trypanosoma cruzi* is the causative agent of Chagas disease, a neglected tropical disease affecting >8 million persons across Mexico and Central and South America. In the United States, estimates of human infection range from 300,000 to >1 million (*1,2*). Although immigrants exposed in Chagas disease-endemic regions constitute the majority of infected persons in the United States, autochthonous transmission is increasingly recognized (*3*), and enzootic cycles involving infected wildlife reservoirs and domestic dogs occur across the southern United States. (*4*). Vectorborne transmission occurs through contamination of the bite site or mucous membranes with feces of infected hematophagous triatomines ("kissing bugs"). In addition, the parasite can be passed through consumption of infected bugs or contaminated food products, through blood transfusions, and congenitally (*4*).

Clinical manifestation in humans and dogs ranges from asymptomatic to acute myocarditis and sudden death to chronic progressive cardiac disease (*5,6*). No vaccine is available for humans or dogs. Drugs used to treat Chagas disease in humans have not been approved by the US Food and Drug Administration and are available in the United States only through investigational protocols. The disease is notifiable in 4 states including Texas, where as of 2013, human and veterinary cases must be reported.

Texas is a high-risk state for transmission of *T. cruzi* to dogs, considering the diversity of triatomine vectors, reservoir hosts, and previous documentation of canine disease (*5,7*). Because dogs arriving at shelters may have high exposure to vectors, we expect that shelter dogs will provide a sensitive spatial index of Chagas disease risk across the landscape. The objective of this study was to measure *T. cruzi* seroprevalence in shelter dog populations across Texas.

Author affiliations: Texas A&M University, College Station, Texas, USA

DOI: http://dx.doi.org/10.3201/eid2008.131843

The Study

To assess exposure to *T. cruzi*, we established a network of 7 canine shelters in major cities and rural areas representing diverse ecoregions across Texas (*8*; Figure). Using a cross-sectional study design, we collected blood samples during May–August 2013 from ≤30 dogs at each shelter, in adherence with client-owned animal use protocols approved by Texas A&M University Institutional Animal Care and Use Committee. Enrollment criteria for our study included dogs >6 months of age within 4 days of admittance to shelters. We detected exposure to *T. cruzi* using Chagas STAT-PAK, a commercially available rapid immunochromatographic test (Chembio Diagnostic Systems, Inc., Medford, NY, USA) that has been validated for use in dogs (*9*) and has high sensitivity and specificity when compared with conventional serologic techniques (*10*). To detect parasite DNA within blood, we followed this process: a 200-mL aliquot of centrifuged blood, including the buffy coat, from a subset of seropositive and seronegative dogs across all shelters was subjected to DNA extraction and used as the template in a real-time quantitative PCR with a TaqMan probe to amplify a 166-bp repetitive sequence of satellite DNA specific to *T. cruzi* (*11*). Using a sequential testing approach, we subjected positive samples in the real-time quantitative PCR to a second confirmatory PCR using previously published TCZ primers to amplify a 188-bp sequence of satellite DNA (*12*). Samples positive by both assays were considered to contain *T. cruzi* DNA within the blood.

A total of 205 blood samples were collected from shelter dogs. Dogs enrolled in the study ranged from 6 months to 13 years of age and represented diverse breed groups. A total of 18 (8.8%) dogs were seropositive for *T. cruzi* antibodies. Seropositive dogs were found at all shelters within the network; shelter prevalence ranged from 6.7% to 13.8% (Table). Using logistic regression, we found no differences in the odds of seropositivity across location, sex, age category, breed group, and dog origin (Table). In a subset of 50 dogs, including 14 seropositive and 36 seronegative dogs, 3 (6%) blood samples were positive for *T. cruzi* DNA by both PCR methods, each with similar cycle threshold values indicative of ≈50 parasite equivalents of DNA per mL of blood. PCR-positive dogs included 2 male and 1 female dog of sporting and toy breeds from shelters A, C, and F, ranging in age from 1.5 to 7 years. Of these 3 dogs, 1 was serologically positive for antibodies against *T. cruzi*.

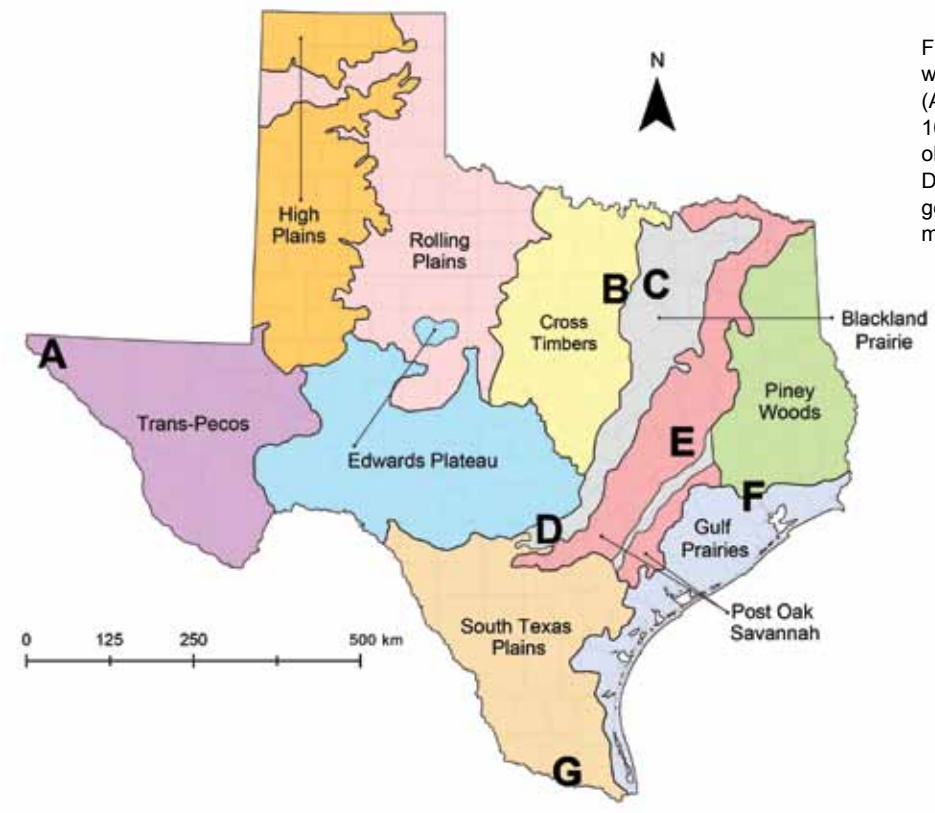


Figure. Locations of canine shelters within Texas, USA, 2013. Shelters (A–G) are distributed across 7 of the 10 Gould Ecoregions of Texas (*9*), Map obtained from Texas Parks and Wildlife Department (http://www.tpwd.texas.gov/publications/pwdpubs/media/pwd_mp_e0100_1070ad_08.pdf).

Additionally, we observed very faint-colored bands on serologic dipsticks in samples from 26 dogs that are not included in the overall seroprevalence estimate. Because we were uncertain of how to interpret antibody presence in these samples, we submitted a subset (n = 11) for indirect fluorescent antibody testing at the Texas Veterinary Medical Diagnostic Laboratory, of which 4 samples (36.3%) tested positive. Although cross-reactivity cannot be ruled out by

Table. Logistic regression model for risk for *Trypanosoma cruzi* seropositivity from 205 dogs across Texas, USA, 2013

Risk factor	No.*	No. seropositive (%)	Odds ratio	95% CI	p value
Location (Figure label)					
El Paso (A)	29	2 (6.9)	0.69	0.11–4.47	0.69
Fort Worth (B)	30	2 (6.7)	0.67	0.10–4.30	0.67
Dallas (C)	30	3 (10.0)	1.04	0.19–5.59	0.97
San Antonio (D)	29	4 (13.8)	1.49	0.30–7.33	0.62
College Station (E)	31	3 (9.7)	Referent	Referent	Referent
Houston (F)	26	2 (7.7)	0.78	0.12–5.05	0.79
Edinburg (G)	30	2 (6.7)	0.67	0.10–4.30	0.67
Sex					
F	105	9 (8.6)	Referent	Referent	Referent
M	96	9 (9.4)	1.10	0.41–2.95	0.84
Age†					
<2 y	115	8 (7.0)	Referent	Referent	Referent
>2 y	84	10 (11.9)	1.81	0.68–4.94	0.23
Breed group‡					
Herding and working	53	5 (9.4)	Referent	Referent	Referent
Hound, nonsporting, and toy	43	4 (9.3)	0.98	0.23–3.96	0.98
Sporting	44	6 (13.6)	1.52	0.43–5.62	0.52
Terrier	56	1 (1.8)	0.17	0.01–1.13	0.12
Origin					
Owner surrender	40	3 (7.5)	Referent	Referent	Referent
Stray	137	10 (7.3)	1.08	0.32–4.94	0.91
Transfer from another shelter	21	4 (19.0)	2.9	0.58–16.13	0.19

*Sample size for some variables does not equal 205 because complete information was not available for all dogs.
†Age category was estimated on the basis of patterns of dentition and tooth wear.
‡Most dogs were mixed breed on the basis of appearance; American Kennel Club breed group assignment is based on dominant breed features.

using the indirect fluorescent antibody technique, these datas uggest that the seroprevalence we report (8.8%) is a conservative estimate.

Conclusions

Shelter dogs had widespread exposure to *T. cruzi* across 7 ecologic regions in Texas, with a conservative statewide average of 8.8% seroprevalence. The presence of seropositive dogs across all sampled regions, age classes, breed groups, and canine origins suggests that ecologic requirements for parasite transmission to dogs are not constrained to focal areas or particular breed groups. Although the travel histories of dogs in our study are unknown, the presence of antibodies in dogs across all age classes, including young dogs that are less likely to have traveled, suggests local exposure. Furthermore, at least some shelters are located in regions in which kissing bugs have previously been reported (*7*). The only published prospective seroprevalence study of dogs from 1 county in south Texas reported a seroprevalence of 7.5% (n = 375 stray dogs) (*13*), similar to our statewide average. Across 2 regions in Mexico, including 1 where Chagas disease was previously considered nonendemic, seroprevalence of *T. cruzi* in canines ranged from 17.5% to 21% and was directly correlated with *T. cruzi* seroprevalence in humans in these regions (*14*). Further research is needed to quantify the association between infection of canines with *T. cruzi* and risk for Chagas disease among humans in the United States.

The widely accepted concept of Chagas disease is that *T. cruzi* infection is lifelong, which is supported by continuous detection of antibody presence within hosts. Despite detection of antibodies in dogs sampled across the state, parasite DNA was detected in the blood of only 3 dogs. Limited experimental investigations of *T. cruzi* infection of dogs indicate that parasitemia is detectable cytologically as soon as 3 days after inoculation and lasts ≤3 weeks (*5*), after which the parasite localizes in tissues and parasitemia is undetectable. Our observations suggest that most seropositive dogs were not in the acute phase of the infection at the time of sampling. Although domestic dogs have been shown to serve as reservoir hosts in some regions of Central and South America (*15*), the importance of dogs relative to wildlife hosts as reservoirs in the United States is unknown.

Dogs that arrive at shelters, especially stray dogs, are likely to have had increased exposure to the outdoors and vectors and have been shown to have higher exposure to vectorborne pathogens than client-owned dogs that are brought to veterinary clinics (*16*). Shelter dogs, therefore, provide a sensitive population for assessment of local canine transmission risk, and we suggest that awareness of kissing bugs and Chagas disease risk among citizens and the medical community should be heightened in areas where seropositive dogs are detected.

Acknowledgments

We thank the staff at the canine shelters for supporting our research; and Lisa Auckland, Miranda Bertram, Frida Cano, Carolyn Hodo, Jessica Rodriguez, and Dalton Webb of Texas A&M University for providing technical assistance.

The Bernice Barbour Foundation funded this research. A veterinary student stipend was provided to T.D.T. by the Office of Research Infrastructure Programs of the National Institutes of Health (5T35OD010991-09) and the College of Veterinary Medicine & Biomedical Sciences, Texas A&M University.

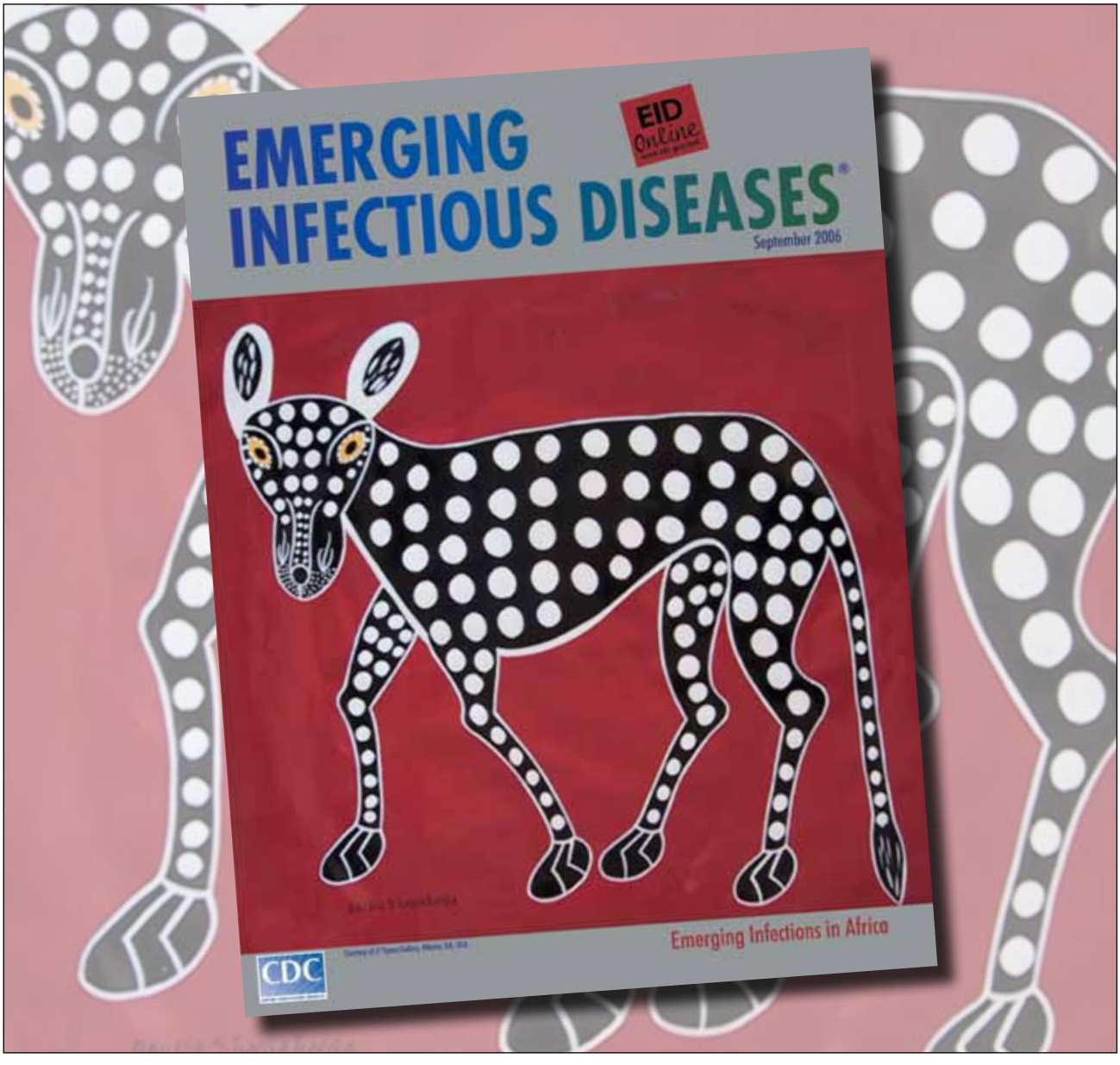
Mr Tenney is a second-year veterinary student at Texas A&M University in College Station, Texas. His research interests include military veterinary medicine and infectious-diseases.

References

1. Bern C, Montgomery SP. An estimate of the burden of Chagas disease in the United States. Clin Infect Dis. 2009;49:e52–4. http://dx.doi.org/10.1086/605091
2. Hanford EJ, Zhan FB, Lu YM, Giordano A. Chagas disease in Texas: recognizing the significance and implications of evidence in the literature. Soc Sci Med. 2007;65:60–79. http://dx.doi.org/10.1016/j.socscimed.2007.02.041
3. Cantey PT, Stramer SL, Townsend RL, Kamel H, Ofafa K, Todd CW, et al. The United States *Trypanosoma cruzi* infection study: evidence for vector-borne transmission of the parasite that causes Chagas disease among United States blood donors. Transfusion. 2012;52:1922–30. http://dx.doi.org/10.1111/j.1537-2995.2012.03581.x
4. Bern C, Kjos S, Yabsley MJ, Montgomery SP. *Trypanosoma cruzi* and Chagas' disease in the United States. Clin Microbiol Rev. 2011;24:655–81. http://dx.doi.org/10.1128/CMR.00005-11
5. Barr SC. Canine Chagas' disease (American Trypanosomiasis) in North America. Vet Clin N Am-Small. 2009 Nov;39(6):1055–64. PubMed http://dx.doi.org/10.1016/j.cvsm.2009.06.004
6. Rassi A, Rassi A, Marin-Neto JA. Chagas disease. Lancet. 2010;375:1388–402. http://dx.doi.org/10.1016/S0140-6736(10)60061-X
7. Kjos SA, Snowden KF, Olson JK. Biogeography and *Trypanosoma cruzi* infection prevalence of Chagas disease vectors in Texas, USA. Vector Borne Zoonotic Dis. 2009;9:41–50. http://dx.doi.org/10.1089/vbz.2008.0026
8. Gould FW, Hoffman, GO, Rechenthin CA. Vegetational areas of Texas; 1960. Texas Agricultural Experiment Station, Texas A&M University (Texas). Leaflet no. 492. http://aggieclover.tamu.edu/map/
9. Nieto PD, Boughton R, Dorn PL, Steurer F, Raychaudhuri S, Esfandiari J, et al. Comparison of two immunochromatographic assays and the indirect immunofluorescence antibody test for diagnosis of *Trypanosoma cruzi* infection in dogs in south central Louisiana. Vet Parasitol. 2009;165:241–7. http://dx.doi.org/10.1016/j.vetpar.2009.07.010
10. Luquetti AO, Ponce C, Ponce E, Esfandiari J, Schijman A, Revollo S, et al. Chagas' disease diagnosis: a multicentric evaluation of Chagas Stat-Pak, a rapid immunochromatographic assay with recombinant proteins of *Trypanosoma cruzi*. Diagn Microbiol Infect Dis. 2003;46:265–71. http://dx.doi.org/10.1016/S0732-8893(03)00051-8

11. Duffy T, Cura CI, Ramirez JC, Abate T, Cayo NM, Parrado R, et al. Analytical performance of a multiplex real-time PCR assay using TaqMan probes for quantification of *Trypanosoma cruzi* satellite DNA in blood samples. PLoS Negl Trop Dis. 2013;7. http://dx.doi.org/10.1371/journal.pntd.0002000
12. Virreira M, Torrico F, Truyens C, Alonso-Vega C, Solano M, Carlier Y, et al. Comparison of polymerase chain reaction methods for reliable and easy detection of congenital *Trypanosoma cruzi* infection. Am J Trop Med Hyg. 2003;68:574–82.
13. Beard CB, Pye G, Steurer FJ, Rodriguez R, Campman R, Peterson AT, et al. Chagas disease in a domestic transmission cycle, southern Texas, USA. Emerg Infect Dis. 2003;9:103–5. http://dx.doi.org/10.3201/eid0901.020217
14. Estrada-Franco JG, Bhatia V, Diaz-Albiter H, Ochoa-Garcia L, Barbabosa A, Vazquez-Chagoyan JC, et al. Human *Trypanosoma cruzi* infection and seropositivity in dogs, Mexico. Emerg Infect Dis. 2006;12:624–30. http://dx.doi.org/10.3201/eid1204. 050450
15. Ramírez JD, Turriago B, Tapia-Calle G, Guhl F. Understanding the role of dogs (*Canis lupus familiaris*) in the transmission dynamics of *Trypanosoma cruzi* genotypes in Colombia. Vet Parasitol. 2013;196:216–9. http://dx.doi.org/10.1016/j.vetpar.2012. 12.054
16. Amusategui I, Tesouro MA, Kakoma I, Sainz A. Serological reactivity to *Ehrlichia canis, Anaplasma phagocytophilum, Neorickettsia risticii, Borrelia burgdorferi* and *Rickettsia conorii* in dogs from northwestern Spain. Vector Borne Zoonotic Dis. 2008;8:797–804. http://dx.doi.org/10.1089/vbz.2007. 0277

Address for correspondence: Sarah A. Hamer, Department of Veterinary Integrative Biosciences, 4458 TAMU, Texas A&M University, College Station, TX 77843, USA; email: shamer@cvm.tamu.edu



Natural Intrauterine Infection with Schmallenberg Virus in Malformed Newborn Calves

Calixte Bayrou,[1] Mutien-Marie Garigliany,[1] Michael Sarlet, Arnaud Sartelet, Dominique Cassart, and Daniel Desmecht

We surveyed morphologic alterations in calves in Belgium that were naturally infected in utero by Schmallenberg virus (SBV) and born with deformities during January–March 2012. SBV-specific RNA was distributed unevenly in different tissues. Natural intrauterine SBV infection of calves might cause serious damage to the central nervous system and muscles.

During summer and fall 2011, a nonspecific febrile syndrome characterized by hyperthermia and decreased milk production was reported in adult dairy cows from farms in the Netherlands and Germany (*1*,*2*). In November 2011, an enzootic outbreak of abortions, stillbirths, and term births of lambs, kids, and calves that exhibited neurologic signs and/or musculoskeletal malformations emerged throughout northwestern Europe (*3*,*4*). Both syndromes were associated with the presence in the blood (adults) or in the central nervous system (CNS) (newborns) of the genome of a new orthobunyavirus, which was named Schmallenberg virus (SBV) after the place where the first positive samples were collected (*3*,*4*). SBV belongs to the Simbu serogroup (*5*) and, like its phylogenetic relatives Akabane and Aino viruses, can cross the placenta (*6*). Because this new viral disease of ruminants emerged 3 years ago, information is limited. We comprehensively surveyed morphologic alterations in calves naturally infected in utero. In addition, we report the distribution of SBV-specific RNA in the different tissues of these calves, which has implications for diagnosis.

The Study

In Belgium each year during January–June, field veterinarians refer ≈30 newborn calves per month for necropsy to the University of Liège Faculty of Veterinary Medicine (Liège, Belgium). During January–March 2012, the consequences of SBV infection on bovine fetuses were not yet known, which prompted the staff to look systematically for the new virus in all deformed calves and in calves that died spontaneously without obvious cause. Among the 67 animals in these categories, SBV genetic material was detected in 15 calves by reverse transcription quantitative PCR, and IgG specific for SBV nucleoprotein was systematically highlighted in their serum by ELISA. In addition, all attempts to retrieve the genetic material of bluetongue virus 8 and bovine viral diarrhea virus from the tissues of these 15 seropositive calves failed. None of these calves carried the mutation responsible for noninfectious arthrogryposis in local livestock. These 15 calves, in which both SBV RNA and antibodies against SBV were detected, are the subject of this study.

Detailed information about the methods used to examine the calves is available in online Technical Appendix 1 (http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp1.pdf). A detailed description of the lesions found in SBV-infected calves is provided in online Technical Appendix 2 (http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp2.pdf).

SBV-positive animals weighed significantly less than expected (32 kg $\pm$ 15 kg vs. 49 kg $\pm$ 4 kg, $p<0.05$). The body mass deficit, severity of deformities in whole-body conformation, and amount of skeletal muscle were obviously correlated (Table 1; online Technical Appendix 3 Figure 1, http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp3.pdf).

We observed overall permanent deviations of the axial skeleton in all 3 planes (online Technical Appendix 3 Figure 2), the most common being a lateral deviation of the cervical spine (torticollis). In the most distorted animals, the torticollis was accompanied by a dorso-ventral deviation of the thoracolumbar spine. Most SBV-infected calves displayed joint fixation of 1 or all joints of $\geq$1 limbs (arthrogryposis). Tendons spanning fixed joints were shorter than expected, and corresponding muscles displayed decreased mass and altered color. Often the animal's head was distorted, having a horse-like or pig-like shape, brachygnathism, prognathism, and/or diverging sagittal axes (online Technical Appendix 3 Figure 3).

We systematically observed major alterations after we opened the skull and spinal canal (Figure 1; online Technical Appendix 3 Figure 4). These changes involved the spinal cord and the telencephalon, whereas the brainstem and cerebellum were kept intact (although 1 cerebellum was hypoplastic). We consistently observed a decrease in the cross-sectional area of the spinal cord (Figure 2), which correlated positively with the magnitude of axial/appendicular musculoskeletal deformities (online Technical Appendix

Author affiliation: University of Liège, Liège, Belgium

DOI: http://dx.doi.org/10.3201/eid2008.121890

[1]These authors contributed equally to this article.

Table 1. Macroscopic characteristics of 15 SBV-infected newborn calves at necropsy, Belgium, January–March 2012*

Characteristic	WBD-0†	WBD-1†	WBD-2†	WBD-3†	Total no. calves
No. calves	2	4	4	5	15
Method of death					
Euthanasia	2	3	0	0	5
Spontaneous	0	1	4	5	10
Bodyweight, kg‡§	49 ± 4	39 ± 3	34 ± 3	21 ± 2	
Axial musculoskeletal system					
Defect location					
Cervical	0	2	4	5	11
Thoracic	0	0	2	5	7
Lumbar	0	0	0	5	5
Type of deviation					
Lateral	0	2	4	5	11
Dorso-ventral	0	0	1	4	5
Helicoidal	0	0	1	4	5
Appendicular musculo-skeletal system					
Arthrogryposis (≥1 limb involved)	0	3	4	5	12
Symetric limb involvement	NA	3	3	5	11
Forelimb/hind limb involvement	NA	0	1	5	6
Forelimbs only	NA	2	3	0	5
Hind limbs only	NA	1	0	0	1
Head					
Coaptation defect					
Prognathism	0	0	0	1	1
Brachygnathism	0	1	1	2	4
Altered profile					
Horse-like	0	1	1	0	2
Pig-like	0	0	0	2	2
Broken sagittal axis	0	1	2	2	5
Central nervous system					
Porencephaly	2	3	3	1	9
Hydranencephaly	1	1	0	1	3
Hydrocephaly	0	0	1	4	5
Cerebellar hypoplasia	0	0	0	1	1
Micromyelia	2	4	4	5	15

*Fifteen newborn calves infected in utero by SBV were categorized according to the extent of their deformities. The table lists the gross morphologic changes observed at necropsy. SBV, Schmallenberg virus; WBD, whole-body deformity score; NA, not applicable.
†Animals with neurologic signs and apparently normal body shape were given a WBD of 0; those with altered body shape were scored 1, 2, or 3 depending on whether 1, 2, or 3 skeletal segments were deformed, respectively (spine, forelimbs, or hind limbs). All values are number of calves unless otherwise indicated.
‡Mean ± SD.
§Bodyweight of age-matched Belgian Blue healthy calves is 49.2 kg ± 7.1 kg (7).

3 Figure 5). The neopallial part of the telencephalon was always decreased, giving 3 distinct morphotypes. In some calves, we detected multiple, bilateral, and randomly located cystic cavities, most of which communicated with the ipsilateral ventricle (porencephaly). In other cases, all that remained from the neopallium was a thin, nearly transparent membrane, sometimes with a few floating smooth-surfaced islets resembling cortex (hydranencephaly). Finally, in a third subset of calves, the brain appeared normal, but we observed a severe, bilateral, and symmetric dilatation of lateral ventricles after section (hydrocephaly).

Microscopic examination of the spinal cord revealed a significant decrease in neuron numbers, the magnitude of which correlated positively with the severity of whole-body deformities (Table 2). Muscle sections displayed a diffuse pattern of increased fiber size variation with connective tissue and adipocyte infiltrations (online Technical Appendix 4 Table 1, http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp4.pdf).

The viral RNA was always present in the CNS and sometimes in the lungs and colon (online Technical Appendix 4 Table 2). When the entire cohort was considered, SBV was detected in all parts of the CNS. When we examined the animals individually, however, the detection rate varied depending on the segment (online Technical Appendix 4 Table 2). The virus was almost always detected in the spinal cord (93%) and the neopallium (87%); often in the midbrain (83%) and pons (67%); and about half the time in the diencephalon, cerebellum, and paleopallium. The practical implications of these findings for routine diagnosis are highlighted separately (online Technical Appendix 5, http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp5.pdf).

Conclusions

Our findings show that natural in utero infection of the bovine fetus by SBV may result in serious damage to the CNS and muscles. Mechanistic hypotheses that could

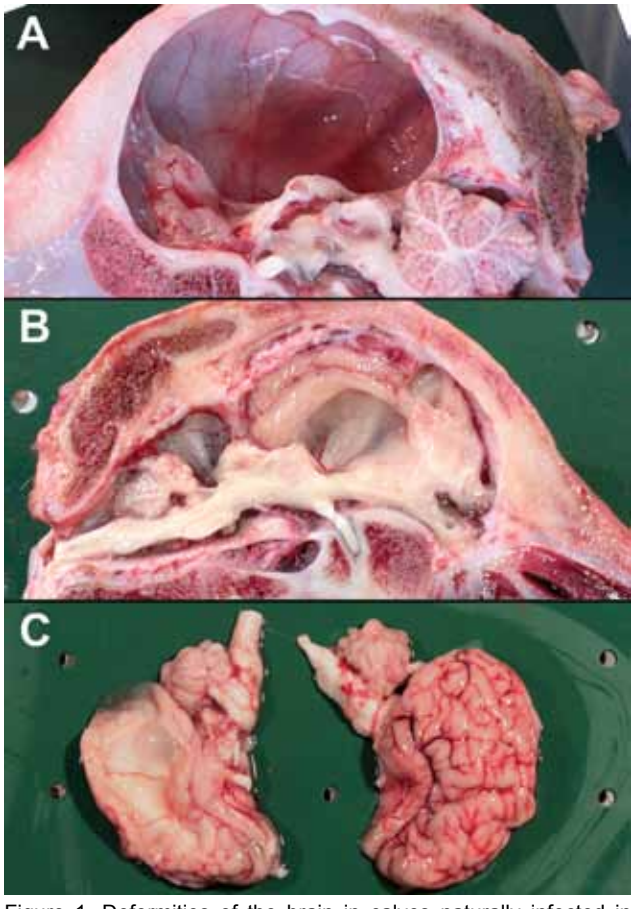


Figure 1. Deformities of the brain in calves naturally infected in utero with Schmallenberg virus, Belgium, January–March 2012. A) Hydranencephaly. B) Hydrocephaly and cerebellar hypoplasia. C) Porencephaly.

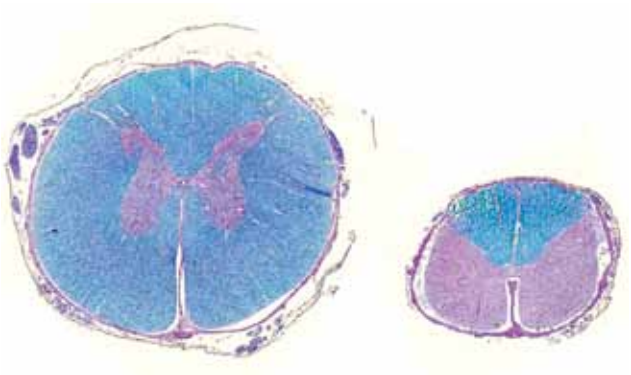


Figure 2. Micromyelia. Age- and site-matched spinal cord transversal histologic section at the level of C4. Left, control calf; right, Schmallenberg virus (SBV)–infected calf. Note atrophy/hypoplasia and prominent deficiency of stainable myelin in ventral and lateral tracts of SBV-infected calf. Luxol fast blue staining.

explain these alterations are discussed in online Technical Appendix 6 (http://wwwnc.cdc.gov/EID/article/20/8/12-1890-Techapp6.pdf). Similar to the situation with Akabane virus infection (*8*), the clinical picture shown by in utero SBV-infected newborn calves is likely to depend largely on the age of the fetus at the time of infection. The infection must be quickly contained if the fetus is infected when immunocompetent (≥120–150 days after conception), and we deduce that damages inflicted by the virus consequently have no or little effect on its further development. Conversely, the infection probably spreads more easily and lasts much longer if the virus contaminates an immunologically immature fetus. Because transplacental infection is possible only when the first placentome is present (30 days after conception in cattle), the window during which the infection of a bovine fetus might lead to a porencephaly/hydranencephaly-micromyelia-arthrogryposis syndrome ranges from 30 to 150 days after conception. The degree of overall body deformity correlated with a progressively greater reduction in the size of the spinal cord (as determined by spinal cord:foramen magnum ratio) and with fewer spinal neurons, suggesting that the lack of movement leading to arthrogryposis results directly from the spinal cord lesions, leading to denervation atrophy of skeletal muscle. This primary role for the spinal cord lesion is further supported by the tendency of forelimbs and hind limbs to be affected bilaterally because muscle involvement might be expected to lead to more randomly distributed lesions.

When SBV virus infects the bovine fetus during the risk window mentioned above and causes neuromuscular defects, its genetic material remains detectable at term—thus 4 months later—at a minimum. The physical form of this persisting virus and the way it persists in the face of the seroconversion are unknown. The hypothesis of the existence of sites of persistence must be addressed, for example, in the CNS (≈90% of cases were virus positive at term) or lungs (≈30%). In practice, a priority is to establish whether SBV persists in calves infected in utero but born asymptomatic.

Acknowledgments

We thank Martin Beer and Bernd Hoffmann for kindly providing help for the setup and validation of the reverse transcription quantitative PCR procedure and for confirming a set of results. We also are grateful to Deborah Kleijnen and Pauline Meirlaen for their skilled technical support through the study.

The study was supported by a grant from the Research Council in Life Sciences of University of Liège.

References

1. Hoffmann B, Scheuch M, Höper D, Jungblut R, Holsteg M, Schirrmeier H, et al. Novel orthobunyavirus in cattle, Europe, 2011. Emerg Infect Dis. 2012;18:469–72. http://dx.doi.org/10.3201/eid1803.111905

Table 2. Correlation between spinal neuron counts and axial muscle histologic changes in 15 SBV-infected newborn calves, Belgium, January–March 2012*

Structure examined	Control calves	WBD/calf ID: WBD-0 A	WBD-0 B	WBD-1 C	WBD-1 D	WBD-1 E	WBD-1 F	WBD-2 G	WBD-2 H	WBD-2 I	WBD-2 J	WBD-3 K	WBD-3 L	WBD-3 M	WBD-3 N	WBD-3 O
Axial muscles, histology																
Musculus semispinalis capitis, cas	0	NT	0	0	0	3	0	3	3	NT	3	3	3	3	3	3
Musculus semispinalis capitis, ces	0	NT	1	0	0	2	0	3	3	NT	3	3	3	3	3	3
Spinal cord																
Dorsal horn neurons, no.																
Left dorsal horn	12 ± 5	NT	11	21	4	5	10	5	1	NT	4	0	0	0	7	3
Right dorsal horn	11 ± 4	NT	23	17	6	5	10	7	3	NT	5	1	0	0	5	0
Ventral horn neurons, no.																
Left ventral horn	50 ± 10	NT	56	57	15	1	45	9	1	NT	0	0	0	0	7	0
Right ventral horn	50 ± 4	NT	47	55	14	3	31	7	0	NT	0	0	0	0	4	0

*Extent of histologic changes was reported semiquantitatively by using a score of 0, 1, 2, or 3 depending on whether the histologically normal tissue extended over 100%, 75%–100%, 25%–75%, or <25% of the area examined and intensity of shading reflects these values. Neurons were enumerated in transverse sections of the spinal cord corresponding to C4. Intensity of shading reflects the magnitude of neuron deficits. SBV, Schmallenberg virus; WBD, whole-body deformity score; NT, not tested; cas, concave side; ces, convex side.

2. Muskens J, Smolenaars AJ, van der Poel WH, Mars MH, van Wuijckhuise L, Holzhauer M, et al. Diarrhea and loss of production on Dutch dairy farms caused by the Schmallenberg virus [in Dutch]. Tijdschr Diergeneeskd. 2012;137:112–5.
3. Garigliany M-M, Hoffmann B, Dive M, Sartelet A, Bayrou C, Cassart C, et al. Schmallenberg virus in calf born at term with porencephaly, Belgium. Emerg Infect Dis. 2012;18:1005–6.
4. van den Brom R, Luttikholt SJ, Lievaart-Peterson K, Peperkamp NH, Mars MH, van der Poel WH, et al. Epizootic of ovine congenital malformations associated with Schmallenberg virus infection. Tijdschr Diergeneeskd. 2012;137:106–11.
5. Goller KV, Höper D, Schirrmeier H, Mettenleiter TC, Beer M. Schmallenberg virus as possible ancestor of Shamonda virus. Emerg Infect Dis. 2012;18:1644–6. http://dx.doi.org/10.3201/eid1810.120835
6. Garigliany MM, Bayrou C, Kleijnen D, Cassart D, Desmecht D. Schmallenberg virus in domestic cattle, Belgium, 2012. Emerg Infect Dis. 2012;18:1512–4. http://dx.doi.org/10.3201/eid1809.120716
7. Kolkman I, Opsomer G, Aerts S, Hoflack G, Laevens H, Lips D. Analysis of body measurements of newborn purebred Belgian Blue calves. Animal. 2010;4:661–71. http://dx.doi.org/10.1017/S1751731109991558
8. Charles JA. Akabane virus. Vet Clin North Am Food Anim Pract. 1994;10:525–46.

Address for correspondence: Daniel Desmecht, Department of Pathology, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium; email: daniel.desmecht@ulg.ac.be

Role of Migratory Birds in Spreading Crimean-Congo Hemorrhagic Fever, Turkey

Hakan Leblebicioglu, Cafer Eroglu, Kiraz Erciyas-Yavuz, Murat Hokelek, Mustafa Acici, and Hava Yilmaz

We investigated migratory birds' role in spreading Crimean-Congo hemorrhagic fever virus (CCHFV) through attached ticks. We detected CCHFV RNA in ticks on migratory birds in Turkey. Two isolates showed similarity with CCHFV genotype 4, suggesting a role for ticks in CCHFV epidemics in Turkey and spread of CCHFV by birds.

Crimean-Congo hemorrhagic fever (CCHF), an illness characterized by fever and hemorrhage, is caused by CCHF virus (CCHFV) (family *Bunyaviridae*, genus *Nairovirus*). CCHFV has been isolated from many species of ticks, primarily *Hyalomma* spp (*1*). In Turkey, CCHFV has been detected mostly in *Hyalomma* spp. ticks (*2*). Although CCHF is common in Turkey, Iran, Pakistan, and Afghanistan, sporadic cases are reported from the neighboring countries and the Balkans (*1*). No case was reported before 2002 from Turkey, but the annual number of cases increased exponentially until 2009. A total of 7,192 CCHF cases were reported during 2002–2012 to the Ministry of Health (Turkish Ministry of Health, unpub. data).

CCHF is encountered in the inner parts of the Black Sea and Middle Anatolia regions, which provide a suitable climate for *Hyalomma* spp. ticks. Infected ticks carrying the virus might have been transported to Turkey on migratory birds. Turkey is a land bridge on this primary migration route for many migratory birds breeding in the Palearctic and wintering in Africa (*3*).

The role of infected ticks carried on migratory birds has not been investigated as a cause for increased CCHF in Turkey. Our aim was to investigate the role of the migratory birds in spreading CCHFV through attached ticks.

Author affiliations: Ondokuz Mayis University Medical School, Samsun, Turkey (H. Leblebicioglu, C. Eroglu, M. Hokelek, H. Yilmaz); Ondokuz Mayis University Ornithology Research Center, Samsun (K. Erciyas-Yavuz); and Ondokuz Mayis University Faculty of Veterinary Medicine, Samsun (M. Acici)

DOI: http://dx.doi.org/10.3201/eid2008.131547

The Study

Birds were caught by mist-nets, banded (ringed), and examined for ticks at the Cernek Bird Ringing Station (41°36'N, 36°05'E) in the Kizilirmak Delta in Turkey, an internationally important wetland area for birds (*4*). We conducted the study during the spring and autumn migration seasons in 2010 and 2011 and in spring 2012. Bird species and number of ticks on each species were recorded. Each tick was speciated by examining morphologic characteristics under stereomicroscope (*5*).

The identified ticks were placed in tubes with steel beads and homogenized at the maximum speed (50 Hz) for 10 min in TissueLyser LT device (QIAGEN, Hilden, Germany). RNA was isolated according to the manufacturer's recommendations by using High Pure Viral Nucleic Acid Kit (Roche Applied Science, Mannheim, Germany), but as a small modification, the homogenized tick mixture was kept at 37°C for 1 h.

In accordance with the manufacturer's recommendations, we obtained viral cDNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, Vilnius, Lithuania). Real-time PCR was performed by using the combination of primer pairs and the FastStart TaqMan Probe Master Kit (Roche Applied Science), as described by Yapar et al. (*6*), for each tick sample. cDNA from patient samples, which previously had been determined as positive, were used as the positive control sample.

We performed conventional PCR only on positive samples obtained from real-time PCR. CCHFV small (S) segment (encoding for the nucleocapsid protein) specific primer pairs (F3: 3′-GAATGTGCATGGGTTAGCTC-5′ and R2: 3′-GACATCACAATTTCACCAGG-5′) and same PCR conditions defined by Schwarz et al. (*7*) were used in the PCR. Sequence analysis was performed on the ≈260-bp PCR product, when positivity was detected, by using the primers of F3 and R2 in the ABI 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Sequences organized by using Chromas Lite Program (http://technelysium.com.au) were entered in GenBank. Our sequences and GenBank sequences were aligned in MEGA 5.1 (http://www.megasoftware.net), and the phylogenetic tree was drawn on the basis of the 260 bp of the S segment of the CCHFV genome. To compare the sequences and phylogenetic analysis, we used the maximum-likelihood method.

We found attached ticks on 65 (0.5%) of the 13,377 captured and banded birds, which represented 17 species. A total of 188 ticks collected on these birds belonged to *Ixodes*, *Hyalomma*, *Haemaphysalis*, and *Rhipicephalus* genera (Table). Only 2 ticks (*Hyalomma* sp. and *Ixodes* sp.) were CCHF positive by PCR.

CCHFV (Samsun T3–2010 and Samsun T18–2010) was detected in *Hyalomma* spp. (nymphs) (21 [4.8%] ticks)

Table. Ticks collected on migratory birds in a study of the role of migration in spreading Crimean-Congo hemorrhagic fever, Turkey

Bird (species)	No. infested/no. captured (%)	Mean Intensity*	Tick characteristic†				
			Species	Age, no.		Sex, no.	
				Larvae	Nymph	F	M
Common blackbird (*Turdus merula*)	17/514 (3.3)	6.8	*Ixodes hexagonus*			3	
			I. ricinus			6	
			Ixodes spp.	9	95		
			Rhipicephalus bursa				1
			Haemaphysalis spp.	2			
Song thrush (*T. philomelos*)	2/238 (0.8)	1	*Ixodes* spp.		2		
Thrush nightingale (*Luscinia luscinia*)	1/150 (0.7)	1	*Hyalomma* sp.			1	
Common redstart (*Phoenicurus phoenicurus*)	3/457 (0.7)	1.7	*I. ricinus*			1	
			Ixodes sp.		1		
			Hyalomma spp.		3		
European robin (*Erithacus rubecula*)	16/3,106 (0.5)	1.4	*I. hexagonus*			2	
			I. ricinus			2	1
			Ixodes spp.‡	1	16		
European pied flycatcher (*Ficedula hypoleuca*)	1/58 (1.7)	11	*Hyalomma* spp.		11		
Common chaffinch (*Fringilla coelebs*)	2/194 (1.0)	1	*I. hexagonus*			1	
			Ixodes sp.		1		
Dunnock (*Prunella modularis*)	1/41 (2.4)	1	*Ixodes* sp.		1		
Eurasian blackcap (*Sylvia atricapilla*)	8/1,478 (0.5)	1	*I. hexagonus*			1	
			I. ricinus				1
			Ixodes spp.	1	5		
Garden warbler (*S. borin*)	2/1,183 (0.2)	1	*Ixodes* spp.		2		
Lesser whitethroat (*S. curruca*)	1/154 (0.7)	1	*Ixodes* sp.	1			
Common chiffchaff (*Phylloscopus collybita)*	1/1,104 (0.1)	2	*Ixodes* spp.	2			
Great reed warbler (Acrocephalus arundinaceus)	3/30 (10.0)	2.7	*Hyalomma* spp.‡		6	2	
Savi's warbler (*Locustella luscinioides*)	2/20 (10.0)	1	*I. ricinus*			1	
			Ixodes sp.		1		
Cetti's warbler (*Cettia cetti*)	3/187 (1.6)	1	*Ixodes* spp.		3		
Eurasian blue tit (*Cyanistes caeruleus*)	1/10 (10.0)	1	*I. hexagonus*			1	
Red-backed shrike (*Lanius collurio*)	1/193 (0.5)	1	*Hyalomma* sp.		1		
Total	65/9,117 (0.7)			16	148	21	3

*Mean overall intensity of infestation per bird species.
†Blank cells indicate no data.
‡One tick was CCHFV-positive by PCR.

and *Ixodes* spp. (nymphs) (127 [0.8%]) on great reed warblers (*Acrocephalus arundinaceus*) (9.7% of banded birds) and on European robins (*Erithacus rubecula*) (0.5% of banded birds), respectively.

The Samsun CCHFV partial sequences of the S segments obtained in this study have been deposited in GenBank under the accession nos. KF727976 and KF727977. CCHFV are distributed within 7 different genotypes in the world. The CCHFV sequences obtained in the present study belong to genotype 4 (Figure 1).

Conclusions

We detected CCHFV RNA in *Hyalomma* spp. (nymphs) collected on great reed warblers and in *Ixodes* spp. (nymphs) on European robins, which migrate across Turkey twice a year en route from their breeding sites to their wintering sites (Figure 2) and back. The probability of CCHFV transportation by ticks among different regions and countries is high during migration of both bird species. Because these birds stop several times during migration (*9*), CCHF in Europe possibly could increase, especially at the stopover sites in southern Europe, which provide suitable ecologic environments.

Although *Hyalomma* ticks are the most commonly encountered ticks that carry CCHFV in Turkey, the virus also was detected in ixodid ticks, such as *Rhipicephalus* spp. and *Haemaphysalis* spp. picked up from humans and animals (*10*). Also, Albayrak et al. (*11*) detected CCHFV in *I. ricinus* ticks. Because the 2 sequences detected showed similarity with CCHFV genotype 4, which was widespread in Turkey (*1*), whether the ticks were infected in Turkey or infected earlier during bird migration is impossible to say. Other studies have shown that CCHFV could be transported by ticks on birds (*12–14*). By itself, transportation of infected ticks by birds might not be sufficient to cause the epidemics in Turkey, but along with this, climate changes, environmental changes, increased number of sensitive animals, and tick and animal movements might play a role in spreading CCHF (*1*).

In ecologically important regions, such as the Kizilirmak Delta, where resident and migratory birds are mixed,

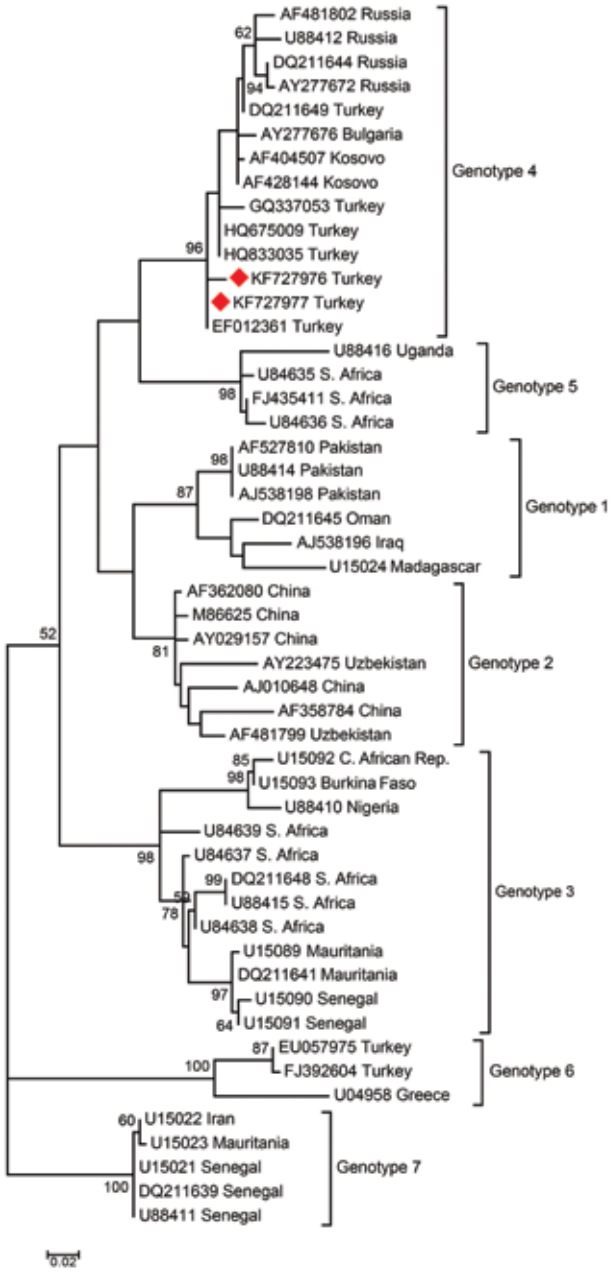


Figure 1. Phylogenetic tree of nucleotide sequences of CCHFV. Phylogenetic tree based on the 260 bp of the small segment of the CCHFV genome. The multiple sequence alignment was obtained by using MEGA 5.1 (http://www.megasoftware.net), and the phylogenetic tree was constructed by the maximum-likelihood method using 1,000 bootstrap replicates of the sequence data. The tree is drawn to scale with branch length in the same unit as those of the evolutionary distance used to infer the phylogenetic tree. The phylogenetic tree includes the 7 genotypes described by Mild et al. (*8*). Bootstrap confidence limits (>50) are shown at each node. The geographic origin is given for each sequence. The CCHFV Samsun Turkey described in this report is shown by the diamond. Scale bar indicates number of nucleotide substitutions per site. CCHFV, Crimean-Congo hemorrhagic fever; S., South; C., Central.

different microorganisms or ticks can be transferred among birds, and then carried by birds for long distances. Therefore, knowing migration routes and what pathogens birds are infected with may help predict future epidemics in various countries and provide highlight the geographic regions where diseases could be detected (*15*).

Acknowledgment

We thank Sancar Baris and Piotr Zduniak for their careful reading of the manuscript and for all their comments.

This work was supported by Ondokuz Mayis University (project no. PYO.TIP.1901.10.002).

The authors declare no conflicts of interest with respect to the authorship and/or publication of this article.

Dr Leblebicioglu is head of the Department of Infectious Diseases and Clinical Microbiology, Ondokuz Mayis University Medical School, in Turkey. His research interests include Crimean-Congo hemorrhagic fever and viral hepatitis.

References

1. Leblebicioglu H. Crimean-Congo haemorrhagic fever in Eurasia. Int J Antimicrob Agents. 2010;36(Suppl 1):S43–6. http://dx.doi.org/10.1016/j.ijantimicag.2010.06.020
2. Uyar Y, Christova I, Papa A. Current situation of Crimean Congo hemorrhagic fever (CCHF) in Anatolia and Balkan Peninsula. Turk Hij Deney Biyol Derg. 2011;68:139–51. http://dx.doi.org/10.5505/TurkHijyen.2011.60352
3. Shirihai H, Kirwan GM, Yosef R. Raptor migration in Israel and the Middle East: a summary of 30 years of field research. Eilat (Israel): International Birding & Research Center in Eliat; 2000.
4. Bariş S, Erciyas K, Gürsoy A, Özsemir C, Nowakowski J. Cernek—a new bird ringing station in Turkey. Ring. 2005;27:113–20. http://dx.doi.org/10.2478/v10050-008-0007-0
5. Walker AR, Bouattour A, Camicas J-L, Estrada-Peña A, Horak IG, Latif AA, et al. Ticks of domestic animals in Africa: a guide to identification of species. Edinburgh (Scotland): Bioscience Reports; 2003.
6. Yapar M, Aydogan H, Pahsa A, Besirbellioglu BA, Bodur H, Basustaoglu AC, et al. Rapid and quantitative detection of Crimean-Congo hemorrhagic fever virus by one-step real-time reverse transcriptase–PCR. Jpn J Infect Dis. 2005;58:358–62.
7. Schwarz TF, Nsanze H, Longson M, Nitschko H, Gilch S, Shurie H, et al. Polymerase chain reaction for diagnosis and identification of distinct variants of Crimean-Congo hemorrhagic fever virus in the United Arab Emirates. Am J Trop Med Hyg. 1996;55:190–6.
8. Mild M, Simon M, Albert J, Mirazimi A. Towards an understanding of the migration of Crimean-Congo hemorrhagic fever virus. J Gen Virol. 2010;91:199–207. http://dx.doi.org/10.1099/vir.0.014878-0
9. Alerstam T, Christie DA. Bird migration. Cambridge (UK): Cambridge University Press; 1993.
10. Tekin S, Bursali A, Mutluay N, Keskin A, Dundar E. Crimean-Congo hemorrhagic fever virus in various ixodid tick species from a highly endemic area. Vet Parasitol. 2012;186:546–52. http://dx.doi.org/10.1016/j.vetpar.2011.11.010
11. Albayrak H, Ozan E, Kurt M. Molecular detection of Crimean-Congo haemorrhagic fever virus (CCHFV) but not West Nile virus (WNV) in hard ticks from provinces in northern Turkey. Zoonoses Public Health. 2010;57:e156–60. http://dx.doi.org/10.1111/j.1863-2378.2009.01316.x

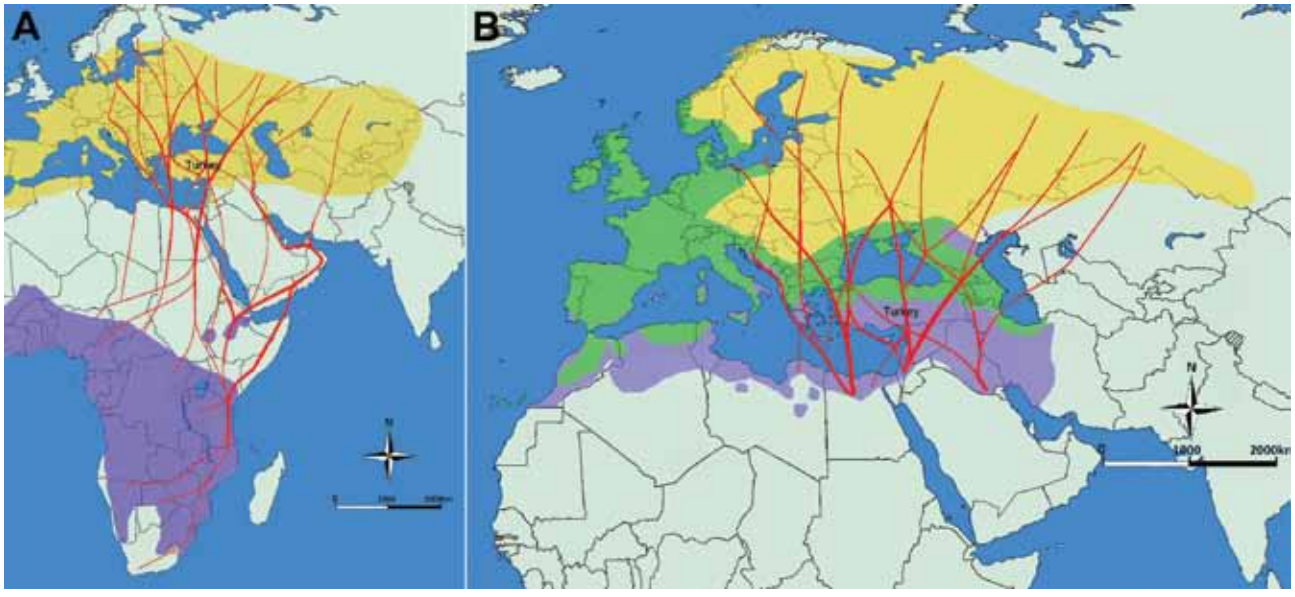


Figure 2. Migration patterns of birds carrying ticks with Crimean-Congo hemorrhagic fever virus. A) Great reed warbler (*Acrocephalus arundinaceus*) eastern migration routes (red lines), breeding grounds (yellow) and wintering areas (purple). Bodies of water are blue, and nonbreeding/nonwintering areas are light green. B) European robin (*Erithacus rubecula*) eastern migration routes (red lines), resident grounds (green), breeding grounds (yellow), and wintering areas (purple). Bodies of water are blue, and nonbreeding/nonwintering areas are light green.

12. Zeller HG, Cornet JP, Camicas JL. Crimean-Congo haemorrhagic fever virus infection in birds: field investigations in Senegal. Res Virol. 1994;145:105–9. http://dx.doi.org/10.1016/S0923-2516(07)80012-4
13. Lindeborg M, Barboutis C, Ehrenborg C, Fransson T, Jaenson TG, Lindgren PE, et al. Migratory birds, ticks, and Crimean-Congo hemorrhagic fever virus. Emerg Infect Dis. 2012;18:2095–7. http://dx.doi.org/10.3201/eid1812.120718
14. Palomar AM, Portillo A, Santibanez P, Mazuelas D, Arizaga J, Crespo A, et al. Crimean-Congo hemorrhagic fever virus in ticks from migratory birds, Morocco. Emerg Infect Dis. 2013;19:260–3. http://dx.doi.org/10.3201/eid1902.121193
15. Tsiodras S, Kelesidis T, Kelesidis I, Bauchinger U, Falagas ME. Human infections associated with wild birds. J Infect. 2008;56:83–98. http://dx.doi.org/10.1016/j.jinf.2007.11.001

Address for correspondence: Hakan Leblebicioglu, Department of Infectious Diseases and Clinical Microbiology, Ondokuz Mayis University Medical School, Samsun, Turkey; email: hakan@omu.edu.tr

Borrelia crocidurae Infection in Acutely Febrile Patients, Senegal

Oleg Mediannikov,[1] Cristina Socolovschi,[1] Hubert Bassene, Georges Diatta, Pavel Ratmanov, Florence Fenollar, Cheikh Sokhna, and Didier Raoult

As malaria cases in Africa decline, other causes of acute febrile illness are being explored. To determine incidence of *Borrelia crocidurae* infection during June 2010–October 2011, we collected 1,566 blood specimens from febrile patients in Senegal. Incidence was high (7.3%). New treatment strategies, possibly doxycycline, might be indicated for febrile patients.

As malaria cases decrease, the need for programs to study the roles of other causes of febrile syndromes in Africa increases (*1*). Other causes of fever in outpatients include typhoid and paratyphoid fevers; pneumococcal bacteremia; and a spectrum of viral infections, including influenza, yellow fever, dengue, chikungunya, and Rift Valley fever (*2*). In preliminary studies, we detected the DNA of *Borrelia crocidurae, Rickettsia* spp., *Tropheryma whipplei*, and *Coxiella burnetii* in environmental samples and blood specimens from febrile patients in Senegal (*3*–*6*).

In western Africa, tick-borne relapsing fever (TBRF) is caused by *B. crocidurae*; this acute febrile illness produces multiple recurrences of nonspecific signs and symptoms, including fever, headache, myalgia, and arthralgia. After decades of neglect, TBRF has again been detected (*7*) and is thought to be one of the major causes of fever in Africa. The reported incidence rate for TBRF in western Africa is high, reaching 25 cases per 100 person-years or accounting for 13% of febrile illnesses treated at rural dispensaries (*4*,*8*). This incidence rate is even higher than that for TBRF caused by *B. duttonii* in eastern Africa (*9*). A study from Tanzania found *B. duttonii* DNA in 3.9% of blood samples (*10*).

In 2008, we began to create a network of rural dispensaries from which to recruit patients (Table; Figure 1). The 5 study sites covered several ecosystems, ranging from dry Sahelian in northern Senegal (Keur Momar Sarr, Niakhar, and Sine-Saloum) to humid sub-Guinean in southern Senegal (Casamance and Kedougou). Two seasons are typical: dry (November–May) and rainy (June–October). The National Ethics Committee of Senegal approved the study (*11*,*12*). Since 2010, the populations of both villages in Sine-Saloum (Dielmo and Ndiop) have benefited from routine rapid point-of-care laboratory diagnostics (*12*).

The Study

From June 2010 through October 2011, fingerstick blood samples (200 mL) were collected from 1,549 febrile patients (axillary temperature >37.5°C) at 14 dispensaries and 91 randomly selected healthy villagers in Senegal. Samples were subjected to DNA extraction. Digesting, binding, and washing were performed directly in the village dispensaries by use of the QIAamp kit (QIAGEN, Hilden, Germany) as previously reported (*6*). DNA elution was performed at Aix-Marseille Université in Marseille, France. Quantitative PCR (qPCR) was performed by using primers and probes specific for the genus *Borrelia*. All samples positive for *Borrelia* spp. were subjected to standard PCR (*flaB* gene) (*4*). To determine the quality of the extracted DNA, we also measured the human actin gene (*5*). The samples were considered positive only if qPCR and *flaB*-based PCR results were positive; the sequencing of all *flaB* amplicons demonstrated that they belonged to *B. crocidurae*.

Isolation of borreliae involved intraperitoneal inoculation of laboratory BALB/c mice with 100 mL of patient capillary blood. Borreliae in mice were detected by microscopic examination of Giemsa-stained peripheral blood smears followed by qPCR of blood samples.

The sex ratio for the 1,549 patients did not differ significantly among sites (772 male and 777 female patients). An analysis of 6 age groups (<12 months, 1–3 years, 4–6 years, 7–15 years, 16–29 years, and ≥30 years) showed no differences among sites. All tested samples from clinically healthy persons had negative qPCR results for borreliae.

The incidence rate was calculated as the number of febrile episodes divided by the person-time multiplied by 1,000 (data available only for the Sine-Saloum site). The incidence rate of febrile episodes was 0.80 in Dielmo and 0.36 in Ndiop ($p<0.05$). Among the 1,566 samples tested, 115 (7.3%) were positive for *B. crocidurae.* The incidence rate for TBRF was 9.7 cases/100 persons in Dielmo and 2.4 cases/100 persons in Ndiop. The first autochthonous cases in Ndiop, which was previously considered borreliosis free, were observed in October 2010; incidence was significantly lower in Ndiop than in Dielmo ($p<0.05$). All cases registered in Ndiop before October 2010 were included in the

Author affiliations: Aix-Marseille Université, Marseille, France, and Dakar, Senegal (O. Mediannikov, C. Socolovschi, H. Bassene, G. Diatta, P. Ratmanov, F. Fenollar, C. Sokhna, D. Raoult); and Far Eastern State Medical University, Khabarovsk, Russia (P. Ratmanov)

DOI: http://dx.doi.org/10.3201/eid2008.130550

[1]These authors contributed equally to this article.

Table. Rural health centers and laboratories participating in study of *Borrelia crocidurae* infection in acutely febrile patients, Senegal, June 2010–October 2011*

Study site	District/dept/ region	District pop	Climate/ vegetation	Precip, mm/y	Health center	Coord	Other	IR
Sine-Saloum	Toubacouta/ Foundiougne/ Fatick	120,554	Sudanian/wooded savannah	939	Dielmo	13°43′N, 16°24′W	POC	10.5 (70/669)
					Ndiop	13°41′N, 16°23′W		
Niakhar	Niakhar/ Fatick and Niakhar/ Fatick	69,446	Sahelo-Sudanian/wooded steppe	757	Toucar	14°32′N, 16°28′W		19.1 (33/173)
					Diohine	14°30′N, 16°30′W		
					Ngayokheme	14°32′N, 16°26′W		
					Niakhar†	14°28′N, 16°23′W	DNA extraction	
Casamance	Loudia-Oulof/ Oussouye/ Ziguinchor	57,505	Sub-Guinean/primary and secondary gallery forests	1,432	Mlomp	12°33′N, 16°34′W	DNA extraction	0.6 (2/315)
					Kagnout	12°33′N, 16°37′W		
					Elinkine	12°30′N, 16°39′W		
Kedougou	Bandafassi/ Kedougou/ Kedougou	20,021	Sudano-Guinean/woodland, wooded savannah	1,189	Bandafassi	12°32′N, 12°18′W	DNA extraction	0.4 (1/246)
					Ibel	12°30′N, 12°22′W		
					Tiabeji	12°38′N, 12°25′W		
Keur Momar Sarr	Keur Momar Sarr/Louga/ Louga	70,743	Sahelian/steppe type	400	Keur Momar Sarr	15°55′N, 15°58′W	DNA extraction	5.5 (9/163)
					Loboudou	15°57′N, 15°55′W		
					Ganket Balla	15°58′N, 15°55′W		

*Climate information obtained from http://www.au-senegal.com/IMG/png/climat.png; demographic data obtained from the National Agency of Statistics and Demography of Senegal (http://www.ansd.sn). Dept, department; pop, population; precip, precipitation; coord, coordinates; IR, *B. crocidurae* incidence rate, % (no. tested/no. positive); POC, point-of-care laboratory.
†No patient recruitment at this site.

epidemiologic investigation and considered to be imported. The proportion of the *Borrelia*-positive samples was significantly higher for northern sites with a drier Sudanian climate; positivity reached 19.1% (33/173) in Niakhar (Table). By analyzing the epidemiologic questionnaires completed by families of ill persons, we determined that the 2 TBRF cases in Casamance were imported from the northern regions of Senegal by seasonal workers.

Patients most frequently infected were 7–15 years of age (13.5%, 43/318), unlike in eastern Africa where younger persons are more frequently infected (*9*). No positive results were found among the 155 children <12 months of age, but positive results were found for 16 (4.8%) of the 352 children 1–3 years of age ($p<0.05$). Unlike in other northern regions, in Sine-Saloum, the proportion of *Borrelia*-positive samples was significantly higher for samples

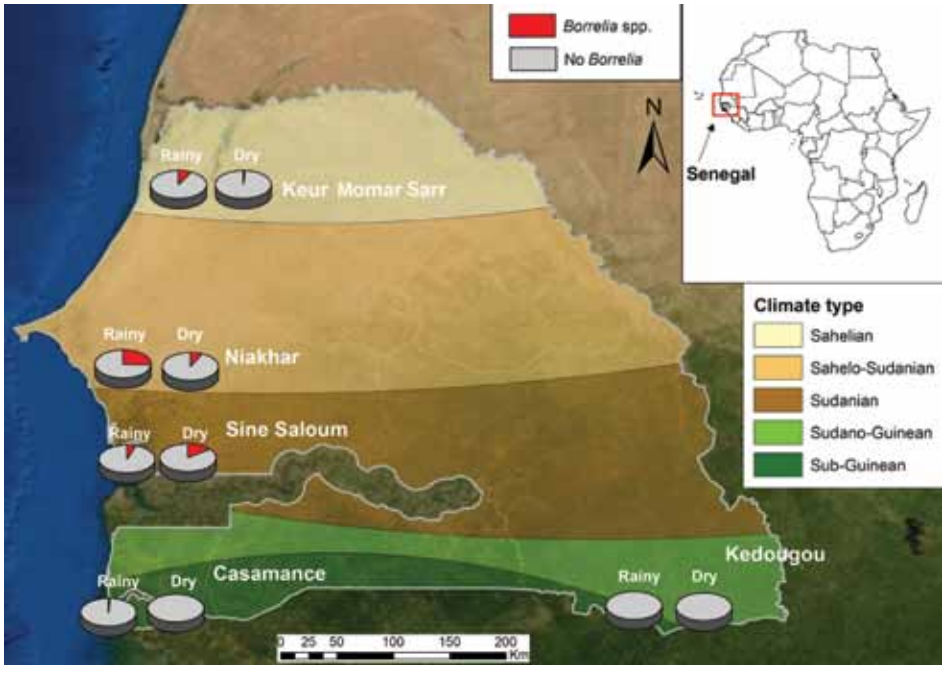


Figure 1. Incidence of tick-borne relapsing fever at study sites, according to season, Senegal, June 2010–October 2011. Map reproduced from Bing Maps (http://www.bing.com/maps/).

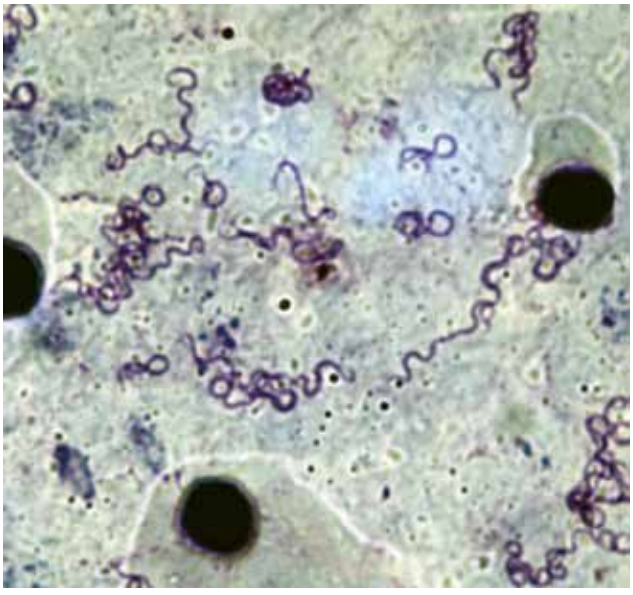


Figure 2. Thick smear of mouse blood showing *Borrelia crocidurae* isolate 02–03. Giemsa stain; original magnification x900.

collected during the dry (16.9%, 40/237) than the rainy season (6.9%, 30/432); p<0.0001 (Figure 1).

We identified 20 patients (49 samples) for whom 2–4 samples were positive for *B. crocidurae*. The interval between the sample collections was short (5–30 days) for 13 persons, average (30–66 days) for 4 persons, and long (102–381 days) for 3 persons. Two *Borrelia* isolates (no. 03–02 from Ndiop and no. 19/31 from Dielmo) were recovered from the peripheral blood of 2 febrile patients. The bacteria had a morphologic appearance that was typical for borreliae (Figure 2). A BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) search for the sequenced *flaB* gene (JX119098) demonstrated that the isolates were nearly identical with the type Achema strain of *B. crocidurae* (CP003426).

Conclusions

We detected an alarmingly high proportion of *Borrelia* DNA in the blood of febrile patients in Senegal. The presence of this DNA is strongly and specifically linked to the fever because no *Borrelia* DNA was identified among the 90 control participants. In Tanzania, however, borreliae have been identified in up to 33% of blood samples obtained from asymptomatic blood donors who lived in similar conditions as ill persons (*13*).

The geographic repartition of TBRF is linked to drier climates (*9*). We observed autochthonous cases only in northern Senegal, roughly north of the 13°30′ parallel. We noted the recent extension of *B. crocidurae* into the village of Ndiop, which had been free of *B. crocidurae*. This extension might be linked to recent climate changes (*14*). The person-year incidence of borreliosis in our study (6.1 cases/100 population) is similar to that reported by Vial et al. (4 cases/100 population) for the interepidemic period (*8*).

We report a unique series of cases in which *Borrelia* DNA was identified several times consecutively in the blood of the same patient. For 17 patients for whom the time between positive samples was short or average (up to 66 days), repeated detection of *Borrelia* DNA during repeated episodes of fever could be explained by relapses. However, reinfection is strongly suspected in 3 patients because the interval between 2 positive samples was >100 days. To the best of our knowledge, reinfection with relapsing fever borreliae has not been previously reported in Africa. The phenomenon of easy reinfection after treatment with tetracycline has been reported for the relapsing-fever group *B. hermsii* in vervet monkeys, which could be reinfected 12–36 weeks after primary infection (*15*).

In conclusion, the incidence of TBRF and the proportion of borreliosis cases among febrile patients in Senegal is very high and, in at least 1 region (Niakhar), exceeds that of malaria. This considerably high incidence rate should lead to the development of new therapeutic strategies that could be based on treating febrile patients in Senegal with doxycycline.

Acknowledgments

We thank the Agence National de Recherche, grant MALEMAF (Research of Emergent Pathogens in Africa) and La Fondation Méditerranée Infection for financial support; all villagers who participated in the study; Annick Bernard, Aliou Diallo, Denis Piak, and Khadim Mbacke Leye for technical support; and Pascal Weber for photography.

Dr Mediannikov is a researcher in epidemiology and microbiology in Dakar, Senegal. His scientific interests include vector-borne diseases and other causes of nonmalarial fever in Africa.

References

1. Gwer S, Newton CR, Berkley JA. Over-diagnosis and co-morbidity of severe malaria in African children: a guide for clinicians. Am J Trop Med Hyg. 2007;77(Suppl):6–13.
2. Hotez PJ, Kamath A. Neglected tropical diseases in sub-Saharan Africa: review of their prevalence, distribution, and disease burden. PLoS Negl Trop Dis. 2009;3:e412. http://dx.doi.org/10.1371/journal.pntd.0000412
3. Mediannikov O, Fenollar F, Socolovschi C, Diatta G, Bassene H, Molez JF, et al. *Coxiella burnetii* in humans and ticks in rural Senegal. PLoS Negl Trop Dis. 2010;4:e654. http://dx.doi.org/10.1371/journal.pntd.0000654
4. Parola P, Diatta G, Socolovschi C, Mediannikov O, Tall A, Bassene H, et al. Tick-borne relapsing fever borreliosis, rural Senegal. Emerg Infect Dis. 2011;17:883–5. http://dx.doi.org/10.3201/eid1705.100573
5. Fenollar F, Mediannikov O, Socolovschi C, Bassene H, Diatta G, Richet H, et al. *Tropheryma whipplei* bacteremia during fever in rural West Africa. Clin Infect Dis. 2010;51:515–21. http://dx.doi.org/10.1086/655677

6. Mediannikov O, Diatta G, Fenollar F, Sokhna C, Trape JF, Raoult D. Tick-borne rickettsioses, neglected emerging diseases in rural Senegal. PLoS Negl Trop Dis. 2010;4. pii: e821. http://dx.doi.org/10.1371/journal.pntd.0000821
7. Bergeret C, Raoult A. Notes sur les formes nerveuses de la fièvre récurrente-–fièvre récurrente à tiques en Afrique Occidentale Française. Bul Med Afrique Occidentale Française 1934;27:593–8.
8. Vial L, Diatta G, Tall A, Ba el H, Bouganali H, Durand P, et al. Incidence of tick-borne relapsing fever in West Africa: longitudinal study. Lancet. 2006;368:37–43. http://dx.doi.org/10.1016/S0140-6736(06)68968-X
9. Cutler SJ. Possibilities for relapsing fever reemergence. Emerg Infect Dis. 2006;12:369–74. http://dx.doi.org/10.3201/eid1203.050899
10. Reller ME, Clemens EG, Schachterle SE, Mtove GA, Sullivan DJ, Dumler JS. Multiplex 5′ nuclease-quantitative PCR for diagnosis of relapsing fever in a large Tanzanian cohort. J Clin Microbiol. 2011;49:3245–9. http://dx.doi.org/10.1128/JCM.00940-11
11. Trape JF, Rogier C, Konate L, Diagne N, Bouganali H, Canque B, et al. The Dielmo project: a longitudinal study of natural malaria infection and the mechanisms of protective immunity in a community living in a holoendemic area of Senegal. Am J Trop Med Hyg. 1994;51:123–37.
12. Sokhna C, Mediannikov O, Fenollar F, Bassene H, Diatta G, Tall A, et al. Point-of-care laboratory of pathogen diagnosis in rural Senegal. PLoS Negl Trop Dis. 2013;7: e1999. doi: 10.1371/journal.pntd.0001999.
13. Cutler SJ, Bonilla EM, Singh RJ. Population structure of East African relapsing fever *Borrelia* spp. Emerg Infect Dis. 2010;16:1076–80. http://dx.doi.org/10.3201/eid1607.091085
14. Trape JF, Godeluck B, Diatta G, Rogier C, Legros F, Albergel J, et al. The spread of tick-borne borreliosis in West Africa and its relationship to sub-Saharan drought. Am J Trop Med Hyg. 1996;54:289–93.
15. Felsenfeld O, Wolf RH. Reinfection of vervet monkeys (*Cercopithecus aethiops*) with *Borrelia hermsii*. Res Commun Chem Pathol Pharmacol. 1975;11:147–50.

Address for correspondence: Didier Raoult, URMITE, IRD 198, UM63, CNRS 7278, INSERM 1095, Aix-Marseille Université, 13005 Marseille, France ; email: didier.raoult@gmail.com

Isolation of MERS Coronavirus from Dromedary Camel, Qatar, 2014

V. Stalin Raj,[1] Elmoubasher A.B.A. Farag,[1] Chantal B.E.M. Reusken, Mart M. Lamers, Suzan D. Pas, Jolanda Voermans, Saskia L. Smits, Albert D.M.E. Osterhaus, Naema Al-Mawlawi, Hamad E. Al-Romaihi, Mohd M. AlHajri, Ahmed M. El-Sayed, Khaled A. Mohran, Hazem Ghobashy, Farhoud Alhajri, Mohamed Al-Thani, Salih A. Al-Marri, Mamdouh M. El-Maghraby, Marion P.G. Koopmans, and Bart L. Haagmans

We obtained the full genome of Middle East respiratory syndrome coronavirus (MERS-CoV) from a camel in Qatar. This virus is highly similar to the human England/Qatar 1 virus isolated in 2012. The MERS-CoV from the camel efficiently replicated in human cells, providing further evidence for the zoonotic potential of MERS-CoV from camels.

Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel coronavirus that can cause severe lower respiratory tract infection in humans (*1*,*2*). MERS-CoV clusters with viruses in the genus *Betacoronavirus*; the closest relative to this virus is bat CoVs clade 2c (*3*). Although bats are believed to carry different CoV ancestors, antibody reactivity against MERS-CoV has been found in serum from dromedary camels from countries within the Arabian Peninsula (*4*–*7*), Egypt (*8*), and the Canary Islands (*4*). More recently, MERS-CoVs that phylogenetically cluster with human MERS-CoVs were detected in camels from Qatar, Saudi Arabia, and Egypt (*7*,*9*–*12*). To further characterize MERS-CoV from camels, we screened nose swab samples from camels in Qatar.

Author affiliations: Erasmus Medical Center, Rotterdam, the Netherlands (V. Stalin Raj, C.B.E.M. Reusken, M.M. Lamers, S.D. Pas, J. Voermans, S.L. Smits, A.D.M.E. Osterhaus, M.P.G. Koopmans, B.L. Haagmans); Supreme Council of Health, Doha, Qatar (E.A.B.A. Farag, H.E. Al-Romaihi, M.M. AlHajri, A.M. El-Sayed, M. Al-Thani, S.A. Al-Marri); Hamad Medical Corporation, Doha (N. Al-Mawlawi); and Ministry of Environment, Doha (K.A. Mohran, H. Ghobashy, F. Alhajri, M.M. El-Maghraby)

DOI: http://dx.doi.org/10.3201/eid2008.140663

The Study

In February 2014, nasal swab samples were collected from 53 healthy dromedary camels in Doha, Qatar. After sampling, swabs were put into tubes containing viral transport medium and stored at -80°C until shipment to the Netherlands on dry ice, as described (*9*).

Total nucleic acids from nasal swabs were isolated by using the MagnaPure 96 total nucleic acid isolation kit (Roche, Mannheim, Germany), and samples were tested for MERS-CoV by using 2 TaqMan assays: 1 for the envelope (upE) and 1 for the nucleocapsid gene (N), as described previously (*9*,*13*). In each assay we detected MERS-CoV RNA in a sample from an 8-month-old camel. The cycle threshold of the positive sample was 12.9 in the upE assay and 11.3 in the N assay.

For further genomic characterization, RNA was isolated from 50 μL of 1 swab sample with the QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), eluted in 60 μL water, and reverse transcribed with the Superscript III First-Strand Synthesis System (Life Technologies (Bleiswijk, the Netherlands) with random hexamers. The MERS-CoV genome was amplified by using MERS-CoV–specific overlapping primer sets as described previously (*3*). Amplified MERS-CoV fragments were sequenced directly on both strands by using the BigDye Terminator version 3.1 Cycle Sequencing kit on an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Bleiswijk, the Netherlands). To obtain the 5′ and 3′ ends, we used the FirstChoice RLM-RACE kit (Ambion, Bleiswijk, the Netherlands) according to the manufacturer's protocols. Using overlapping sequence fragments, we assembled the complete MERS-CoV genome, except for 1 nt potentially missing at the 5′ end.

The genome was 30,117 nt long, including 12 nt at the 3′ poly A tail (MERS-CoV camel/Qatar_2_2014, GenBank accession no. KJ650098). Similar to the genome of human MERS-CoV isolates, the genome of camel MERS-CoV isolates contains 10 complete open reading frames (ORFs) (ORF 1ab, spike, ORF3, ORF4a, ORF4b, ORF5, envelope, membrane, nucleocapsid, and ORF8b), 8 transcription-regulatory sequences, and 2 terminal untranslated regions. The alignment of the camel MERS-CoV with known human MERS-CoVs, including1 near-complete camel MERS-CoV (NRCE_HKU205) sequence, showed overall nucleotide identities of 99.5%–99.9% between camel and human MERS-CoV isolates from different geographic regions.

Phylogenetic analysis of the complete genome clearly showed that MERS-CoV camel/Qatar_2_2014 is highly similar to human MERS-CoV; the closest relative to camel MERS-CoV was England/Qatar1 2012 (99.9% identity) (Figure 1), and it was clearly distinct from the camel

[1]These authors contributed equally to this article.

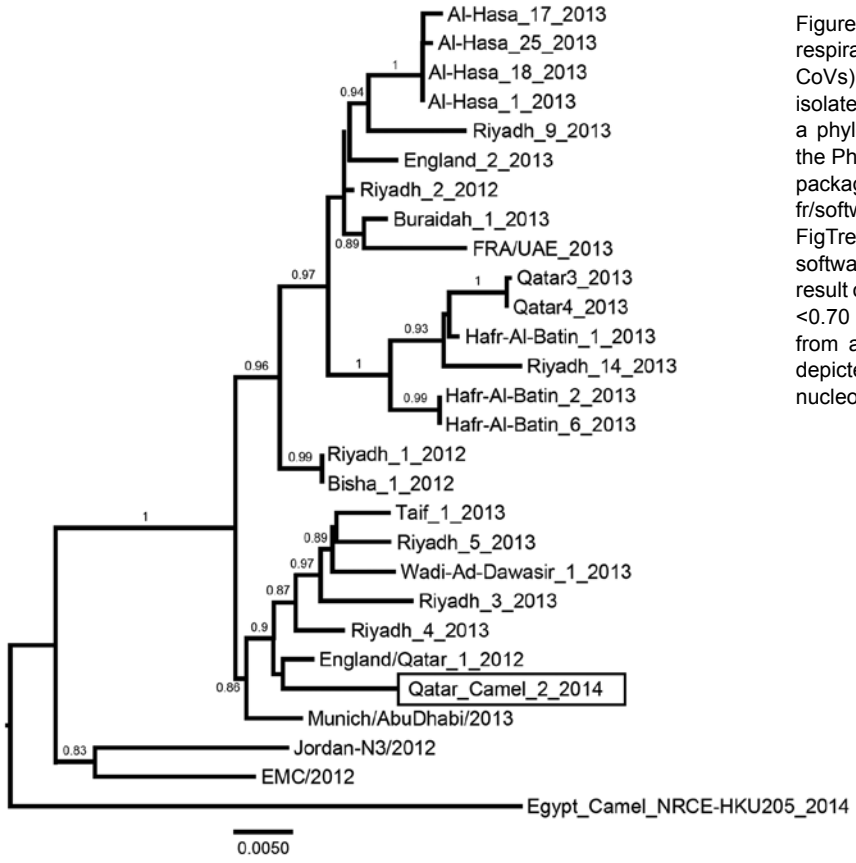


Figure 1. Phylogenetic analysis of Middle East respiratory syndrome coronaviruses (MERS-CoVs). Genome sequences of representative isolates were aligned by using ClustalW, and a phylogenetic tree was constructed by using the PhyML method in Seaview 4 (all 3 software packages can be found at http://pbil.univ-lyon1.fr/software/seaview) and was visualized in FigTree version 1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Values at branches show the result of the approximate likelihood ratio; values <0.70 are not shown. The MERS-CoV isolated from a dromedary camel in Qatar in 2014 is depicted in a rectangle. Scale bar indicates nucleotide substitutions per site.

MERS-CoV (99.5% identity) isolated from camels at a different location in Qatar and in Egypt (*9*,*11*). Comparison of spike protein amino acid sequences from various human and camel isolates showed that this protein is highly conserved between this camel virus and other human isolates (online Technical Appendix Table, wwwnc.cdc.gov/EID/article/20/8/14-0663-Techapp1.pdf).

In addition, most amino acid residues critical for receptor binding (*14*) are identical in human and camel isolates, except for L506F in England/Qatar_1_2012. The biologic relevance of this mutation has not been investigated. The presence of arginine at position 1020 in the camel virus isolate might indicate that selective pressure at this site has probably not taken place as previously postulated. The fact that a MERS-CoV from a camel is highly similar to that from a human patient who probably became infected >1 year earlier in the same region suggests that this virus is maintained within camel populations and further supports the hypothesis that MERS-CoV can be transmitted from camels to humans.

To test for the presence of infectious virus, we titrated the swab sample on Vero cells (ATCC no. CCL-81). After 48 hours, we observed cytopathic changes in cells (320 50% tissue culture infectious dose/mL). After isolation, the passage-3 virus stock was used for all subsequent experiments.

To check for adaptive mutations obtained during cell culture, we used 454 deep-sequencing technology (Roche, Indianapolis, IN, USA) to analyze the full-genome sequence as described elsewhere (*3*). A total of 57,655 sequence reads were obtained, of which 17,056 were specific for MERS-CoV, revealing ≈99.77% of the virus genome. Genome coverage ranged from 1 to 2,082 reads at single nucleotide positions. Gaps or regions with coverage of <4 reads were confirmed by Sanger sequencing. When the genome of the passaged virus was aligned with the genome of the initial clinical isolate, we did not observe any mutations acquired during passaging.

To further functionally characterize this virus isolate, we subsequently inoculated human hepatoma (Huh-7) cells with MERS-CoV camel/Qatar_2_2014. After 2 days, virus-induced cytopathic effects were observed in the inoculated cell cultures (online Technical Appendix Figure). In addition, a strong increase in virus titer was measured in the cell supernatant (Figure 2, panel A); produced virus could be passaged (not shown). Virus production in Huh-7 cells was blocked by preincubating camel MERS-CoV with a 1:200 dilution of serum from MERS-CoV antibody–positive camels (*9*) but not with seronegative camel serum (*4*) (Figure 2, panel A). Infection of Huh-7 cells could also be blocked by preincubation of cells with polyclonal

antiserum against human DPP4 but not with control serum (Figure 2, panel A). Furthermore, transfection of nonsusceptible MDCK cells with human DPP4 (Figure 2, panel B), but not with empty vector, conferred susceptibility to infection with camel MERS-CoV (Figure 2, panel C). These data demonstrate that the MERS-CoV obtained from a dromedary camel is able to replicate in human cells and uses DPP4 as entry receptor, similar to MERS-CoV isolates obtained from human patients (*15*).

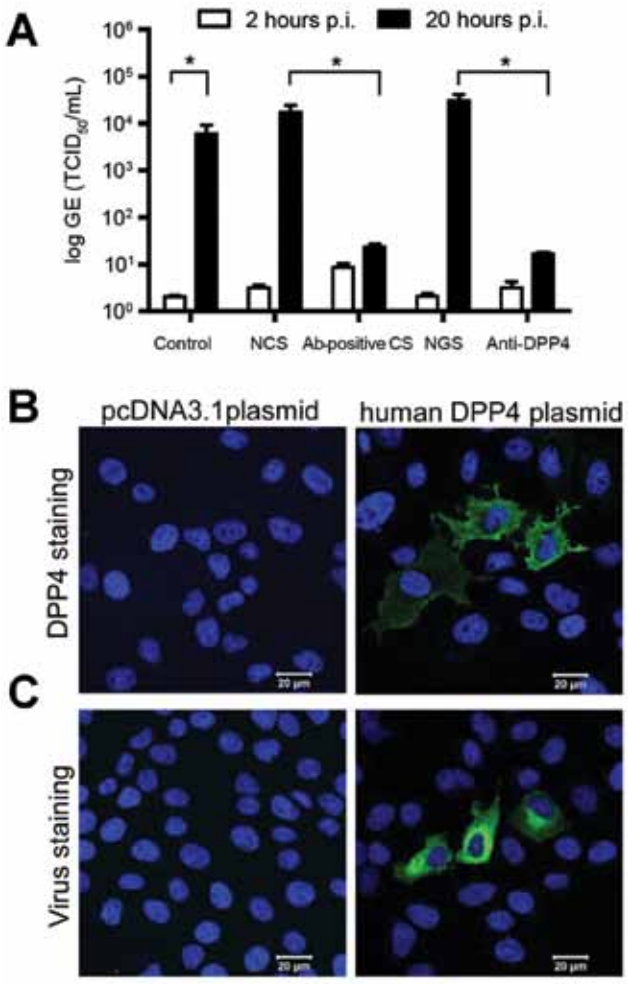


Figure 2. Middle East respiratory syndrome coronavirus (MERS-CoV) from camel replicates in human hepatoma (Huh-7) cells and uses human DPP4 as entry receptor. Huh-7 cells were inoculated with camel MERS-CoV and left for 1 h. Next, cells were washed twice, and supernatant was collected at 2 h (open bars) and 20 h (closed bars) before being tested for MERS-CoV RNA by using a TaqMan assay. We analyzed control camel MERS-CoV–infected cells, cells inoculated with camel MERS-CoV in the presence of normal camel serum (NCS), MERS-CoV–antibody positive camel serum (Ab-positive CS), normal goat serum (NGS), and anti-DPP4 polyclonal antibody–treated cells. Results are expressed as genome equivalents (GE), 50% tissue culture infective dose ($TCID_{50}$/mL) (A). MDCK cells transfected with plasmid-encoding human DPP4 or a control plasmid (pcDNA) were stained with polyclonal antibody against human DPP4 (B) or inoculated with camel MERS-CoV and fixed 20 h after inoculation (p.i.) and stained for viral antigen (C).

Conclusions

We isolated MERS-CoV from the nasal cavity of 1 dromedary camel and demonstrated its infectiousness. Further studies are needed to test whether camels infected at a young age are more likely than adult dromedary camels to excrete infectious virus, possibly because of the MERS-CoV seronegative status of the younger camels. In addition, our results add to recent findings that MERS-CoVs from camels and humans are nearly identical (*9*–*11*). As might be expected from the high level of conservation in the critical interacting amino acids in the receptor-binding domain of the camel and human MERS-CoV isolates (online Technical Appendix Table), we show that camel MERS-CoV can infect human Huh-7 cells by using the same entry receptor as the human MERS-CoV isolates (*15*). Collectively, combined with the observation that the sequence of this virus was most closely related to that of a virus from a human patient who acquired MERS-CoV in Qatar a year earlier, these data support the hypothesis that dromedary camels are a reservoir for MERS-CoV and can transmit the infection to humans. However, whether exposure of humans to camels directly can lead to human infection cannot be concluded from our data. We are not aware of a connection between the camel population sampled in this study and the patient infected with MERS-CoV England/Qatar 1. Future epidemiologic studies are needed to investigate whether contact with camels or camel products constitutes a risk factor for MERS-CoV infection.

Acknowledgments

We are grateful to the Joint Supreme Council of Health and Animal Resources Department of Ministry of Environment field investigation team for exceptional research assistance; to the Doha Camel slaughterhouse veterinarians, staff, and workers for their help; and to the Supreme Council of Health Administration for funding this study through a grant from the Health Promotion Department and the Communicable Disease Control Department routine budget. We also thank Thomas P.Samuel, Redentor Cuizon, Ronald R. Manaor, Khalid Yousif, and Farid Abdoudia for help with collecting samples.

This work was funded by a grant from the Dutch Scientific Research (no. 40-00812-98-13066), the European Community's Seventh Framework Program (FP7/2007–2013) under the project "European Management Platform for Emerging and Re-emerging Infectious disease Entities"; European Commission (agreement no. 223498); and the project "Anticipating Global Onset of Epidemics" (agreement no. 102938).

Dr Stalin Raj is a postdoctoral scientist at the Department of Viroscience, Erasmus Medical Center, Rotterdam, the

Netherlands. His research interests are the molecular characterization and pathogenesis of emerging viruses.

References

1. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367:1814–20. http://dx.doi.org/10.1056/NEJMoa1211721
2. World Health Organization, MERS-CoV Research Group. State of knowledge and data gaps of Middle East respiratory syndrome coronavirus (MERS-CoV) in humans. PLoS Curr. 2013;5:pii: ecurrents.outbreaks.0bf719e352e7478f8ad85fa30127ddb8.
3. van Boheemen S, de Graaf M, Lauber C, Bestebroer TM, Raj VS, Zaki AM, et al. Genomic characterization of a newly discovered coronavirus associated with acute respiratory distress syndrome in humans. MBiol 2012;3:e00473–12.
4. Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis. 2013;13:859–66. http://dx.doi.org/10.1016/S1473-3099(13)70164-6
5. Hemida MG, Perera R, Wang P, Alhammadi MA, Siu LY, Poon LL, et al. Middle East respiratory syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. Euro Surveill. 2013;18:20659.
6. Meyer B, Müller MA, Corman VM, Reusken CBEM, Ritz D, Godeke G-D. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014;20:552–9. http://dx.doi.org/10.3201/eid2004.131746
7. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, de Wit E, et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. MBio. 2014;5:e00884–14. http://dx/doi.org/10.1128/mBio.00884–14.
8. Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagoto O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 2013;18:20574.
9. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infect Dis. 2014;14:140–5. http://dx.doi.org/10.1016/S1473-3099(13)70690-X. Epub 2013 Dec 17.
10. Memish ZA, Cotton M, Meyer B, Watson SJ, Alsahafi AJ, Al Rabeeah AA, et al. Human infection with MERS coronavirus after exposure to infected camels, Saudi Arabia, 2013. Emerg Infect Dis. 2014; [Epub ahead of print]. http://dx.doi.org/10.3201/eid2006.140402
11. Chu DKW, Poon LLM, Gomaa MM, Shehata MM, Perera RAPM, Zeid DAE. MERS coronaviruses in dromedary camels, Egypt. Emerg Infect Dis. 2014; [Epub ahead of print]. http://dx.doi.org/10.3201/eid2006.140299
12. Hemida MG, Chu DKW, Poon LLM, Perera RAPM, Alhammadi MA, Ng H-Y, et al. MERS coronavirus in dromedary camel herd, Saudi Arabia. Emerg Infect Dis. 2014 [Epub ahead of print].
13. Corman VM, Eckerle I, Bleicker T, Zaki A, Landt O, Eschbach-Bludau M, et al. Detection of a novel human coronavirus by real-time reverse-transcription polymerase chain reaction. Euro Surveill. 2012;17:20285.
14. Lu G, Hu Y, Wang Q, Qi J, Gao F, Li Y, et al. Molecular basis of binding between novel human coronavirus MERS-CoV and its receptor CD26. Nature. 2013;500:227–31. http://dx.doi.org/10.1038/nature12328. Epub 2013 Jul 7.
15. Raj VS, Mou H, Smits SL, Dekkers DH, Müller MA, Dijkman R, et al. Dipeptidyl peptidase 4 is a functional receptor for the emerging human coronavirus-EMC. Nature. 2013;495:251–4. http://dx.doi.org/10.1038/nature12005.

Address for correspondence: Bart L. Haagmans, Department of Viroscience, Erasmus Medical Center, P.O. Box 2040, 3000 CA Rotterdam, the Netherlands; email: b.haagmans@erasmusmc.nl

New Introductions of Enterovirus 71 Subgenogroup C4 Strains, France, 2012

Isabelle Schuffenecker, Cécile Henquell, Audrey Mirand, Marianne Coste-Burel, Stéphanie Marque-Juillet, Delphine Desbois, Gisèle Lagathu, Laure Bornebusch, Jean-Luc Bailly, and Bruno Lina

In France during 2012, human enterovirus 71 (EV-A71) subgenogroup C4 strains were detected in 4 children hospitalized for neonatal fever or meningitis. Phylogenetic analysis showed novel and independent EV-A71 introductions, presumably from China, and suggested circulation of C4 strains throughout France. This observation emphasizes the need for monitoring EV-A71 infections in Europe.

Human enterovirus 71 (EV-A71) is a member of the enterovirus species A in the family *Picornaviridae*, genus *Enterovirus*. On the basis of the 1D gene sequences encoding the VP1 capsid protein ($1D^{VP1}$), EV-A71 has been classified into 3 genogroups (A–C) and 12 subgenogroups (A, B0–B5, C1–C5) (*1*); in addition, 3 new genogroups (D–F) were recently identified (*2–4*). In children, EV-A71 mainly causes asymptomatic or benign infections, such as neonatal fever and hand-foot and mouth disease (HFMD); less frequently, EV-A71 causes neurologic complications, such as encephalitis and poliomyelitis-like paralysis (*1*).

In the Asia–Pacific region, EV-A71 has emerged as a major public health concern over the past 15 years. Large outbreaks have been reported, associated with the emergence of new genogroups and subgenogroups, high rates of illness, and fatal cases of encephalitis (*1*,*5*). The largest epidemic expansion of EV-A71 occurred in China, mainly caused by EV-A71 subgenogroup C4 (EV-A71 C4) strains (*5*,*6*). By contrast, epidemic activity is low in Europe, where only 4 outbreaks of EV-A71 infection have been reported over the past 40 years: Bulgaria (1975), Hungary (1978), and the Netherlands (1986, 2007) (*5*,*7*). Most of the cases of EV-A71 infection reported since 1986 have been caused by subgenogroup C1 and C2 strains (*7–9*). In 2004, EV-A71 C4 strains were rarely detected in France, Germany, and Austria (*8–11*), and no other EV-A71 C4 cases were reported in Europe until 2012, when we detected C4 strains in 4 hospitalized patients, suggesting that dissemination of the C4 strains was restricted during 2004–2011. We describe the clinical cases caused by the EV-A71 C4 strains detected in 2012 and address the origin of these newly detected viruses.

Author affiliations: Hospices Civils de Lyon, Lyon, France (I. Schuffenecker, B. Lina); Centre Hospitalier Universitaire de Clermont-Ferrand, Clermont-Ferrand, France (C. Henquell, A. Mirand); Université d'Auvergne, Clermont-Ferrand (C. Henquell, A. Mirand, J.-L. Bailly); Centre Hospitalier Universitaire de Nantes, Nantes, France (M. Coste-Burel); Centre Hospitalier de Versailles, Le Chesnay, France (S. Marque-Juillet); Centre Hospitalier de Rambouillet, Rambouillet, France (D. Desbois); Centre Hospitalier Universitaire de Rennes, Rennes, France (G. Lagathu); and Centre Hospitalier de Grasse, Grasse, France (L. Bornebusch)

DOI: http://dx.doi.org/10.3201/eid2008.131858

The Study

In France, EV infections diagnosed in hospital settings have been voluntarily reported to the National Institute for Public Health by a network of hospital laboratories since 2000 (*9*). In 2012, a total of 2,088 EV infections were reported by the laboratory network. In addition, in 2010, a total of 158 community cases of HFMD and herpangina were reported through a sentinel surveillance system implemented in Clermont-Ferrand, France (*12*). As part of the national surveillance, 1,249 EV strains were analyzed by 6 laboratories in the EV network (including the 2 National Enterovirus Reference Center laboratories in Lyon and Clermont-Ferrand). Of the 1,249 EV strains, 1,105 (88.5%) were successfully genotyped. Most of the genotyped strains were detected in cerebrospinal fluid (CSF) samples from patients with neonatal fever, meningitis, or meningoencephalitis or in samples from patients with HFMD or herpangina. Of the 1,105 genotyped EV strains, 16 (1.4%) were EV-A71 strains. Among these 16 cases of EV-A71 infection, a fatal case of rhombencephalitis was diagnosed in an adult who had been treated with rituximab (*13*). On the basis of the complete 1D gene sequences encoding the VP1 capsid protein ($1D^{VP1}$), 12 of the 16 EV-A71 strains were assigned to subgenogroup C2, and 4 were assigned to subgenogroup C4.

We conducted a retrospective review of medical records for the 4 patients with EV-A71 C4 infection to document the patients' ages at diagnosis, clinical symptoms, length of hospitalization, and laboratory findings. The EV-A71 C4 infections were detected throughout the year in 3 regions (Brittany, Ile de France, and Provence-Alpes-Côte d'Azur). Of the 4 patients, 3 (6, 17, and 21 days of age) had neonatal fever when medical care was sought, and 1 patient (4 years of age) had meningitis (Table). The 21-day-old infant had persisting irritability and was hospitalized for 6 days. No severe neurologic complications were observed, and all 4 children had a favorable outcome. Bacterial culture results for CSF, blood, and urine samples from all 4

Table. Characteristics of 4 patients with enterovirus 71 subgenogroup C4 infections, France, 2012*

Patient no., hospital location†	Date admitted	Age at diagnosis	Clinical signs	Laboratory values for CSF		Enterovirus RT-PCR results, C_t‡		Duration, d	
				Leukocytes/ mm^3	Protein, g/L	CSF	NP	Hospital stay	Antimicrobial drug use
1, Nantes	Feb 12	6 d	Fever syndrome	<2	0.54	Pos, 36.0	Pos, 36.2	4.0	4.0
2, Versailles	Mar 12	17 d	Fever syndrome	53	0.52	Pos, 31.7	NA	2.5	2.5
3, Grasse	Jun 12	4.5 y	Fever syndrome, meningitis	83§	0.92§	Neg	Pos, 27.4	1.0	0
4, Rennes	Oct 12	21 d	Fever syndrome, rhinitis	234	0.77	Pos, 30.0	NA	6.0	3.0

*For all 4 children, molecular testing results were negative for the detection of herpes simplex virus types 1 and 2 and varicella-zoster virus in CSF. In addition, for patients 1 and 4, molecular testing results were also negative for the detection of human herpesvirus type 6, cytomegalovirus, and Epstein-Barr virus. Results were also negative for CSF bacterial cultures. CSF, cerebrospinal fluid sample; C_t, cycle threshold; NA, not available; Neg, negative NP, nasopharyngeal sample; Pos, positive; RT-PCR, reverse transcription PCR.
†Nantes (47°13′N; 1°33′W; Brittany); Versailles (48°48′N; 2°08′E; Ile de France); Grasse (43°40′N; 6°55′E; Provence-Alpes-Côte d'Azur); Rennes (48°06′N; 1°40′W; Brittany).
‡The upper Ct for positivity for each of the RT-PCR assays used was 45.0. An in-house enterovirus real-time RT-PCR was used to test samples from patient 1. A commercial RT-PCR assay produced by Cepheid and commercialized by Orgentec (GP-EV-040, Orgentec, Trappes, France) was used to test samples from patients 2 and 3. Another commercial RT-PCR assay, Dia-ENT-050 (Diagenode, Seraing, Belgium) was used to test samples from patient 4.
§CSF sample was hemorrhagic (10,300 erythrocytes/mm^3).

children were negative, and molecular detection results for herpes simplex virus types 1 and 2 and varicella-zoster virus in CSF were also negative. For the 3 children with neonatal fever, reverse transcription PCR was positive for EV-A71 in CSF specimens. For the child with meningitis, reverse transcription PCR was negative for EV-A71 in the CSF specimen, but an EV-A71 strain was isolated from a throat swab specimen.

Phylogenetic analyses based on a Bayesian approach were performed with a set of 97 $1D^{VP1}$ sequences (Figure; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/13-1858-Techapp1.pdf). The chronogram clearly shows that the EV-A71 C4 strains detected in France in 2012 (Figure, red branches) and those detected in France, Austria, and Germany in 2004 (Figure, green branches) belong to 2 separate lineages. The 4 strains detected in 2012 in France clustered with EV-A71 C4 strains detected in China during 2008–2011. We estimated that the most recent common ancestor of this cluster (Figure, blue branches) emerged, presumably in China, during 2006 (95% highest posterior density interval 2005–2007). Of the 4 EV-A71 C4 strains detected within a 4-month period in geographically distant (≈1,100 km) French regions, 2 displayed direct clustering (posterior probability 0.999) but substantial variation (genetic distance of 0.018 nt substitution per site between the corresponding $1D^{VP1}$ sequences). The quick evolution of the 2 EV-A71 C4 strains from their common ancestor suggests local spread of the viruses. The other 2 strains displayed consistent clustering (posterior probability >0.8) with different strains isolated in China, suggesting independent introductions of virus.

Conclusions

In 2012, EV-A71 C4 strains were detected in France in 4 children hospitalized for neonatal fever or meningitis. Although EV-A71 C4 strains have circulated extensively in China since 2008, this virus has rarely been detected in Europe. In France, 133 cases of EV-A71 infections were reported during January 2000–May 2013 (*9*) (I. Schuffenecker, unpub. data). EV-A71 C2 infections have been predominant since 2007; however, only 5 cases of EV-A71 C4 infection have been identified in the country since 2004. Our Bayesian analyses excluded a direct evolution of the 2012 EV-A71 C4 strains from the earlier 2004 European virus lineage. The phylogenetic data are consistent with 3 independent virus introductions, presumably from China, and are compatible with a more global circulation of subgenogroup C4 enteroviruses. In 2013, the C4 subgenogroup also emerged in Russia, where it was associated with an outbreak of 78 reported cases, including 1 fatal case of meningoencephalitis (*14*).

Many cases of fatal encephalitis have been associated with EV-A71 C4 infection outbreaks in China (*6*), which highlights the neurovirulence of EV-A71 strains. Rare acute flaccid paralysis cases have also been reported in Australia through the national poliomyelitis surveillance program (*15*). Although the prevalence of neurologic cases associated with EV-A71 infection is currently low in Europe, the recent circulation of EV-A71 C4 in France and in Rostov, Russia (along the eastern border with Europe), underscores the need for improved surveillance of neurologic manifestations associated with EV infection and of the incidence of HFMD within communities. In addition, careful monitoring for the possible introduction and circulation of new EV-A71 genogroups and subgenogroups should be conducted.

Acknowledgments

We are grateful to Laurence Josset for critical review of the manuscript and revision of the English. We also acknowledge Delphine Falcon and Katy Pinet for their technical contributions.

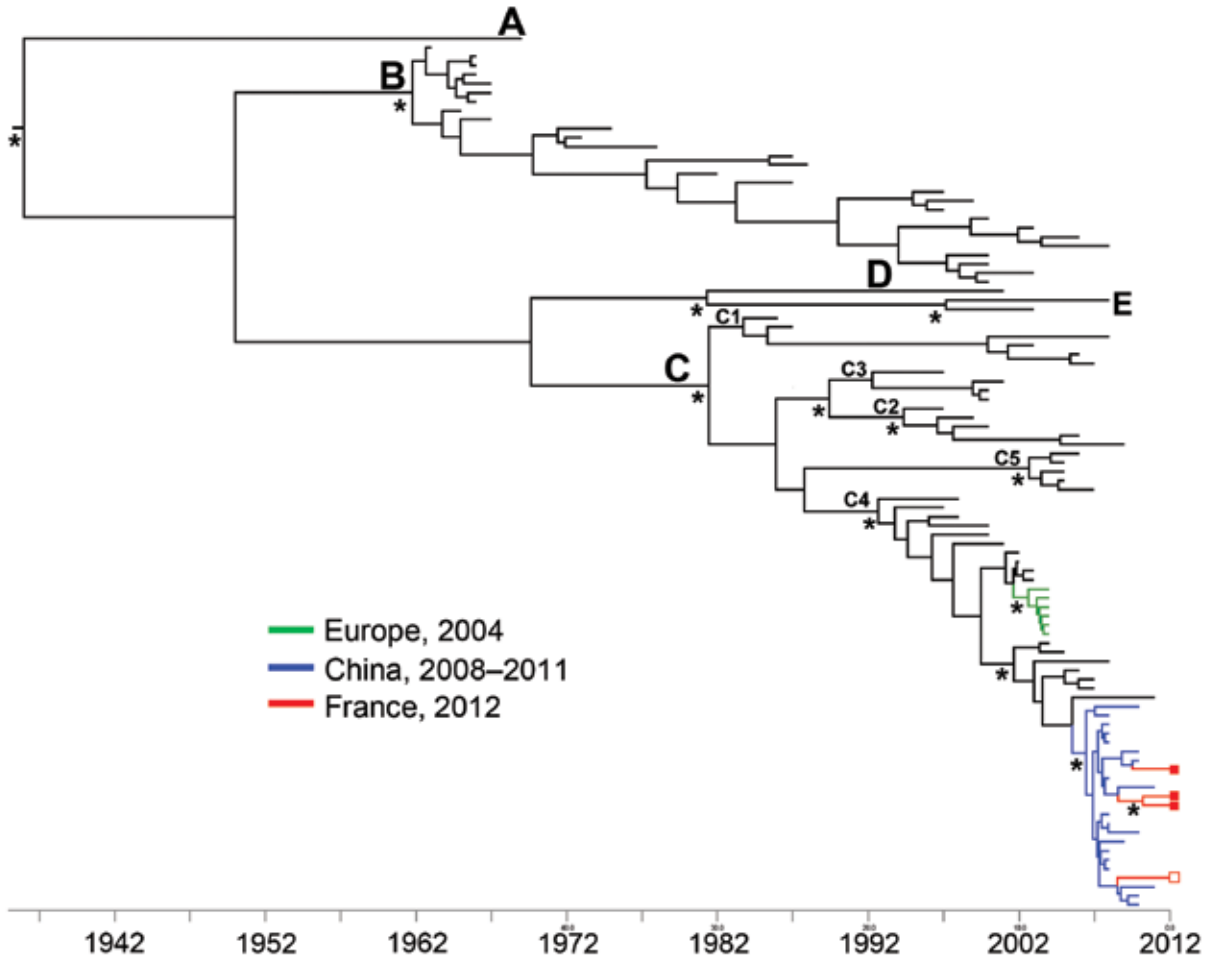


Figure. Dated phylogeny inferred by using 97 enterovirus 71 (EV-A71) 1D gene sequences encoding the VP1 capsid proteins ($1D^{VP1}$). The dataset included the 4 sequences determined in this study; 37 sequences from EV-A71 C4 strains detected in Austria, China, Korea, France, Germany, Japan, and Taiwan during 1998–2011; and 57 sequences from prototype and clinical strains representative of the genogroups and subgenogroups A, B1–B5, C1–C5. The tree topology shows the relationships between the strains isolated in France during 2012 and the strains circulating in China. The *x*-axis represents sampling years. The phylogenetic relationships were inferred with complete $1D^{VP1}$ gene sequences (891 nt) by using a Bayesian method (BEAST software; http://beast.bio.ed.ac.uk). The tree was reconstructed using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree). Asterisks at key nodes indicate posterior probability values ≥0.99. Red indicates the $1D^{VP1}$ sequences determined in this study: complete sequences (GenBank accession nos. KF900159–61) are represented by closed squares on the right side of the figure; a partial sequence (298 nt) (GenBank accession no. KF900162) is represented by an open square). Green indicates the $1D^{VP1}$ sequences from EV-A71 C4 strains detected in France, Germany, and Austria in 2004. Blue indicates the $1D^{VP1}$ sequences from EV-A71 C4 strains detected in China during 2008–2011. Letters A–E indicate genogroups; C1–C5 indicate subgenogroups.

Dr Schuffenecker is a virologist working at the National Reference Center for Enterovirus and Parechovirus. She is involved in diagnosis and surveillance of enterovirus and parechovirus infections.

References

1. Solomon T, Lewthwaite P, Perera D, Cardosa MJ, McMinn P, Ooi MH. Virology, epidemiology, pathogenesis, and control of enterovirus 71. Lancet Infect Dis. 2010;10:778–90. http://dx.doi.org/10.1016/S1473-3099(10)70194-8
2. Deshpande JM, Nadkarni SS, Francis PP. Enterovirus 71 isolated from a case of acute flaccid paralysis in India represents a new genotype. Curr Sci. 2003;84:1350–3.
3. Bessaud M, Pillet S, Ibrahim W, Joffret ML, Pozzetto B, Delpeyroux F, et al. Molecular characterization of human enteroviruses in the Central African Republic: uncovering of wide diversity and identification of a new human enterovirus A71 genogroup. J Clin Microbiol. 2012;50:1650–8. http://dx.doi.org/10.1128/JCM.06657-11
4. Bessaud M, Razafindratsimandresy R, Nougairede A, Joffret ML, Deshpande JM, Dubot-Peres A, et al. Molecular comparison and evolutionary analyses of VP1 nucleotide sequences of new African human enterovirus 71 isolates reveal a wide genetic diversity. PLoS ONE. 2014;9:e90624. http://dx.doi.org/10.1371/journal.pone.0090624
5. Yip CCY, Lau SKP, Woo PCY, Yuen K-Y. Human enterovirus 71 epidemics: what's next? Emerg Health Threats J. 2013;6:19780–97.

6. Zhang Y, Tan X, Cui A, Mao N, Xu S, Zhu Z, et al. Complete genome analysis of the C4 subgenotype strains of enterovirus 71: predominant recombination C4 viruses circulating in China for 14 years. PLoS ONE. 2013;8:e56341. http://dx.doi.org/10.1371/journal.pone.0056341
7. van der Sanden S, van der Avoort H, Lemey P, Uslu G, Koopmans M. Evolutionary trajectory of the VP1 gene of human enterovirus 71 genogroup B and C viruses. J Gen Virol. 2010;91:1949–58. http://dx.doi.org/10.1099/vir.0.019695-0
8. Mirand A, Schuffenecker I, Henquell C, Billaud G, Jugie G, Falcon D, et al. Phylogenetic evidence for a recent spread of two populations of human enterovirus 71 in European countries. J Gen Virol. 2010;91:2263–77. http://dx.doi.org/10.1099/vir.0.021741-0
9. Schuffenecker I, Mirand A, Antona D, Henquell C, Chomel JJ, Archimbaud C, et al. Epidemiology of human enterovirus 71 infections in France, 2000–2009. J Clin Virol. 2011;50:50–6. http://dx.doi.org/10.1016/j.jcv.2010.09.019
10. Ortner B, Huang CW, Schmid D, Mutz I, Wewalka G, Allerberger F, et al. Epidemiology of enterovirus types causing neurological diseases in Austria 1999–2007: detection of clusters of echovirus 30 and enterovirus 71 and analysis of prevalent genotypes. J Med Virol. 2009;81:317–24. http://dx.doi.org/10.1002/jmv.21374
11. Diedrich S, Weinbrecht A, Schreier E. Seroprevalence and molecular epidemiology of enterovirus 71 in Germany. Arch Virol. 2009;154:1139–42. http://dx.doi.org/10.1007/s00705-009-0413-x
12. Mirand A, Henquell C, Archimbaud C, Ughetto S, Antona D, Bailly J-L, et al. Outbreak of hand, foot and mouth disease/ herpangina associated with coxsackievirus A6 and A10 infections in 2010, France: a large citywide, prospective observational study. Clin Microbiol Infect. 2012;18:E110–8. http://dx.doi.org/10.1111/j.1469-0691.2012.03789.x
13. Kassab S, Saghi T, Boyer A, Lafon M-E, Gruson D, Lina B, et al. A fatal case of enterovirus 71 infection complicating rituximab therapy, France, 2012. Emerg Infect Dis. 2013;19:1345–7. http://dx.doi.org/10.3201/eid1908.130202
14. Akhmadishina LV, Eremeeva TP, Trotsenko OE, Ivanova OE, Mikhailov MI, Lukashev AN. Seroepidemiology and molecular epidemiology of enterovirus 71 in Russia. PLoS ONE. 2014;9:e97404. http://dx.doi.org/10.1371/journal.pone.0097404
15. Thorley B. Detection of enterovirus 71 sub-genogroup C4a in specimens from cases of acute flaccid paralysis, Australia. ProMed. 2013 May 26 [cited 2013 Dec 1]. http://www.promedmail.org, archive no. 20130526.1738087.

Address for correspondence: Isabelle Schuffenecker, Centre National de Référence des Enterovirus et Parechovirus, Laboratoire de Virologie, Groupement Hospitalier Est, 59 Bd Pinel, 69677 Bron, France; email: isabelle.schuffenecker@chu-lyon.fr

Rapid Detection, Complete Genome Sequencing, and Phylogenetic Analysis of Porcine Deltacoronavirus

Douglas Marthaler, Lindsey Raymond, Yin Jiang, James Collins, Kurt Rossow, and Albert Rovira

In February 2014, porcine deltacoronavirus (PDCoV) was identified in the United States. We developed a PDCoV real-time reverse transcription PCR that identified PDCoV in 30% of samples tested. Four additional PDCoV genomes from the United States were sequenced; these had ≈99%–100% nt similarity to the other US PDCoV strains.

Coronaviruses belonging to the *Coronavirinae* subfamily are divided into 3 genera, *Alphacoronavirus*, *Betacoronavirus*, and *Gammacoronavirus* (*1*). Woo et al. investigated the presence of coronaviruses in birds and mammals from Hong Kong and identified a new *Coronavirinae* genus, *Deltacoronavirus* (*2,3*). Of 169 swine samples tested, 10% were positive for porcine deltacoronavirus (PDCoV), and 2 complete PDCoV genomes were generated and analyzed (*3*).

On February 11, 2014, the Ohio Department of Agriculture officially announced the identification of PDCoV in the United States. Furthermore, the University of Minnesota Veterinary Diagnostic Laboratory (Saint Paul, MN, USA) and Iowa State University Veterinary Diagnostic Laboratory (Ames, IA, USA) sequenced a US PDCoV strain, which had an ≈99% nt identity to the 2 China PDCoV strains. In addition, the Ohio Department of Agriculture released 2 more complete PDCoV genomes from the United States (GenBank accession nos. KJ569769 and KJ462462), for a total of 4 complete PDCoV genomes (*4*,*5*). We designed a real-time reverse transcription PCR (rRT-PCR) to rapidly identify PDCoV, and 4 additional PDCoV strains were sequenced to further characterize PDCoV in the United States.

Author affiliation: University of Minnesota Veterinary Diagnostic Laboratory, Saint Paul, Minnesota, USA

DOI: http://dx.doi.org/10.3201/eid2008.14-0526

The Study

During January 6–February 27, 2014, we tested a total of 293 porcine samples—90 fecal swab samples, 75 fecal samples, 54 saliva samples, 52 intestinal homogenate samples, 2 vomit samples, 19 feed samples, and 1 environmental sample—from Ohio (108 samples), Michigan (63), Illinois (38), Minnesota (24), Nebraska (25), South Dakota (24), Missouri (3), and Canada (8) with the new PDCoV rRT-PCR (Table 1). The PDCoV rRT-PCR design,

Table 1. Characteristics and results of samples tested for PDCoV, United States, January 6–February 27, 2014*

Characteristic	Positive samples, no. (%)
Sample type tested	
Fecal swab, n = 90	15 (17)
Feces, n = 75	30 (40)
Saliva, n = 54	10 (19)
Intestines, n = 52	27 (52)
Feed, n = 19	6 (32)
Vomit, n = 2	1 (50)
Environment, n = 1	0
Total, n = 293	89 (30)
Location	
Ohio, n = 108	41 (38)
Illinois, n = 38	27 (71)
Minnesota, n = 24	7 (29)
Nebraska, n = 25	14 (56)
Michigan, n = 63	0
South Dakota, n = 24	0
Canada, n = 8	0
Missouri, n = 3	0
Total, n = 293	89 (30)
rRT-PCR results for PDCoV-positive samples	
Total, n = 89	
PEDV	29 (33)
RVA	35 (39)
RVB	33 (37)
RVC	52 (58)
PDCoV only	20 (22)
PDCoV + any co-infections	69 (78)
PDCoV + 1 pathogen	
PEDV	5 (19)
RVA	6 (22)
RVB	4 (15)
RVC	12 (44)
Total	27 (30)
PDCoV + 2 pathogens	
PEDV + RVA	0
PEDV + RVB	1 (5)
PEDV + RVC	3 (16)
RVA + RVB	0
RVA + RVC	9 (47)
RVB + RVC	6 (32)
Total	19 (21)
PDCoV + 3 pathogens	
PEDV + RVA + RVB	1 (13)
PEDV + RVA + RVC	1 (13)
PEDV + RVB + RVC	3 (38)
RVA + RVB + RVC	3 (38)
Total	8 (9)
PDCoV + 4 pathogens	
PEDV + RVA + RVB + RVC	15 (17)

*PDCoV, porcine deltacoronavirus; rRT-PCR, real-time reverse transcription PCR; PEDV, porcine epidemic diarrhea virus; RVA, rotavirus A; RVB, rotavirus B; RVC, rotavirus C.

Table 2. Nucleotide identities of porcine deltacoronavirus strains*

Strain	Nucleotide identity, %							
	Genome	ORF1	Spike	Envelope	Membrane	Nucleocapsid	NS6	NS7
HKU strains	99.1	99.1	98.8	100	99.4	99.6	98.9	99.3
HKU vs. US	98.9–99.2	98.9–99.4	98.5–98.8	99.2–99.6	98.9–99.2	98.8–99	98.9–100	98.8–99.0
US	99.9–100	99.9–100	99.7–100	99.6–100	99.8–100	99.4–100	99.3–100	99.2–100

*ORF, open reading frame; NS, nonstructural.

comparison, sensitivity, and specificity are described in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/20/8/14-0526-Techapp1.pdf). We selected samples for PDCoV testing in accordance with veterinarians' requests to investigate the presence of PDCoV in pigs with diarrhea. The samples were homogenized and the RNA extracted by using previously described methods (*6*,*7*). Of 293 porcine samples tested, 89 (30%) were PDCoV positive; we did not detect transmissible gastroenteritis virus (TGEV) in any samples tested (Table 1). Of the 89 PDCoV-positive samples, 20 samples (22% [11 fecal, 6 intestinal homogenate, 2 fecal swab, and 1 feed]) were negative for TGEV, porcine epidemic diarrhea virus (PEDV), rotavirus A (RVA), rotavirus B (RVB), and rotavirus C (RVC); 69 (78%) PDCoV-positive samples were positive for PEDV, RVA, RVB, or RVC. Co-infection with PDCoV and RVC were most common (52 [58%] samples). Although most (27 [30%]) PDCoV co-infections were with only 1 viral pathogen, 15 (17%) were positive for PEDV, RVA, RVB, and RVC.

We detected PDCoV in 52% of intestinal samples and 40% of fecal samples; 32% of feed samples and 19% of saliva samples tested positive for PDCoV. Of the 8 different locations tested for PDCoV, samples from Ohio (n = 41), Illinois (n = 27), Minnesota (n = 17), and Nebraska (n = 14) were positive for PDCoV; samples from Michigan, South Dakota, Missouri, and Canada were negative for PDCoV (Table 1).

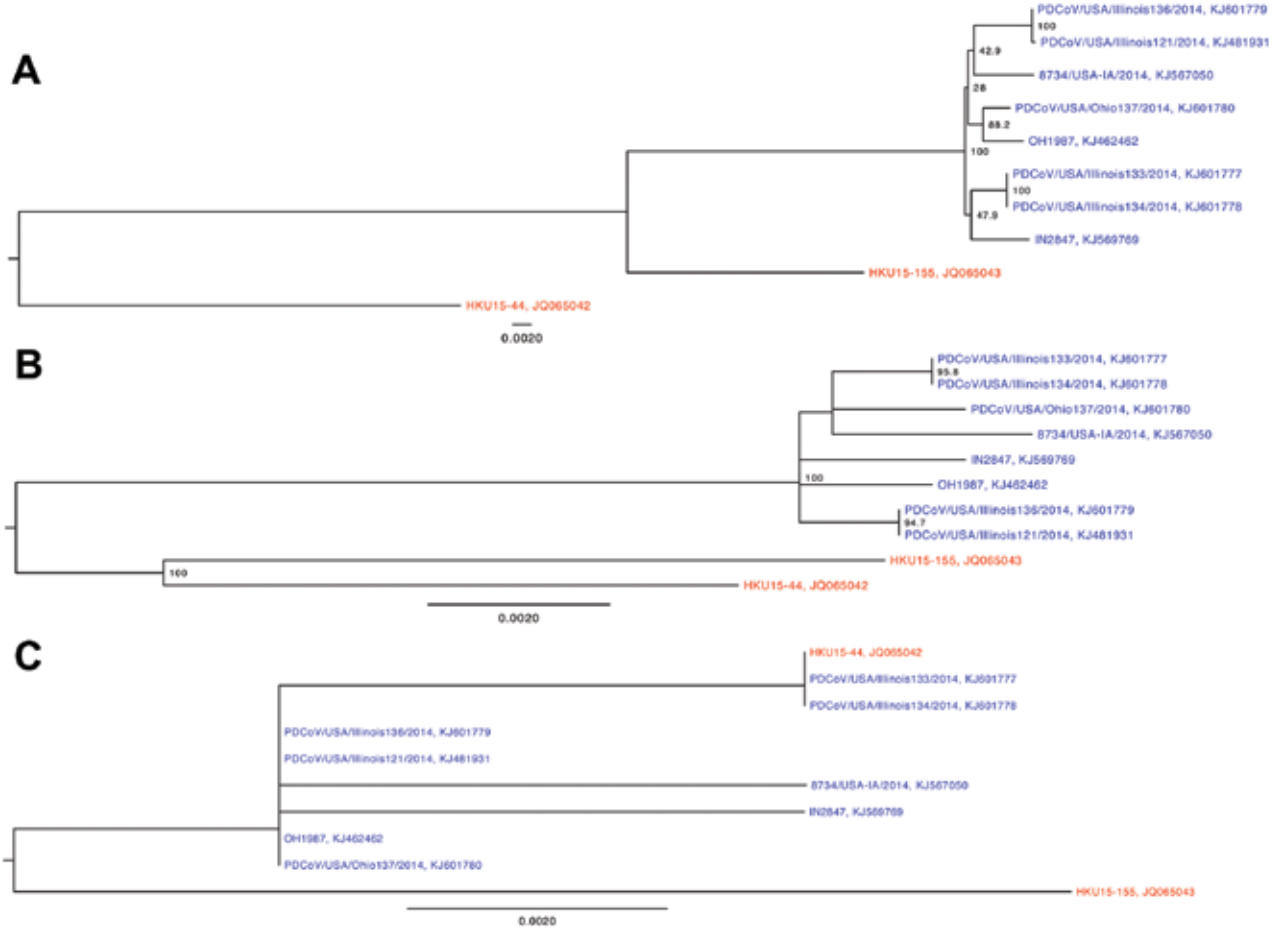


Figure. Phylogenetic trees of the complete porcine deltacoronavirus (PDCoV) genome (A), spike gene (B), and nonstructural protein 6 (NS6) accessory gene (C). US strains are in blue; China strains are in red. Bootstrap values >70% are illustrated. Scale bar indicates nucleotide substitutions per site.

From the PDCoV-positive samples, we selected 4 for complete genome sequencing (online Technical Appendix). The 8 US PDCoV complete genome sequences were 99.9%–100% nt identical to each other and 98.9%–100% nt identical to the China PDCoV strains (Table 2). The envelope and membrane gene segments were the most conserved and had a 100% nt identity, and the nonstructural (NS) 6 accessory gene had the lowest nucleotide identity (98.9%–100%) within the US strains. Compared with segments of the China strains, the envelope gene segment was the most conserved (99.6% nt identity), and the spike gene segment was the most diverse (98.5%–98.8% nt identity). The China PDCoV strain HKU15-155 contained two 3-nt deletions in the spike gene and 3′ untranslated region; the China strain HKU15 44 and US strains lacked this deletion. Inversely, PDCoV China strain HKU15–44 contained a nucleotide deletion in the 3′ untranslated region that was not present in the US PDCoV strains.

In the complete genome phylogenetic tree, the 8 US PDCoV strains clustered with China PDCoV strain HKU15-155 instead of HKU15-44 (Figure, panel A). With the open reading frame 1, spike, envelope, membrane, and nucleocapsid gene segments and NS7 accessory gene phylogenetic trees, the US PDCoV strains clustered separately from the China PDCoV strains (Figure, panel B). The phylogenetic tree for the NS6 accessory gene had a different clustering pattern from the China strains (Figure, panel C). China PDCoV strain HKU15-44 clustered with Illinois133 and Illinois134, and China PDCoV strain HKU15–155 clustered independently.

Conclusions

The PDCoV rRT-PCR is a fast and accurate detection method that can be used to diagnose PDCoV infection. Identification of PDCoV in 30% of samples tested indicates that PDCoV is a common viral pathogen of pigs in the midwestern United States. We identified positive PDCoV in 20 (22%) samples that were negative for TGEV, PEDV, RVA, RVB, and RVC, but PDCoV co-infections were more common (69 [78%] samples), especially with RVC (52 [58%]). Although the samples from Canada were negative for PDCoV, the Animal Health Laboratory has confirmed that 6 Ontario farms contain PDCoV. Because we selected samples on the basis of clinical diarrhea and geographic location was limited, the results do not accurately reflect the prevalence of PDCoV in North America. In addition, the presence of PDCoV RNA in feed does not indicate infectivity of the virus. The prevalence of PDCoV in North America is unknown, and the new PDCoV rRT-PCR can be used to access the prevalence in the United States and Canada.

Phylogenetic analysis of the US PDCoV strains indicates a common ancestor with the China PDCoV strains. The China PDCoV strains are the only available sequences, and we cannot state that the US PDCoV strains originated in China. Because little is known about PDCoV, the US PDCoV parental strain may never be discovered. The NS6 phylogenetic tree branched differently from the other PDCoV gene segments; therefore, the NS6 accessory gene may evolve differently from the other gene segments. Complete genomes of PDCoV from other countries are needed to increase understanding of the origin, phylogenetic relationship, and evolution of the US PDCoV strains.

The date that PDCoV was introduced into the United States is unknown. Because the sequenced PDCoV samples were from a similar geographic location, the 99.9%–100% nt identity does not correlate with the possible genetic diversity within the United States. An alternate hypothesis would indicate that PDCoV has been an undiagnosed pathogen of pigs in the United States and, like RVB, has been circulating there for an extended period (*6*) or that PDCoV might be a secondary infection to other enteric pathogens. PDCoV pathogenesis and retrospective surveillance studies are needed to answer these epidemiologic questions in the United States and to determine PDCoV prevalence worldwide.

In conclusion, a PDCoV rRT-PCR was designed to accurately detect PDCoV in a variety of samples. Complete genome analysis of the US PDCoV strains showed that they share 99.9%–100% nt identity and a common ancestor with the only available PDCoV sequences, the China PDCoV strains.

The University of Minnesota Veterinary Diagnostic Laboratory provided funding for this study.

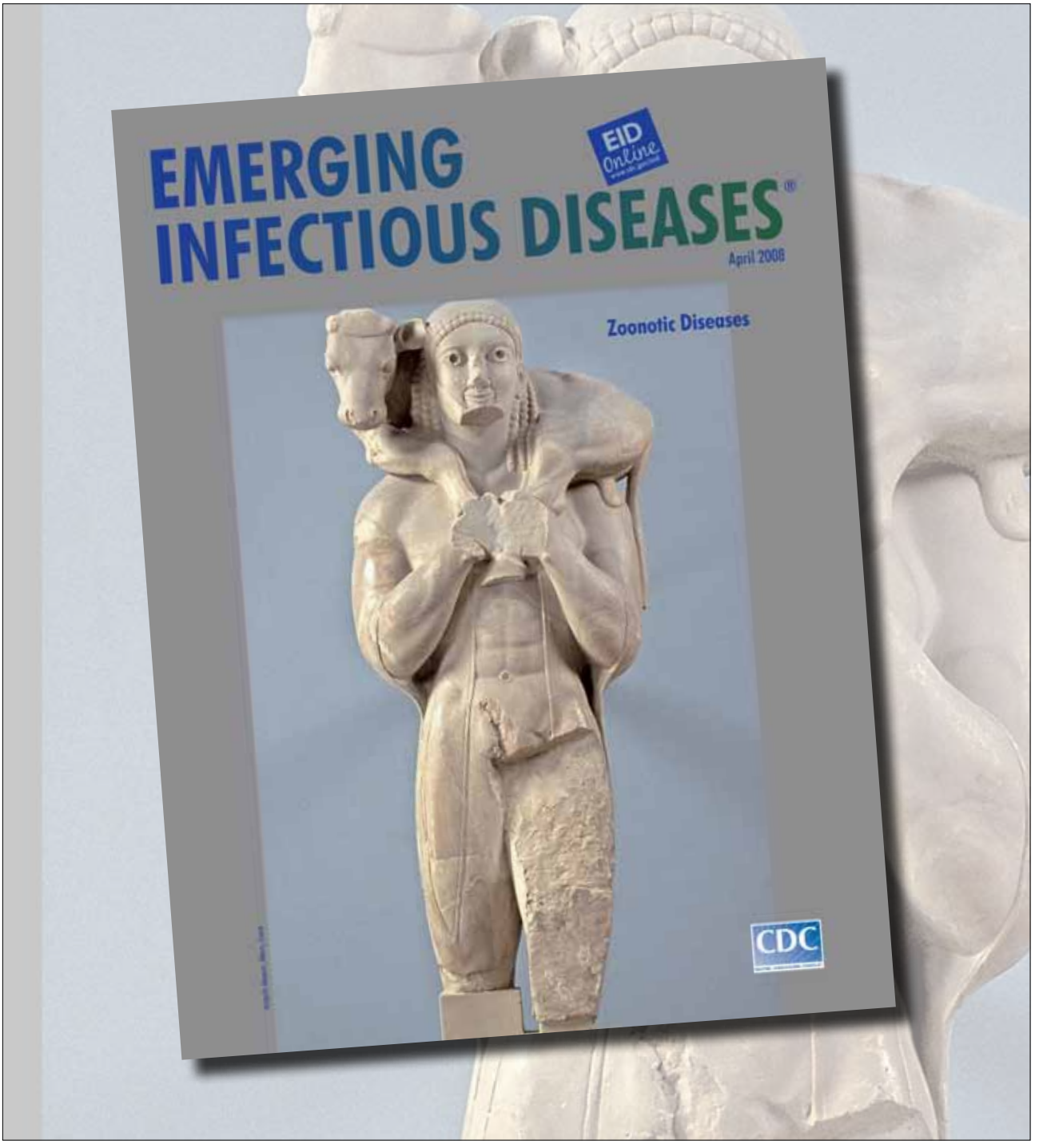
Dr Marthaler is a scientist at the University of Minnesota Veterinary Diagnostic Laboratory. His primary research interests are rotavirus and other pathogens of swine.

References

1. Masters P, Perlman S. Coronaviridae. In: Knipe D, Howley P, editors. Fields virology. 5th ed. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins; 2013. p. 825–58.
2. King A. Family *Coronaviridae*. In: Virus taxonomy. Ninth report of the International Committee on Taxonomy of Viruses. Oxford (UK): Elsevier; 2011. p. 806–28.
3. Woo PC, Lau SK, Lam CS, Lau CC, Tsang AK, Lau JH, et al. Discovery of seven novel mammalian and avian coronaviruses in the genus *Deltacoronavirus* supports bat coronaviruses as the gene source of *Alphacoronavirus* and *Betacoronavirus* and avian coronaviruses as the gene source of *Gammacoronavirus* and *Deltacoronavirus*. J Virol. 2012;86:3995–4008. http://dx.doi.org/10.1128/JVI.06540-11
4. Marthaler D, Jiang Y, Collins J, Rossow K. Complete genome sequence of strain SDCV/USA/Illinois121/2014, a porcine deltacoronavirus from the United States. Genome Announc. 2014;2:e00218–14. http://dx.doi.org/10.1128/genomeA.00218-14

5. Li G, Chen Q, Harmon KM, Yoon KJ, Schwartz KJ, Hoogland MJ, et al. Full-length genome sequence of porcine deltacoronavirus strain USA/IA/2014/8734. Genome Announc. 2014;2:e00278–14. http://dx.doi.org/10.1128/genomeA.00278-14
6. Marthaler D, Rossow K, Gramer M, Collins J, Goyal S, Tsunemitsu H, et al. Detection of substantial porcine group B rotavirus genetic diversity in the United States, resulting in a modified classification proposal for G genotypes. Virology. 2012;433:85–96. http://dx.doi.org/10.1016/j.virol.2012.07.006
7. Marthaler D, Rossow K, Culhane M, Collins J, Goyal S, Ciarlet M, et al. Identification, phylogenetic analysis and classification of porcine group C rotavirus VP7 sequences from the United States and Canada. Virology. 2013;446:189–98. http://dx.doi.org/10.1016/j.virol.2013.08.001

Address for correspondence: Douglas Marthaler, Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Minnesota, 1333 Gortner Ave, Saint Paul, MN 55108, USA; email: marth027@umn.edu



Co-circulation of Dengue and Chikungunya Viruses, Al Hudaydah, Yemen, 2012

Giovanni Rezza, Gamal El-Sawaf, Giovanni Faggioni, Fenicia Vescio, Ranya Al Ameri, Riccardo De Santis, Ghada Helaly, Alice Pomponi, Dalia Metwally, Massimo Fantini, Hussein Qadi, Massimo Ciccozzi, and Florigio Lista

We investigated 400 cases of dengue-like illness in persons hospitalized during an outbreak in Al Hudaydah, Yemen, in 2012. Overall, 116 dengue and 49 chikungunya cases were diagnosed. Dengue virus type 2 was the predominant serotype. The co-circulation of these viruses indicates that mosquitoborne infections represent a public health threat in Yemen.

Vectorborne infections are not uncommon in the Middle East (*1*). In particular, recurrent outbreaks of dengue fever have been reported on the Arabian Peninsula since 1990 (*2*). In Yemen, dengue virus (DENV) infections have reemerged with higher frequency during the last decade (*3*); in 2010, during a dengue outbreak that occurred in the southern governorate of Hadramout (*4*), cases of dengue hemorrhagic fever were identified (*5*). In 2010–2011, another mosquitoborne virus, the chikungunya virus (CHIKV), was detected in febrile patients in Al Hudaydah, Yemen (*6*). To evaluate to what extent these arboviruses are involved in dengue-like illness outbreaks in Yemen, we conducted a study in Al Hudaydah.

The Study

The study site was represented by 5 hospital centers (Renal Center, Maritime College, Al Rasheed, Al-Thawra, Al Salakhana) located in Al Hudaydah (Figure 1). Patients hospitalized during 2012 were recruited for the study if they had fever (>37.5°C) and at ≥2 of the following signs or symptoms at the time of admission: headache, joint pain, muscle pain, skin rash. Serum samples were collected within 4 days from the date of hospital admission and stored and shipped at -20°C.

We used the Maxwell 16 Viral Total Nucleic Acid Purification Kit with the Maxwell 16 instrument (Promega, Madison, WI, USA) according to the manufacturer's instructions to extract nucleic acids from the serum samples. We then analyzed the nucleic acids by using DENV- and CHIKV-specific PCR sequences, as previously described for DENV and adapted for CHIKV (*7*,*8*). To further confirm the results, we amplified and then sequenced *NS1* and *E1* genes from DENV- and/or CHIKV-positive serum samples; the sequences were deposited in GenBank (accession nos. KJ742803–19).

We used the NovaLisa Dengue IgM- and IgG-ELISAs and the NovaLisa Chikungunya IgM- and IgG-capture ELISAs (NovaTec lmmundiagnostica GmbH, Dietzenbach, Germany) according to the manufacturer's instructions to analyze serum samples for IgM and IgG. Study participants with IgM ELISA– and/or PCR-positive results were defined as recently or acutely infected with DENV or CHIKV.

Overall, 400 persons were enrolled in the study. The median age was 30 years (range 1–60). The median interval between fever onset and sample collection was 4 days (range 2–9).

Of the 400 study participants, 116 (29%) were IgM or PCR positive for DENV RNA. Of those 116 persons, 61 (53%) had IgM, 44 (38%) were positive by PCR, and 11 (9%) had IgM- and PCR-positive results. Of the 55 PCR-positive samples, 41 were DENV-2 and 2 DENV-1. The remaining 12 samples were not typed because the virus titer was low, and it was not possible to achieve a positive signal by using a DENV serotype–specific PCR. Of the 400 study participants, 290 (72.5%) had IgG against DENV. Their distribution by diagnostic category is shown in Table 1.

Of the 400 participants, 49 (12%) were IgM and/or PCR positive for CHIKV RNA: 38 (77%) were IgM positive, 10 (20%) were PCR positive, and 1 was positive by both methods. Of the 351 patients with negative IgM/PCR results, 33 (9.4%) had IgG against CHIKV.

No samples were PCR positive for both viruses. However, 13 samples had IgM against both viruses, and 1 had positive results for DENV by PCR and CHIKV IgM by ELISA.

The monthly distribution and the proportion of DENV- and CHIKV-positive cases are shown in Figure 2. Peaks were observed during February, when the highest number of chikungunya cases was observed, and especially May, when the highest number of dengue fever cases was

Author affiliations: Istituto Superiore di Sanità, Rome, Italy (G. Rezza, F. Vescio, M. Ciccozzi); Alexandria University, Alexandria, Egypt (G. El-Sawaf, G. Helaly, D. Metwally); Army Medical and Veterinary Research Center, Rome (G. Faggioni, R. De Santis, A. Pomponi, F. Lista); University of Sana'a, Yemen (R. Al Ameri, H. Qadi); and University of Rome Tor Vergata, Rome (M. Fantini)

DOI: http://dx.doi.org/10.3201/eid2008.131615

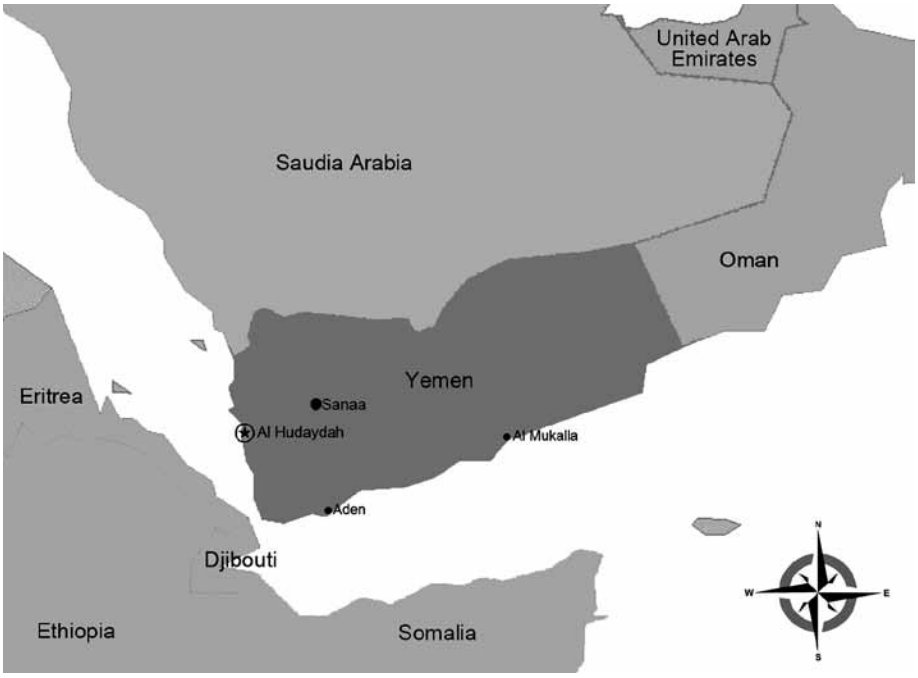


Figure 1. Location of Al Hudaydah, Yemen, where the co-circulation of dengue virus, chikungunya virus, and other dengue-like viruses was studied in 2012. Other important towns, Sanaa, Aden, and Al Mukalla (the capital of Hadramout governorate), are also shown.

observed. A low number of cases were reported in November and December.

Study participants with CHIKV infection (mean age 30.67 years, ± SD 14.17 years) were slightly younger than with DENV infection (mean age 27.53 years, ± standard deviation 17.34 years), but the median age (30 years) and range (1–60 years) were the same for the 2 groups. The distribution of signs and symptoms for recent/acute cases of dengue and chikungunya are shown in Table 2. The most common signs symptoms for both infections were joint pain (98%), myalgia (95% and 94%, respectively), and headache (94% and 88%, respectively). Persons with chikungunya were more likely than those with dengue to report vomiting (41% vs. 25%). No other major difference in the frequency of specific signs or symptoms was found.

Conclusions

Our data provide evidence of co-circulation of CHIKV and 2 DENV serotypes in the governorate of Al Hudaydah. DENV-2 was the predominant serotype in our study population. Whether this serotype was newly introduced into Yemen in 2012 is unknown. In 2011 in the same area, a small study was conducted with 47 patients with dengue-like illness, and only 3 were PCR positive for DENV: 2 for DENV-1 and 1 for DENV-3 (data not shown). In 2010 in Al Mukalla, the capital of the district of Hadramout, DENV-3 was also detected in patients with dengue fever and dengue hemorrhagic fever (*5*,*9*).

The high prevalence (>70%) of DENV IgG in our study population suggests past exposure to DENV. Relatively high rates of IgG were also found during the outbreak that occurred in 2011 in Hadramout, where 28% of DENV IgM–positive and 43% of DENV IgM–negative patients with dengue-like illness were positive for DENV IgG (*4*). The identification of dengue hemorrhagic fever cases caused by DENV-3 in Al Mukalla is also suggestive of exposure to different DENV serotypes (*5*). The hypothesis of continued reintroduction of different DENV serotypes in Yemen dates back to 1983, when a secondary heterotypic DENV infection was suspected in a man who had traveled from Dalah (160 miles from Aden), an area endemic for DENV. The traveler had dengue hemorrhagic fever and high antibody titers against all 4 DENV serotypes (*10*). It is well known that travels to and from DENV-endemic areas are common, and

Table 1. Dengue virus–positive study participants by diagnostic category, Al Hudaydah, Yemen, 2012*

	No. (%) participants by diagnostic category, infection history					
Study participants	IgM+/PCR–, recent infection	PCR+/IgM–, acute infection	IgM+/PCR+, acute infection	IgM+ and/or PCR+, acute or recent infection	IgM–/PCR–, no acute or recent infection	Total
Total†	61 (15.2)	44 (11.0)	11 (2.7)	116 (29.0)	284 (71.0)	400 (100.0)
Positive for dengue virus IgG‡	48 (78.7)	22 (50.0)	6 (54.5)	76 (65.5)	214 (75.3)	290 (72.5)

*+, positive; –, negative.
†The denominator is represented by the total study population.
‡The denominator is represented by the number of individuals in each category.

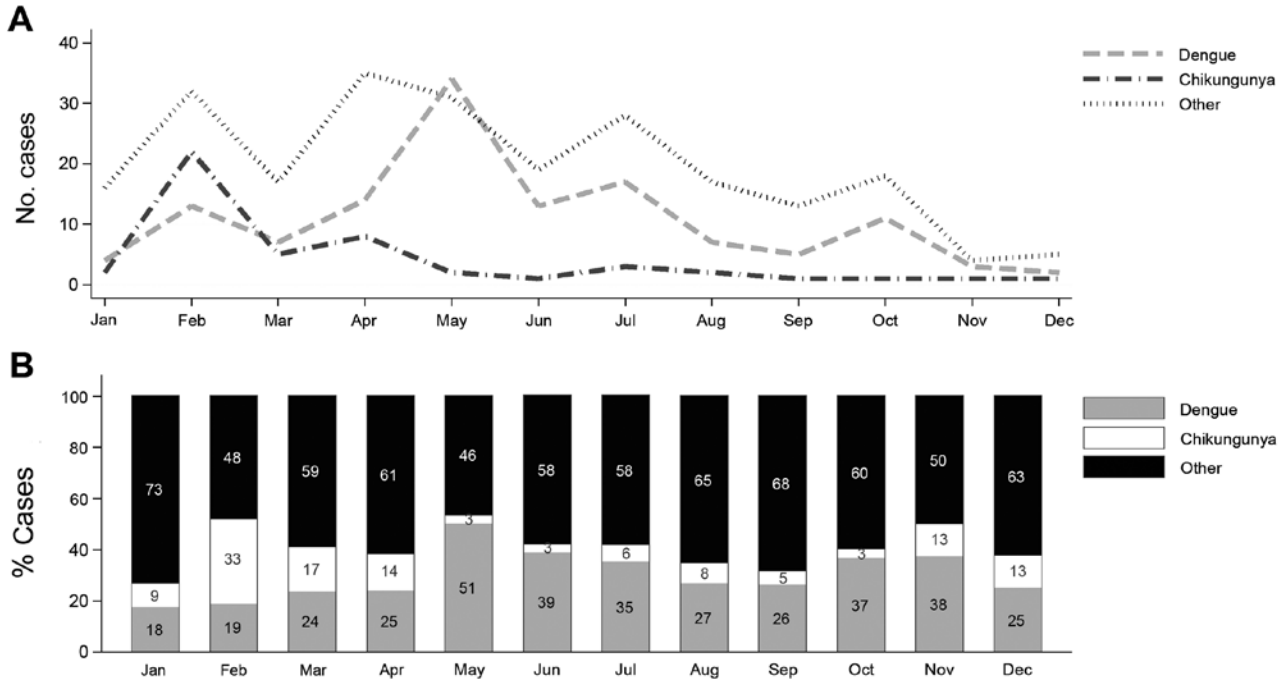


Figure 2. Trends for cases of dengue virus, chikungunya virus, and other dengue-like viruses, Al Hudaydah, Yemen, 2012. A) Number of cases by month. B) Monthly percentages of cases by virus type.

imported cases from eastern Africa (Zanzibar) were reported in Yemen as early as the nineteenth century (*11*,*12*). Circulation of different DENV serotypes has also been reported in Saudi Arabia: DENV-3 emerged as the predominant serotype after 1997, and DENV-2 and DENV-1 serotypes had been associated with earlier outbreaks (*13*).

We identified a rather large number of cases of recent or acute CHIKV infection. This finding is consistent with a previous estimate of 1,542 cases during October 2010–January 2011 (*6*) and with the detection of CHIKV RNA in *Aedes aegypti* mosquitoes, the dominant type of mosquito in entomologic investigations conducted in Al Hudaydah (*14*).

The circulation of mosquitoborne viruses in a dry area like Yemen is not surprising. Water scarcity and lack of infrastructure in periurban areas require regular storage of water for household and potable use, and water containers favor *A. aegypti* mosquito reproduction. Moreover, increasing migration and urbanization may favor the introduction and spread of these infections (*15*).

Our study did have some limitations. First, the study was hospital based. Thus, the findings may not be representative of the whole epidemic in the community. Second, some misclassification of the cases caused by the sensitivity and specificity of the tests or to a time lag between symptom onset and sample collection (i.e., PCR- or IgM-negative results caused by testing intervals that were too long or too short, respectively) cannot be ruled out. Last, the prevalence of IgG against DENV might have been overestimated because of possible cross-reactivity with other flavivirus infections.

Table 2. Clinical characteristics of study participants with dengue and chikungunya, Al Hudaydah, Yemen, 2012

	Participants with signs or symptoms of dengue, n = 116		Participants with signs or symptoms of chikungunya, n = 49				
Sign or symptom	No. (%)	SE	No. (%)	SE	Difference*	SE	p value
Fever	116 (100)	0	49 (100)	0	0		
Arthralgia	114 (98)	0.01	48 (98)	0.02	0	0.02	0.890
Myalgia	110 (95)	0.02	46 (94)	0.03	0.01	0.04	0.807
Headache	109 (94)	0.02	43 (88)	0.05	0.06	0.05	0.178
Abdominal pain	93 (80)	0.04	35 (71)	0.07	0.09	0.07	0.221
Backbone pain	85 (73)	0.04	32 (65)	0.07	0.08	0.08	0.306
Watery diarrhea	56 (48)	0.05	27 (55)	0.07	–0.07	0.09	0.426
Rash	35 (30)	0.04	13 (27)	0.06	0.04	0.08	0.640
Vomiting	29 (25)	0.04	20 (41)	0.07	–0.16	0.08	0.042

*Difference between proportions.

In conclusion, CHIKV and various DENV serotypes co-circulate in Yemen's port city of Al Hudaydah. The detection of CHIKV and DENV IgG–positive persons suggests that these viruses are either endemic or continuously reintroduced to the area. Mosquito control activities are needed to reduce the effect of arbovirus infections on public health.

Acknowledgment

We thank Anna Carinci for editorial assistance in the revision of the manuscript.

Dr Rezza is an infectious diseases epidemiologist and currently Head of the Department of Infectious, Parasitic and Immunomediated Diseases of the Italian National Institute of Health. He is an expert in the field of emerging infections, with particular regard to viral infections, such as HIV, human herpesvirus type 8, pandemic influenza, and arboviruses.

References

1. Hotez PJ, Savioli L, Fenwick A. Neglected tropical diseases of the Middle East and North Africa: review of their prevalence, distribution, and opportunities for control. PLoS Negl Trop Dis. 2012;6:e1475. http://dx.doi.org/10.1371/journal.pntd.0001475
2. Ooi EE, Gubler DJ. Dengue and dengue hemorrhagic fever. In: Guerrant RL, Walker DH, Weller PF, editors. Tropical infectious diseases: principles, pathogens and practice. 3rd ed. Toronto: Saunders Elsevier; 2011.
3. World Health Organization and the Special Programme for Research and Training in Tropical Diseases. Dengue: guidelines for diagnosis, treatment, prevention and control: new edition. Geneva: The Organization; 2009.
4. Bin Ghouth AS, Amarasingh A, Letson W. Dengue outbreak in Hadramout, Yemen, 2010: an epidemiological perspective. Am J Trop Med Hyg. 2012;86:1072–6. http://dx.doi.org/10.4269/ajtmh.2012.11-0723
5. Madani TA, Abuelzein ETM, Al-Bar HM, Azhar EI, Kao M, Alshoeb HO, et al. Outbreak of viral hemorrhagic fever caused by dengue virus type 3 in Al-Mukalla, Yemen. BMC lnfect Dis. 2013;13:136.
6. EpiSouth Network. EpiSouth Weekly Epi Bulletin (e-WEB), no. 155. Report of new health events occurring outside the EpiSouth area (not occurring in one or several EpiSouth countries): chikungunya, Yemen; 2011 Mar 10 [cited 2013 Aug 21]. http://www.episouthnetwork.org/content/episouth-weekly-epi-bulletin-e-web
7. Leparc-Goffart I, Baragatti M, Temmam S, Tuiskunen A, Moureau G, Charrel R, et al. Development and validation of real-time one-step reverse transcription-PCR for the detection and typing of dengue viruses. J Clin Virol. 2009;45:61–6. http://dx.doi.org/10.1016/j.jcv.2009.02.010
8. Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African chikungunya viruses. J Virol Methods. 2005;124:65–71. http://dx.doi.org/10.1016/j.jviromet.2004.11.002
9. Ravanini P, Huhtamo E, Hasu E, Rosa F, Costantino S, Crobu MG, et al. lmported dengue virus serotype 3, Yemen to Italy, 2010. Emerg Infect Dis. 2011;17:929–31. http://dx.doi.org/10.3201/eid1705.101626
10. Jimenez-Lucho VE, Fisher EJ, Saravolatz LD. Dengue with hemorrhagic manifestations: an imported case from the Middle East. Am J Trop Med Hyg. 1984;33:650–3.
11. Christie CJ. Dengue. BMJ. 1872;2:244.
12. Radcliffe JN. Dengue. BMJ. 1877;1:25–6.
13. Shibl A, Senok A, Memish Z. Infectious diseases in the Arabian Peninsula and Egypt. Clin Microbiol Infect. 2012;18:1068–80. http://dx.doi.org/10.1111/1469-0691.12010
14. Zayed A, Awash AA, Esmal MA, Al-Mohamadi HA, Al-Salwai M, Al-Jasari A, et al. Detection of chikungunya virus in *Aedes aegypti* during the 2011 outbreak in Al Hodayda, Yemen. Acta Trop. 2012;123:62–6. http://dx.doi.org/10.1016/j.actatropica.2012.03.004
15. Amarasinghe A, Letson GW. Dengue in the Middle East: a neglected, emerging disease of importance. Trans R Soc Trop Med Hyg. 2012;106:1–2. http://dx.doi.org/10.1016/j.trstmh.2011.08.014

Address for correspondence: Giovanni Rezza, Department of Infectious, Parasitic and Immunomediated Diseases, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161 Roma, Italy; email: giovanni.rezza@iss.it

Antibodies against Severe Fever with Thrombocytopenia Syndrome Virus in Healthy Persons, China, 2013

Lei Zhang,[1] Jimin Sun,[1] Jie Yan, Huakun Lv, Chengliang Chai, Yi Sun, Bin Shao, Jianmin Jiang, Zhiping Chen, Jeroen Kortekaas, and Yanjun Zhang

In June 2013, a subclinical infection with severe fever with thrombocytopenia syndrome virus (SFTSV) was detected in Zhejiang Province, China, prompting seroprevalence studies in 6 districts within the province. Of 986 healthy persons tested, 71 had IgG antibodies against SFTSV. This finding suggests that most natural infections with SFTSV are mild or subclinical.

Severe fever with thrombocytopenia syndrome (SFTS), a serious emerging infectious disease, was first reported in rural areas of central China in 2009 (*1*). The characteristic signs and symptoms of SFTS include fever, thrombocytopenia, and leukocytopenia, and the disease has a case-fatality rate of up to 30%. SFTS is caused by infection with SFTS virus (SFTSV; family *Bunyaviridae*, genus *Phlebovirus*). The virus was first isolated in 2010 from patients with SFTS (*1*); since then, additional cases have been reported from many areas of China (*2*,*3*). After the occurrence of SFTS cases in Zhejiang Province, China, in 2013, enhanced surveillance for the disease was implemented (*4*). We report on the first human case of SFTS in a rural area of Pujiang district in Zhejiang Province and on an apparently associated mild or subclinical case of SFTSV infection in a family member of the patient. In addition, to determine if other mild or subclinical infections had occurred, we conducted seroprevalence studies in the patient's village and 5 other Zhejiang Province districts. The study was approved by the Ethics Committee of the Zhejiang Provincial Center for Disease Control and Prevention.

Author affiliations: Zhejiang Provincial Center for Disease Control and Prevention, Hangzhou, China (L. Zhang, J. Sun, H. Lv, C. Chai, Y. Sun, B. Shao, J. Jiang, Z. Chen, Y. Zhang); Central Veterinary Institute, part of Wageningen University and Research Centre, Lelystad, the Netherlands (J. Kortekaas); and Zhejiang University School of Medicine, Hangzhou (J. Yan)

DOI: http://dx.doi.org/10.3201/eid2008.131796

The Study

A human case of SFTS was identified in a rural area of the Pujiang district (Figure 1). The patient, a 60-year-old local male subsistence farmer, sought treatment at the Zhejiang Provincial People's Hospital on June 1, 2012, after a 6-day history of fever (maximum axillary temperature 40°C), malaise, chills, gingival bleeding, hyperemia of conjunctivae, and diarrhea (10 or fewer times per day). Initial laboratory testing revealed thrombocytopenia (13×10^9 platelets/L; reference range $100–300 \times 10^9$ platelets/L) and leukocytopenia (0.93×10^9 leukocytes/L; reference range $4–10 \times 10^9$/L). Supportive therapy was provided, and the patient's condition seemed to improve on the second day: platelet count rose to 34×10^9 platelets/L, and leukocyte count rose to 7.28×10^9 leukocytes/L. However, on the third day, the patient became weak and died of multiple organ failure.

Serum samples from the patient were tested for the presence of SFTSV RNA by quantitative real-time reverse transcription PCR as previously described (*1*). The QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) was used for RNA extraction. Detection of all 3 viral RNA segments by quantitative real-time reverse transcription PCR and isolation of the virus from Vero cell culture confirmed the association between the clinical syndrome and SFTSV infection. In addition, SFTSV-specific IgG and low levels of viral RNA were detected in a blood sample from a family member of the patient. The family member did not report exposure to potential animal hosts or vectors, so SFTSV transmission is believed to have occurred through personal contact when the family member was caring for the patient. Signs of illness did not develop in the family member.

To investigate if additional mild subclinical infections occurred, we, with the support of the local disease control department, conducted a seroprevalence study in the patient's village in Pujiang district. A total of 54 blood samples were collected from 54 healthy volunteers. We used an ELISA kit provided by the National Center for Disease Control and Prevention to prepare and test serum samples for the presence of SFTSV-specific IgM and IgG (*5*,*6*). This ELISA compares well with serum neutralization assays for SFTSV (*6*). All serum samples were negative for SFTSV-specific IgM, whereas 4 (7.4%) of the serum samples were positive for SFTSV-specific IgG (Figure 2). None of the IgG-positive participants reported any disease symptoms that are associated with SFTSV infections.

To further investigate the occurrence of mild or subclinical SFTSV infections, we collected serum samples

[1]These authors contributed equally to this article.

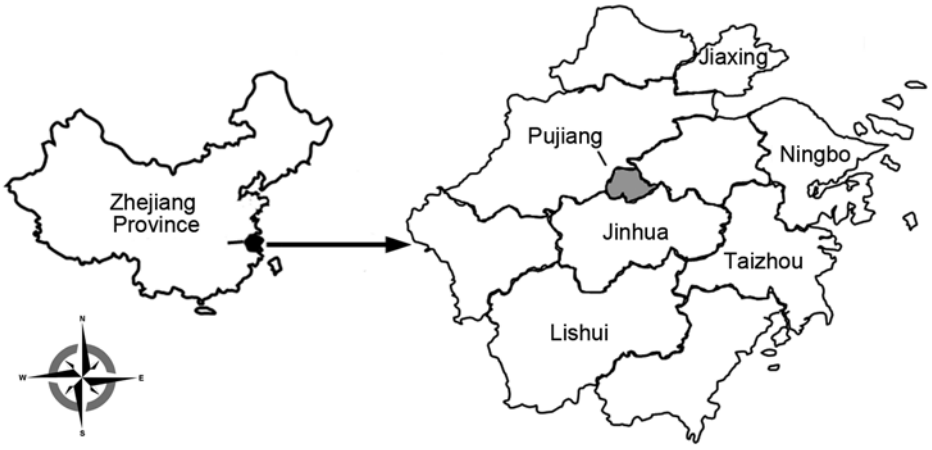


Figure 1. Location of Zhejiang Province in China (left) and the location of selected districts (right) within the province where serum samples of healthy persons were collected and tested in 2013 for the presence of severe fever with thrombocytopenia syndrome virus–specific IgG and IgM.

from healthy volunteers in 5 additional districts in Zhejiang Province and tested the samples for SFTSV-specific IgG. The percentages of positive samples, by district, follow (the no. positive/total no. tested are shown in parentheses): Lishui, 10.2% (18/176); Jinhua, 3.5% (7/200); Ningbo, 10.9 (28/256); Taizhou, 3% (3/100); and Jiaxing, 5.5% (11/200) (Figure 2). Results were confirmed by immunofluorescence assay conducted as previously reported (*1*). In brief, SFTSV-infected Vero cells were fixed with cold acetone, washed with distilled water, air-dried, and then stored at −70°C. Serum samples were diluted 1:20 in phosphate-buffered saline supplemented with 0.01% Evans blue (Sigma-Aldrich Co. LLC, St. Louis, MO, USA). Slides were then incubated with the diluted serum samples in a humidified chamber at 37°C for 30 min and then washed with phosphate-buffered saline. The wells were incubated with fluorescein isothiocyanate–labeled goat anti–human IgG conjugate (Boshide, Wuhan, China), washed, and analyzed by the use of fluorescence microscopy. The results of these experiments confirmed the presence of SFTSV-specific IgG in 84.5% (60/71) of the samples with ELISA-positive results. Serum samples that were negative for SFTSV by ELISA were also negative by immunofluorescence assay.

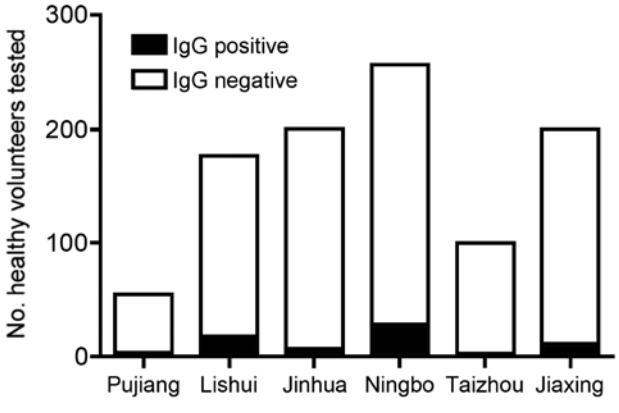


Figure 2. Seroprevalence of IgG to severe fever with thrombocytopenia syndrome virus in healthy persons from selected districts in Zhejiang Province, China, 2013.

Conclusions

SFTSV can cause severe disease and high rates of death among infected hospitalized patients. The virus also has the limited ability to be transmited from person to person through contact with contaminated blood, but secondary cases are generally less severe and have so far not resulted in fatalities (*7*–*9*). Nonetheless, there is great public health concern regarding SFTSV.

Our seroprevalence study was prompted by the identification of a subclinical, secondary infection that was most likely caused by person-to-person transmission of the virus from an infected family member with a fatal case of SFTS. We found an overall SFTSV seroprevalence of 7.2% among 986 healthy persons who reported no symptoms associated with SFTS. Because the seropositive participants in our study did not have contact with persons with diagnosed cases of SFTS, their infections most likely occurred through natural exposure. From this, we conclude that SFTSV infections are widespread in rural areas of Zhejiang Province, and only a small percentage of the infections result in clinical disease.

Acknowledgments

We thank staff members at the Provincial Centers for Disease Control and Prevention in Lishui, Jiaxing, Ningbo, Taizhou, Jinhua, and Pujiang districts for collecting serum samples from volunteers. We thank Quanfu Zhang for providing the slides for immunofluorescence.

This work was supported by research funds from Science and Technology Department of Zhejiang (grant no. 2012C13016-2), Zhejiang Provincial Natural Science Funding (grant no. LY14H260005) and Zhejiang Provincial Program for the Cultivation of High-Level Innovative Health Talents.

Mr Zhang is an SFTSV researcher in the Zhejiang Provincial Center for Disease Control and Prevention. His research interest is the epidemiology and pathogenesis of viral infections.

References

1. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 2011;364:1523–32. http://dx.doi.org/10.1056/NEJMoa1010095
2. Bao CJ, Guo XL, Qi X, Hu JL, Zhou MH, Varma JK, et al. A family cluster of infections by a newly recognized bunyavirus in eastern China, 2007: further evidence of person-to-person transmission. Clin Infect Dis. 2011;53:1208–14. http://dx.doi.org/10.1093/cid/cir732
3. Wen HL, Zhao L, Zhai S, Chi Y, Cui F, Wang D, et al. Severe fever with thrombocytopenia syndrome, Shandong Province, China, 2011. Emerg Infect Dis. 2014;20:1–5. http://dx.doi.org/10.3201/eid2001.120532
4. Zhang L, Wang X, Zhang Y, Zhang Y, Wang H, Wang C, et al. Severe fever with thrombocytopenia syndrome bunyavirus (SFTSV) infections in Zhejiang Province, China. Int J Infect Dis. 2013;17:e137–8. http://dx.doi.org/10.1016/j.ijid.2012.07.007
5. Niu G, Li J, Liang M, Jiang X, Jiang M, Yin H, et al. Severe fever with thrombocytopenia syndrome virus among domesticated animals, China. Emerg Infect Dis. 2013;19:756–63.
6. Jiao Y, Zeng X, Guo X, Qi X, Zhang X, Shi Z, et al. Preparation and evaluation of recombinant severe fever with thrombocytopenia syndrome virus nucleocapsid protein for detection of total antibodies in human and animal sera by double-antigen sandwich enzyme-linked immunosorbent assay. J Clin Microbiol. 2012;50:372–7. http://dx.doi.org/10.1128/JCM.01319-11
7. Gai Z, Liang M, Zhang Y, Zhang S, Jin C, Wang SW, et al. Person-to-person transmission of severe fever with thrombocytopenia syndrome bunyavirus through blood contact. Clin Infect Dis. 2012;54:249–52. http://dx.doi.org/10.1093/cid/cir776
8. Tang X, Wu W, Wang H, Du Y, Liu L, Kang K, et al. Human-to-human transmission of severe fever with thrombocytopenia syndrome bunyavirus through contact with infectious blood. J Infect Dis. 2013;207:736–9. http://dx.doi.org/10.1093/infdis/jis748
9. Chen H, Hu K, Zou J, Xiao J. A cluster of cases of human-to-human transmission caused by severe fever with thrombocytopenia syndrome bunyavirus. Int J Infect Dis. 2013;17:e206–8. http://dx.doi.org/10.1016/j.ijid.2012.11.006

Addresses for correspondence: Jeroen Kortekaas, Department of Virology, Central Veterinary Institute of Wageningen University Research Centre, Edelhertweg 15, 8219 PH Lelystad, the Netherlands; email: jeroen.kortekaas@wur.nl or Yanjun Zhang, Zhejiang Provincial Center for Disease Control and Prevention, 3399 Binsheng Rd, Hangzhou, Zhejiang 310051, P.R. China; email: yjzhang@cdc.zj.cn

Severe Fever with Thrombocytopenia Syndrome Virus in Ticks Collected from Humans, South Korea, 2013

Seok-Min Yun,[1] Wook-Gyo Lee,[1] Jungsang Ryou, Sung-Chan Yang, Sun-Whan Park, Jong Yeol Roh, Ye-Ji Lee, Chan Park, and Myung Guk Han

We investigated the infection rate for severe fever with thrombocytopenia syndrome virus (SFTSV) among ticks collected from humans during May–October 2013 in South Korea. *Haemaphysalis longicornis* ticks have been considered the SFTSV vector. However, we detected the virus in *H. longicornis*, *Amblyomma testudinarium*, and *Ixodes nipponensis* ticks, indicating additional potential SFTSV vectors.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging disease characterized by fever and thrombocytopenia. The syndrome is caused by SFTS virus (SFTSV), a member of the family *Bunyaviridae*, genus *Phlebovirus* (*1*). SFTSV is related to, but distinctly different from, Heartland viruses, which were isolated in the United States (*2*).

The first case of SFTS was reported in China during 2010 (*1*), and in 2013, SFTSV infections were reported in South Korea and Japan (*3*–*5*). In South Korea, the first human case of SFTS was confirmed in May, 2013 (*3*). Although person-to-person transmission of SFTSV through contact with the blood or mucus of an infected person has been reported (*6*,*7*), the virus is primarily transmitted to humans by the bite of SFTSV-infected ticks. The virus has been detected in *Haemaphysalis longicornis* Neumann (bush tick) and *Rhipicephalus microplus* Canestrini (southern cattle tick) ticks (*1*,*8*).

H. longicornis ticks comprise the major population of ticks in the environment and have been considered the main vector for SFTSV (*9*,*10*). SFTSV has been detected in *H. longicornis* ticks collected from the environment by using the dragging or sweeping methods and from mammals. However, to our knowledge, the prevalence of SFTSV in ticks collected from humans has not been reported. To increase our understanding of SFTSV and its possible vectors, we determined the prevalence of SFTSV infection among various ticks collected from humans nationwide in South Korea during May–October 2013.

Author affiliation: Korea Centers for Disease Control and Prevention, Cheongwon-gun, South Korea

DOI: http://dx.doi.org/10.3201/eid2008.131857

The Study

We collected a total of 261 ticks (113 nymphal, 7 adult male, and 141 adult female ticks) from humans during May–October 2013. The ticks were placed in plastic tubes and transported to our laboratory for identification to species and developmental stage (*11*); we used a dissecting microscope for identification purposes. Tick samples were homogenized in 600 μL of phosphate-buffered saline (pH 7.0) containing 10% fetal bovine serum (GIBCO BRL, Grand Island, NY, USA), penicillin (500 IU/mL, GIBCO BRL) and streptomycin (500 μg/mL, GIBCO BRL) by using the Precellys 24 tissue homogenizer (Bertin Technologies, Bretonneux, France) and 2.8-mm stainless steel beads. We used a viral RNA extraction kit (iNtRON Biotechnology, Seongnam, South Korea) to extract RNA from the supernatant of the tick homogenates. To detect SFTSV RNA, we performed a 1-step reverse transcription PCR (RT-PCR) using a DiaStar 2× OneStep RT-PCR Pre-Mix Kit (SolGent, Daejeon, South Korea) with designed primers, MF3 (5′-GATGAGATGGTCCATGCTGATTCT-3′) and MR2 (5′-CTCATGGGGTGGAATGTCCTCAC-3′), under the following conditions: an initial step of 30 min at 50°C for reverse transcription and 15 min at 95°C for denaturation, followed by 35 cycles of 20 s at 95°C, 40 s at 58°C, and 30 s at 72°C, and a final extension step of 5 min at 72°C.

Of the 261 identified ticks, 4 nymphal ticks and 18 adult ticks had fed just before collection. The most abundant tick was *H. longicornis* (81.2%, 212/261), followed by *Amblyomma testudinarium* Koch (6.5%, 17/261); *Ixodes nipponensis* Kitaoka and Saito (5.7%, 15/261); *H. flava* Neumann (5.4%, 14/261); and *H. japonica* Nutt and Warburton, *Ix. persulcatus* Schulze, and *Ix. granulatus* Supino (0.4% each, 1/261) (Table).

We divided the 261 ticks into 189 pools to detect the medium (M) segment gene of SFTSV by RT-PCR: 18 SFTSV-positive pools were detected. The SFTSV minimum infection rate per 100 ticks (MIR) was 5.7% in *H. longicornis* (12 pools), 23.5% in *A. testudinarium* (4 pools), and 13.3% in *Ix. nipponensis* (2 pools) ticks. In fed ticks, the MIR was 18.2% (4 pools), and in unfed ticks, the MIR was 5.9% (14 pools). The mean prevalence of SFTSV in the ticks in the study was 6.9%.

We identified the sequences for the SFTSV-positive tick pools by using the TA cloning method and a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA). Because of

[1]These authors contributed equally to this article.

a cloning failure, the sequence of 1 positive tick pool was not determined. The sequences obtained from 17 SFTSV-positive tick pools were submitted to GenBank (accession nos. KF781489–513).

Sequences from the SFTSVs detected showed 92.3%–98.8% identity with the partial sequence of the M segment from 17 SFTSV strains from South Korea and from 17 other SFTSV strains from China and Japan. Using the neighbor-joining method, we constructed a phylogenetic tree based on the partial M segment sequences (560 bp) obtained in this study and from SFTSV sequences in GenBank. The 17 strains from South Korea were closely

Table.Ticks collected from humans in South Korea during a study of severe fever with thrombocytopenia syndrome virus, 2013*

Tick species, developmental stage	No. ticks	No. pools	No. SFTSV-positive pools	MIR, %
Haemaphysalis longicornis				
Larvae	0	0	0	0
Nymphs	85	34	1	1.2
Adults, sex				
M	5	5	0	0
F	122	109	11	9.0
Subtotal	212	148	12	5.7
Haemaphysalis flava				
Larvae	0	0	0	0
Nymphs	9	4	0	0
Adults, sex				
M	2	2	0	0
F	3	3	0	0
Subtotal	14	9	0	0
Haemaphysalis japonica				
Larvae	0	0	0	0
Nymphs	0	0	0	0
Adults, sex				
M	0	0	0	0
F	1	1	0	0
Subtotal	1	1	0	0
Amblyomma testudinarium				
Larvae	0	0	0	0
Nymphs	16	13	4	25.0
Adults, sex				
M	0	0	0	0
F	1	1	0	0
Subtotal	17	14	4	23.5
Ixodes nipponensis				
Larvae	0	0	0	0
Nymphs	3	3	2	66.7
Adults, sex				
M	0	0	0	0
F	12	12	0	0
Subtotal	15	15	2	13.3
Ixodes persulcatus				
Larvae	0	0	0	0
Nymphs	0	0	0	0
Adults, sex				
M	0	0	0	0
F	1	1	0	0
Subtotal	1	1	0	0
Ixodes granulatus				
Larvae	0	0	0	0
Nymphs	0	0	0	0
Adults, sex				
M	0	0	0	0
F	1	1	0	0
Subtotal	1	1	0	0
Total				
Larvae	0	0	0	0
Nymphs	113	54	7	6.2
Adults, sex				
M	7	7	0	0
F	141	128	11	7.8
Total	261	189	18	6.9

*Pools of nymphal and adult ticks contained 1–30 ticks/pool and 1–5 ticks/pool, respectively. MIR, SFTSV minimum infection rate per 100 ticks (no. positive pools/total no. ticks assayed); SFTSV, severe fever with thrombocytopenia syndrome virus.

related to the SFTSV strains from humans and ticks in China and Japan (Figure).

Conclusions

H. longicornis ticks have been considered the principal tick vector of SFTSV; there is limited information on SFTSV infection by other tick species. On the basis of our findings, we propose that *A. testudinarium* and *Ix. nipponensis* ticks, from which we detected SFTSV, might serve as potential vectors of this virus in South Korea. However, the presence of viral RNA in a tick does not confirm that the tick can transmit the virus. To our knowledge, *A. testudinarium* and *Ix. nipponensis* ticks have not previously been considered as SFTSV vectors.

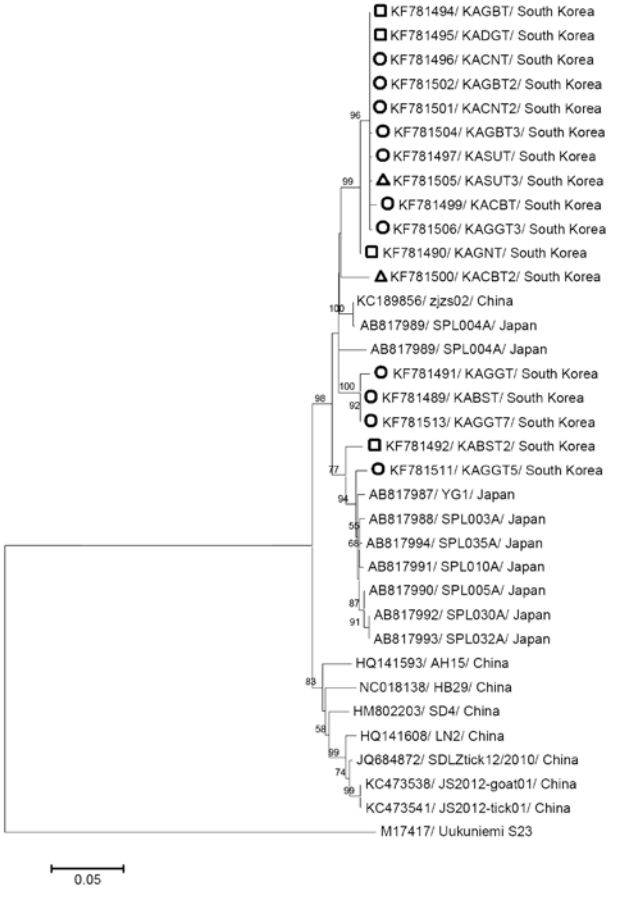


Figure. Phylogenetic analysis of severe fever with thrombocytopenia syndrome viruses based on the partial medium segment sequences (560 bp). The tree was constructed by using the neighbor-joining method based on the p-distance model in MEGA5 (*12*) (5,000 bootstrap replicates). Uukuniemi virus was used as the outgroup. Scale bar indicates the nucleotide substitutions per position. Among the 17 South Korean strains identified in this study, the Korean strains detected from *Haemaphysalis longicornis*, *Amblyomma testudinarium*, and *Ixodes nipponensis* ticks are marked with open circles, squares, and triangles, respectively. Numbers at nodes indicate bootstrap values.

Further studies, including studies to isolate SFTSV from infected tick vectors and laboratory vector competence studies, are needed to confirm whether *A. testudinarium* and *Ix. nipponensis* ticks transmit SFTSV to humans. We attempted to isolate SFTSV from the SFTSV-positive ticks in our study by using Vero E6 cells but were unable to do so.

Adults and nymphs of *Haemaphysalis* and *Ixodes* spp. have been collected most frequently from humans, but larvae have not been collected. We did not detect SFTSV from adult male ticks in our study; however, the number of collected ticks was small. In another study in South Korea, ticks were collected from medium- and large-sized mammals (*9*), and the species and developmental stage of ticks in that study were similar to those in our study. In that study, *H. longicornis* and *Ix. nipponensis* ticks were most frequently found on wild boar, water deer, roe deer, raccoon dog, Siberian weasel, Asian badger, and leopard cat. Although *A. testudinarium* ticks were not collected from mammals in that study, we did collected them from humans in our study.

The prevalence of SFTSV in *H. longicornis* ticks in our study was 5.7%. In 27 other localities in 9 South Korean provinces, the mean SFTSV MIR of *H. longicornis* ticks collected by dragging or sweeping in the environment was 0.5% (S.-W. Park et al., unpub. data). In a study of SFTSV in China, 0.7%–5.4% of the tick population was positive for SFTSV (*13*). In the United States, 0.02% of field-collected ticks were positive for Heartland virus (*14*). The SFTSV MIR for ticks in Japan has not been reported.

Farmers and agricultural and forest workers from rural areas are at high risk for SFTS because their work environment increases their risk of contact with SFTSV-infected ticks (*15*). In our study, most ticks were collected from persons who lived in rural areas. Although 18 persons were bitten by ticks infected with SFTSV, not all of them had signs or symptoms of SFTS. This finding suggests that further studies are needed to obtain a detailed understanding of SFTS as an emerging tickborne viral disease and to develop preventive measures for the disease.

Acknowledgments

We thank the staff members at the Regional Health Institutes for the help they provided with this study.

This study was supported by the National Institute of Health, Korea Centers for Disease Control and Prevention (program no. 4800-4851-300-210-13).

Mr Yun is a senior researcher at the Division of Arboviruses, National Institute of Health, Korea Centers for Disease Control and Prevention. His research interests focus on tickborne viral diseases.

References

1. Yu XJ, Liang MF, Zhang SY, Liu Y, Li JD, Sun YL, et al. Fever with thrombocytopenia associated with a novel bunyavirus in China. N Engl J Med. 2011;364:1523–32. http://dx.doi.org/10.1056/NEJMoa1010095
2. McMullan LK, Folk SM, Kelly AJ, MacNeil A, Goldsmith CS, Metcalfe MG, et al. A new phlebovirus associated with severe febrile illness in Missouri. N Engl J Med. 2012;367:834–41. http://dx.doi.org/10.1056/NEJMoa1203378
3. Kim K-H, Yi J, Kim G, Choi SJ, Jun KI, Kim N-H, et al. Severe fever with thrombocytopenia syndrome, South Korea, 2012. Emerg Infect Dis. 2013;19:1892–4. http://dx.doi.org/10.3201/eid1911.130792
4. Takahashi T, Maeda K, Suzuki T, Ishido A, Shigeoka T, Tominaga T, et al. The first identification and retrospective study of severe fever with thrombocytopenia syndrome in Japan. J Infect Dis. 2014;209:816–27. http://dx.doi.org/10.1093/infdis/jit603
5. ProMEDmail. Severe fever with thrombocytopenia syndrome—South Korea (05): additional fatalities. ProMed. 2013 Jul 6 [cited 2013 Oct 10]. http://www.promedmail.org, archive no. 20130706.1810682.
6. Liu Y, Li Q, Hu W, Wu J, Wang Y, Mei L, et al. Person-to-person transmission of severe fever with thrombocytopenia syndrome virus. Vector Borne Zoonotic Dis. 2012;12:156–60. http://dx.doi.org/10.1089/vbz.2011.0758
7. Bao CJ, Guo XL, Qi X, Hu JL, Zhou MH, Varma JK, et al. A family cluster of infections by a newly recognized bunyavirus in eastern China, 2007: further evidence of person-to-person transmission. Clin Infect Dis. 2011;53:1208–14. http://dx.doi.org/10.1093/cid/cir732
8. Zhang YZ, Zhou DJ, Qin XC, Tian JH, Xiong Y, Wang JB, et al. The ecology, genetic diversity, and phylogeny of Huaiyangshan virus in China. J Virol. 2012;86:2864–8. http://dx.doi.org/10.1128/JVI.06192-11
9. Kim HC, Han SH, Chong ST, Klein TA, Choi CY, Nam HY, et al. Ticks collected from selected mammalian hosts surveyed in the Republic of Korea during 2008–2009. Korean J Parasitol. 2011;49:331–5. http://dx.doi.org/10.3347/kjp.2011.49.3.331
10. Chong ST, Kim HC, Lee IY, Kollars TM Jr, Sancho AR, Sames WJ, et al. Seasonal distribution of ticks in four habitats near the demilitarized zone, Gyeonggi-do (Province), Republic of Korea. Korean J Parasitol. 2013;51:319–25. http://dx.doi.org/10.3347/kjp.2013.51.3.319
11. Yamaguti N, Tipton VJ, Keegan HL, Toshioka S. Ticks of Japan, Korea, and the Ryukyu Islands. Brigham Young Univ Sci Bull Biol Ser. 1971;15:1–226.
12. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121
13. Liu S, Chai C, Wang C, Am S, Lv H, He H, et al. Systematic review of severe fever with thrombocytopenia syndrome: virology, epidemiology, and clinical characteristics. Rev Med Virol. 2014;24:90–102. http://dx.doi.org/10.1002/rmv.1776
14. Savage HM, Godsey MS Jr, Lambert A, Panella NA, Burkhalter KL, Harmon JR, et al. First detection of Heartland virus (Bunyaviridae: *Phlebovirus*) from field collected arthropods. Am J Trop Med Hyg. 2013;89:445–52. http://dx.doi.org/10.4269/ajtmh.13-0209
15. Ding F, Zhang W, Wang L, Hu W, Soares Magalhaes RJ, Sun H, et al. Epidemiologic features of severe fever with thrombocytopenia syndrome in China, 2011–2012. Clin Infect Dis. 2013;56:1682–3. http://dx.doi.org/10.1093/cid/cit100

Address for correspondence: Myung Guk Han, Division of Arboviruses, National Institute of Health, Korea Centers for Disease Control and Prevention, 187 Osongsaengmyeong 2-ro, Osong-eup, Cheongwon-gun, Chungcheongbuk-do, 363-951 South Korea; email: mghan@nih.go.kr

Infection with Possible Precursor of Avian Influenza A(H7N9) Virus in a Child, China, 2013

Lili Ren,[1] Xuelian Yu,[1] Baihui Zhao,[1] Fan Wu, Qi Jin,[2] Xi Zhang,[2] and Jianwei Wang[2]

During the early stage of the avian influenza A(H7N9) epidemic in China in March 2013, a strain of the virus was identified in a 4-year-old boy with mild influenza symptoms. Phylogenetic analysis indicated that this strain, which has similarity to avian subtype H9N2 viruses, may represent a precursor of more-evolved H7N9 subtypes co-circulating among humans.

Influenza A(H7N9) virus infected >400 persons in China during March 2013–April 2014 (*1*–*3*) in China. Although this virus does not appear to be readily transmitted from person to person, its identification in a wide geographic area of China and discovery of amino acid changes associated with mammalian adaption of the virus have caused increased concerns for a pandemic (*1*,*2*).

The origin and evolution of the H7N9 subtype have been discussed intensively based on the results of phylogenetic analysis of the available sequences (*1*,*4*–*8*). The hemagglutinin (HA) and neuraminidase (NA) genes of the H7N9 subtype that circulated among humans during 2013 were possibly introduced from wild birds that carried differing subtype H9N2 strains and then reassorted in domestic birds such as chickens (*4*–*6*). A/brambling/Beijing/16/2012(H9N2) (BJ16)–like virus and/or other related avian virus H9N2 strains are proposed to be the sources of the internal genes of the 2013 H7N9 subtypes (*5*,*7*). However, the precise source and evolution route of strains in human H7N9 subtypes have not been well established (*4*–*6*). Intermediate or precursor strains are extrapolated to exist at the interface between avian and human H7N9 subtypes (*5*,*6*), but such strains have not been identified.

Author affiliations: Ministry of Health Key Laboratory for Systems Biology of Pathogens, Beijing, China (L. Ren, Q. Jin, J. Wang); Institute of Pathogen Biology, Beijing (L. Ren, Q. Jin, J. Wang); Shanghai Municipal Center for Disease Control and Prevention, Shanghai, China (X. Yu, B. Zhao, F. Wu, X. Zhang); and Shanghai Jiao Tong University, Shanghai (B. Zhao)

DOI: http://dx.doi.org/10.3201/eid2008.140325

Here we report the identification of a distinct strain, A/Shanghai/JS01/2013(H7N9) (SH/JS01), which was detected in a patient with mild influenza symptoms in Shanghai during March 2013, during the very early stage of the influenza A(H7N9) epidemic. Phylogenetic analysis indicates that this strain may represent an earlier precursor of the more evolved H7N9 subtypes co-circulating at low levels at the time of isolation in March 2013 thus providing insight into the evolution of H7N9 subtypes.

The Study

A mild case of influenza A(H7N9) virus infection was identified in a 4-year-old boy in a rural area of Jinshan District, Shanghai, reported on March 31. The patient had been exposed to poultry. His signs and symptoms included acute fever (maximum 39° axillary), cough, nasal drainage, and tonsillitis. A diagnosis of upper respiratory tract infection was made, and the child recovered after 5 days of antiviral drug therapy (*9*,*10*). Nasal swab specimens were positive for influenza A(H7N9) virus by using real-time RT-PCR (*11*), as recommended by the World Health Organization. Although his family members, unrelated workers, and chickens he may have had contact with were tested, none tested positive for influenza virus.

The whole genome sequence of the SH/JS01strain was amplified from the nasal swab specimen by using RT-PCR (primer sequences available upon request). Strict controls were used during PCR amplification; results were confirmed by another laboratory to exclude contamination with laboratory strains. We constructed maximum likelihood trees of each gene segment sequence using the general time-reversible model implemented in MEGA 5.1 (*12*), and estimated divergence time using the Bayesian Markov chain Monte Carlo method implemented in BEAST (v1.6.1) (*13*). We compared all known strains of the 2013 H7N9 subtype and other referenced influenza virus sequences deposited in GISAID (http://platform.gisaid.org/epi3/frontend#57f951) and GenBank (Table 1 and online Technical Appendix Figures 1–8; http://wwwnc.cdc.gov/EID/article/20/8/14-0325-Techapp.pdf).

The critical mutations in the SH/JS01 strain associated with virulence and mammalian adaption were compared to 3 prevalent H7N9 subtype reference strains: A/Shanghai/1/2013 (SH/1), A/Shanghai/2/2013 (SH/2), and A/Anhui/1/2013 (AH/1). In the HA gene of SH/JS01, the only mammalian adapting substitution observed was 138A (H3 numbering); amino acid residues involved in receptor-binding specificity showed avian-like signatures, including 186G and 226Q, which were similar to SH/1 but distinctive from SH/2 and

[1]These authors contributed equally to this work.

[2]These authors contributed equally to this work and share senior authorship.

Table 1. The GenBank accession numbers, gene clade, and estimated divergent time of the sequences for A/Shanghai/JS01/2013*

Gene segment	GenBank accession no.	Gene clade†	Estimated time of divergence‡
PB2	KF609508	Minor	Jul 2010
PB1	KF609509	Major	Jun 2002
PA	KF609510	Minor	Mar 2012
HA	KF609511	ND	Oct 2005
NP	KF609512	Major	Jan 2001
NA	KF609513	ND	Sep 2010
M	KF609514	Minor	May 2011
NS	KF609515	ND	Oct 1996

*PB, polymerase basic subunit; PA, RNA polymerase acidic subunit; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; M, matrix gene; NS, nonstructural gene.
†Defined according to reference (*5*); ND, not divided: HA, NA, and NS genes were not divided into clades.
‡Calculations based on sequences in online Technical Appendix (http:wwwnc.cdc.gov/EID/article/20/8/14-0325-Techapp1.pdf).

AH/1. In the internal genes of SH/JS01, we observed some human-like and mammalian-adapting signatures, including 89V in polymerase basic subunit (PB)2, 368V in PB1, 356R in the RNA polymerase acidic subunit, 42S in nonstructural gene 1, and 30D and 215A in matrix gene 1; however, some hallmark changes involved in mammalian adaptation still showed avian signatures, including 627E and 701D in PB2 and 100V and 409S in the RNA polymerase acidic subunit (Table 2). Most strikingly, SH/JS01 retained aa 69–73 (N9 numbering) in the stalk region of the NA gene. In contrast, deletion of aa 69–73, which is considered to occur when viruses adapt to terrestrial birds, prevailed in all the known 2013 H7N9 subtype isolates (*14*) (Table 2, and Figure). These findings indicate that SH/JS01 is genetically distinct from all the known human influenza A(H7N9) strains and carries more avian influenza-like signatures.

Phylogenetic analysis and divergence time estimation showed that the SH/JS01 HA gene diverged in October 2005 and was closely related to SH/1; nucleotide similarity was 99.7% (online Technical Appendix Figure 1). However, the NA gene, which is most closely related to A/northern shoveler/Hong Kong/MPL133/2010(H2N9) and A/duck/Jiangxi/21714/2011(H11N9) with nucleotide similarity of 99% and 99.3%, respectively, are estimated

Table 2. Analysis on critical mammalian-adapting amino acid substitutions in H7N9 virus strains*†

Gene	Site	SH/JS01	SH/1	SH/2	AH/1	Known mutations	Relationship to mammalian adaption
HA	138	**A**	S	**A**	**A**	S138A	Mammalian host adaption
	186	G	G	**V**	**V**	G186V	Unknown
	226	Q	Q	**L**	**L**	Q226L	Unknown
	228	G	G	G	G	G228S	Unknown
NA	292	R	**K**	R	R	R292K	Osteltamivir and zanamivir resistance
	69–73 deletion	No	**Yes**	**Yes**	**Yes**	Not applicable	Deletion of 69–73 Increased virulence in mice
PB2	63	I	I	I	I	I63T	Co-mediate with PB1 677M, to reduce pathogenicity of H5N1 viruses
	89	**V**	**V**	**V**	**V**	L89V	Enhanced polymerase activity and increased virulence in mice
	471	T	T	T	T	T471M	Viral replication, virulence, and pathogenicity
	591	Q	Q	Q	Q	Q591K	Adapt in mammals that compensates for the lack of PB2–627K
	627	E	**K**	**K**	**K**	E627K	Enhanced polymerase activity and increased virulence in mice
	701	D	D	D	D	D701N	Enhanced transmission in guinea pigs
PB1	99	H	H	H	H	H99Y	Results in transmissible of H5 virus among ferrets
	353	K	K	K	K	K353R	Determine viral replication, virulence, and pathogenicity
	368	**V**	I	**V**	**V**	I368V	Results in transmissible of H5 virus among ferrets
	566	T	T	T	T	T566A	Determine viral replication, virulence, and pathogenicity
	677	T	T	T	T	T677M	Co-mediate with PB2 I63T to reduce pathogenicity of H5N1 viruses
PA	100	V	**A**	**A**	**A**	V100A	Related to human adaption
	356	**R**	**R**	**R**	**R**	356R	Related to human adaption
	409	S	**N**	**N**	**N**	S409N	Enhances transmission in mammals
M1	30	**D**	**D**	**D**	**D**	N30D	Increased virulence in mice
	215	**A**	**A**	**A**	**A**	T215A	Increased virulence in mice
M2	31	**N**	**N**	**N**	**N**	S31N	Reduced susceptibility to amantadine and rimantadine
NS1	42	**S**	**S**	**S**	**S**	P42S	Increased virulence in mice

*HA, hemagglutinin; NA, neuraminidase; SH/JS01, A/Shanghai/JS01/2013(H7N9); SH/1, A/Shanghai/1/2013 (H7N9); SH/2, A/Shanghai/2/2013 (H7N9); AH/1, A/Anhui/1/2013 (H7N9); PB, RNA polymerase basic subunit; PA, RNA polymerase acidic subunit; M, matrix gene; NS, nonstructural gene.
†Boldface text indicates the mutant amino acids sites related to mammalian host adaption and increased virulence.

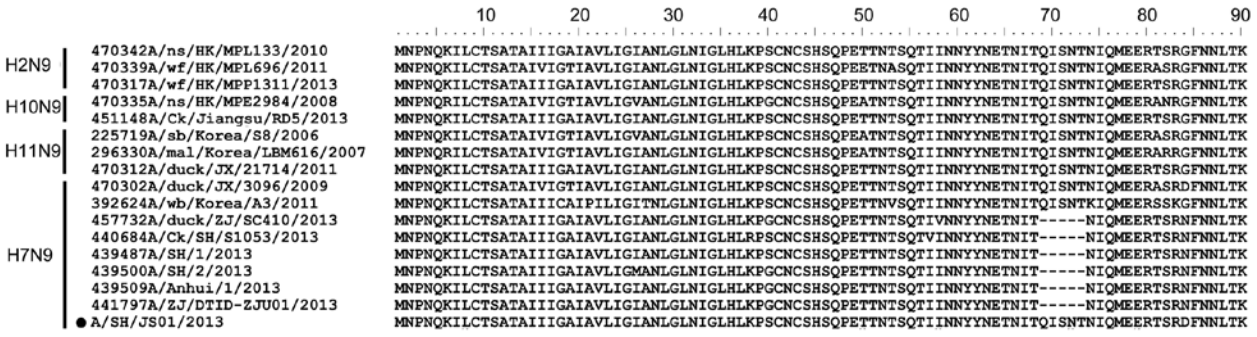


Figure. Amino acid sequence alignment of the neuraminidase (NA) stalk region. The dark circle indicates the sequence characterized in this study. The abbreviations of the sequence names are as follows: ns, northern shoveler; wf, wild waterfowl; Ck, Chicken; Sb, shorebird; mal, mallard; wb, wild bird; HK, Hong Kong; JX, Jiangxi; ZJ, Zhejiang; SH, Shanghai.

to have diverged in September 2010, earlier than that of known strains of the 2013 H7N9 subtype (estimated to have occurred in January 2011) (Table 1; online Technical Appendix Figure 2).

On the basis of internal genes, 2013 H7N9 viruses have been divided into minor (m) and major (M) clades in the phylogenetic trees, and first 9, then 27 genotypes (*5*,*6*,*8*). Our data showed that SH/JS01 belongs to the m-PB2|PA|M or G3 genotype (Table 1; online Technical Appendix Figures 3–8); its NS gene and that of A/Chicken/Dawang/1/2011(H9N2) (DW1), shared the highest nt identity (99.4%). The SH/JS01 NS gene had an estimated divergence time as early as October 1996 (Table 1; online Technical Appendix Figure 3). The closest relatives of the SH/JS01 M gene were poultry H9N2 strains A/chicken/Jiangsu/CZ1/2012 and A/chicken/Jiangsu/NTTZ/2013 (nt identity 99.7%); BJ16 (98.6%) was not closely related. The divergence time of the SH/JS01 M gene is estimated as May 2011, which is earlier than most 2013 H7N9 isolates (Table 1; online Technical Appendix Figure 4). The PB2 and PA genes of SH/JS01 were positioned in minor clades and showed higher identities to A/chicken/Jiangsu/MYJMF/2012(H9N2) (99.2% and 99.3%, respectively) than to BJ16 (97.1% and 98%) (Table 1; online Technical Appendix Figures 5, 6). In contrast, the PB1 and NP genes of SH/JS01 belong to the major lineage (Table 1; online Technical Appendix Figures 7, 8). Collectively, these data indicate that, except for PB1 and NP, the internal genes of SH/JS01 are more closely related to those in poultry H9N2 viruses identified before 2013 than to BJ16, which was an H9N2 virus considered to be the donor of most of internal genes of the 2013 H7N9 virus.

Conclusions

SH/JS01, a distinct H7N9 virus strain identified in 2013 during the early stage of the influenza A(H7N9) epidemic in China, provided information to define the evolution of the H7N9 subtype. Although identified in an infected human, SH/JS01 has more avian-prone properties and fewer mammalian-adapting mutations than other known human 2013 H7N9 subtypes. SH/JS01 has a waterfowl-like NA gene characterized by the absence of a deletion in the NA stalk and most of its internal genes are more closely related to avian H9N2 subtype strains isolated during the 2011–2012 influenza season than to other recently emerged strains of the H7N9 subtype. Molecular clock analysis further predicted an earlier divergence time in most genes of SH/JS01. These findings indicate that SH/JS01 might be a precursor strain of the H7N9 virus that co-circulated with more evolved viruses, although we cannot exclude that SH/JS01 may have been generated independently from the other H7N9 strains by reassortment of waterfowl strains with avian H9N2 strains and then transmitted directly to a human.

The sequences of SH/JS01 contained more avian-like signatures than those of other H7N9 isolates from humans; this underscores the potential of these viruses to infect humans. The phenotypic characteristics of SH/JS01, which might describe its zoonotic potential, remain to be investigated.

It is unclear whether other SH/JS01–like viruses are still circulating in poultry in China and if so, what the potential is for their evolution and ability to infect humans. Intensive influenza surveillance and additional influenza A virus genome sequences isolated from poultry and from humans with severe and mild manifestations of infection are needed to clarify the population dynamics of H7N9 viruses.

Acknowledgments

We thank Adolfo García-Sastre for his helpful discussions and critical-reading of this manuscript.

This study was supported in part by grants from Chinese Ministry of Science and Technology (KJYJ-2013-01-01-01), National Major Science and Technology Project for Control and Prevention of Major Infectious Diseases of China (2012ZX10004-206), Shanghai Health and Family Planning Commission (12GWZX081, 2013QLG007, 2013QLG001, 2013QLG003),

National Funds for Distinguished Young Scientists (81225014), Program for Changjiang Scholars and Innovative Research Team in University (IRT13007), and Foundation Merieux.

Dr. Ren is an associate professor at the Institute of Pathogen Biology, Chinese Academy of Medical Sciences, Beijing. Her research is focused on the etiology and pathogenesis of respiratory viruses.

References

1. Gao R, Cao B, Hu Y, Feng Z, Wang D, Hu W, et al. Human infection with a novel avian-origin influenza A (H7N9) virus. N Engl J Med. 2013;368:1888–97. http://dx.doi.org/10.1056/NEJMoa1304459
2. Uyeki TM, Cox NJ. Global concerns regarding novel influenza A (H7N9) virus infections. N Engl J Med. 2013;368:1862–4. http://dx.doi.org/10.1056/NEJMp1304661
3. World Health Organization. Avian influenza A(H7N9) virus [cited 2014 Apr 8]. http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/
4. Liu D, Shi W, Shi Y, Wang D, Xiao H, Li W, et al. Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet. 2013;381:1926–32. http://dx.doi.org/10.1016/S0140-6736(13)60938-1
5. Wu A, Su C, Wang D, Peng Y, Liu M, Hua S, et al. Sequential reassortments underlie diverse influenza H7N9 genotypes in China. Cell Host Microbe. 2013;14:446–52. http://dx.doi.org/10.1016/j.chom.2013.09.001
6. Lam TT, Wang J, Shen Y, Zhou B, Duan L, Cheung CL, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. Nature. 2013;502:241–4. http://dx.doi.org/10.1038/nature12515
7. Feng Y, Mao H, Xu C, Jiang J, Chen Y, Yan J, et al. Origin and characteristics of internal genes affect infectivity of the novel avian-origin influenza A(H7N9) virus. PLoS ONE. 2013;8:e81136. http://dx.doi.org/10.1371/journal.pone.0081136
8. Cui L, Liu D, Shi W, Pan J, Qi X, Li X, et al. Dynamic reassortments and genetic heterogeneity of the human-infecting influenza A (H7N9) virus. Nat Commun. 2014;5:3142–50. http://dx.doi.org/10.1038/ncomms4142
9. Ip DK, Liao Q, Wu P, Gao Z, Cao B, Feng L, et al. Detection of mild to moderate influenza A/H7N9 infection by China's national sentinel surveillance system for influenza-like illness: case series. BMJ. 2013;346:f3693. http://dx.doi.org/10.1136/bmj.f3693
10. Yu X, Zhang X, He Y, Wu HY, Gao X, Pan QC, et al. Mild infection of a novel H7N9 avian influenza virus in children in Shanghai. Emerg Microbes Infect. 2013;2:e41. http://dx.doi.org/10.1038/emi.2013.41
11. World Health Organization. Real-time RT-PCR protocol for the detection of avian influenza A(H7N9) virus [cited 2013 Apr 15]. http://www.who.int/influenza/gisrs_laboratory/cnic_realtime_rt_pcr_protocol_a_h7n9.pdf.
12. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol. 2011;28:2731–9. http://dx.doi.org/10.1093/molbev/msr121
13. Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. BMC Evol Biol. 2007;7:214. http://dx.doi.org/10.1186/1471-2148-7-214
14. Cheung CL, Vijaykrishna D, Smith GJ, Fan XH, Zhang JX, Bahl J, et al. Establishment of influenza A virus (H6N1) in minor poultry species in southern China. J Virol. 2007;81:10402–12. http://dx.doi.org/10.1128/JVI.01157-07

Address for correspondence: Jianwei Wang, MOH Key Laboratory for Systems Biology of Pathogens and Christophe Mérieux Laboratory, Institute of Pathogen Biology, Chinese Academy of Medical Sciences, #9 Dong Dan San Tiao, Dongcheng District, Beijing, 100730, P.R. China; email: wangjw28@163.com

Dengue Virus Transmission by Blood Stem Cell Donor after Travel to Sri Lanka, 2013

Michael Punzel, Gülay Korukluoğlu, Dilek Yagci Caglayik, Dilek Menemenlioglu, Sinem Civriz Bozdag, Emre Tekgündüz, Fevzi Altuntaş, Renata de Mendonca Campos, Bernd Burde, Stephan Günther, Dennis Tappe, Daniel Cadar, and Jonas Schmidt-Chanasit

Three days after donation of peripheral blood stem cells to a recipient with acute myeloblastic leukemia, dengue virus was detected in the donor, who had recently traveled to Sri Lanka. Transmission to the recipient, who died 9 days after transplant, was confirmed.

Dengue virus (DENV), an arthropod-borne RNA virus of the *Flaviviridae* family, has 4 serotypes that cause dengue fever or dengue hemorrhagic fever in humans. DENV has become a worldwide public health problem: current estimates indicate 390 million DENV infections and 96 million clinically apparent cases in 2010 (*1*). The virus is found in tropical and subtropical regions around the world and is hyperendemic to areas in Asia and Latin America (*1*).

Hematopoietic stem cell transplantation has become a major treatment option for patients with hematopoietic malignancies and immune deficiencies. Each year, >50,000 allogeneic transplants are performed worldwide (*2*). Despite mandatory testing of donors and strict exclusion criteria to prevent transmission, risk remains for transmission of communicable diseases, including tropical diseases for which screening is not usually performed. To the best of our knowledge, only the transmission of malarial parasites has been reported during stem cell transplantation (*3*,*4*). Here, we report transmission of DENV to a peripheral blood stem cell recipient by a donor who had recently traveled to an area to which the virus is endemic. We recommend testing of recent travelers returning from areas to which DENV is endemic before allowing such donations.

Author affiliations: MediaPark Klinik, Cologne, Germany (M. Punzel); Public Health Institutions of Turkey, Ankara, Turkey (G. Korukluoğlu, D.Y. Caglayik, D. Menemenlioglu); Abdurrahman Yurtarslan Ankara Oncology Hospital, Ankara (S.C. Bozdag, E. Tekgündüz, F. Altuntaş); Federal University of Rio de Janeiro, Rio de Janeiro, Brazil (R. de Mendonca Campos); Medizinisches Versorgungszentrum Labor Dr. Quade & Kollegen, Cologne (Bernd Burde); Bernhard Nocht Institute for Tropical Medicine, World Health Organization Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg, Germany (S. Günther, D. Tappe, D. Cadar, J. Schmidt-Chanasit); and German Centre for Infection Research, Hamburg-Luebeck-Borstel, Hamburg (J. Schmidt-Chanasit)

DOI: http://dx.doi.org/10.3201/eid2008.140508

The Study

Acute myeloblastic leukemia was diagnosed in a 51-year-old man in Germany in September 2012. According to international standards, cytogenetic and molecular examination determined that this form of leukemia was "poor risk" at the time of diagnosis. Because of the patient's risk status, the physicians intended to perform allogeneic stem cell transplantation after induction and consolidation chemotherapy, which was scheduled to end in January 2013, and a conditioning chemotherapy regimen, which was planned to be given in March. Because of lack of a related HLA-matched donor, an international donor search was performed; 1 fully matched unrelated female donor was identified in the German National Registry. The 24-year-old woman, who was registered as a volunteer donor in the German Bone Marrow Donor Registry, was selected.

The donor had scheduled a trip to Sri Lanka, and was to return 3 days before the scheduled start of granulocyte-colony-stimulating factor (G-CSF) application. According to German (http://www.zrkd.de/de/_pdf/ZRKD_Standards-V9_deutsch.pdf) and international (http://www.worldmarrow.org/fileadmin/Committees/STDC/20140101-STDC-WMDA_Standards.pdf) guidelines, such travel should have led to the postponement of donation because many infectious diseases are endemic to Sri Lanka. However, the donor was unable to postpone her trip, and the recipient was in urgent need of the transplant. The transplant physicians agreed to keep the dates as scheduled and confirmed the exception as Declaration of Urgent Medical Need of the transplant.

Five days before the scheduled transplant (day –5), the recipient tested positive for *Klebsiella pneumoniae* infection of the central venous catheter. The catheter was removed and piperacillin/tazobactam treatment was initiated.

The donor had returned from her trip 3 days before the start of G-CSF-injections without any signs of infection. On the day of apheresis (day 0), the donor showed signs of a respiratory infection with axillary temperature of ≤39°C, bone pain, and headache. Oral azithromycin

Table. Timeline of events before and after allogeneic stem cell transplantation for patient and donor, Germany*

Time before (–) and after (+) transplantation	Recipient event	Donor event
Month –5	Diagnosis of poor risk acute myeloblastic leukemia and introduction of chemotherapy	Related donor assessment
Months –5 to –2	Consolidation chemotherapy	Unrelated donor search
Month –2	NA	Request of confirmatory typing (CT) and workup of the donor
Day –28	NA	Medical examination without any abnormalities
Day –25 to day –9	NA	Traveled to Sri Lanka
Day –8	Conditioning regimen started (defibrotide prophylaxis)	NA
Day –5	*Klebsiella pneumoniae* infection of the central venous catheter; catheter was removed and piperacillin/tazobactam was given	NA
Day -6 to day –2	NA	G-CSF mobilization of the donor
Day –1	NA	Stem cell collection; fever of unknown origin developed
Day 0	Transplantation of the stem cell graft; information about fever of the donor	Donor condition worsened suspect of tropical disease with thombocytopenia, skin rash and fever ≤41°C
Day +3	Fever and clinical signs of hepatic veno-occlusive disease with subsequent treatment	DENV infection confirmed by laboratory
Day +7	*Staphylococcus epidermidis* in blood cultures with subsequent vancomycin treatment	NA
Day +8	Hematochezia, metabolic acidosis, hypoxia, abdominal pain, and intensive care unit	Donor slowly recovered from DENV infection
Day +9	Patient died from enterocolitis/ hepatic veno-occlusive disease	NA

*NA, not applicable

(2 × 250 mg/d) and ibuprofen 400 mg were given. The stem cell mobilization result was poor: 11 CD34+ cells/mL peripheral blood (0.04% of leukocytes) were measured at the beginning of apheresis. Before apheresis, the donor's blood count showed mild thrombocytopenia (134,000 cells/mL) after G-CSF mobilization. Standard leukopheresis processing of 14 L of blood from the donor was performed without problems. Approximately 90×10^6 CD34+ cells, corresponding to 10^6 CD34+ progenitor cells per kg bodyweight of the recipient, could be collected.

A second apheresis or a bone marrow collection was considered, but neither was performed because the clinical condition of the donor worsened. Her temperature increased to 41°C, the platelet count dropped from 84,000 cells/mL on day 0 to 74,000/mL the day after. In the morning of the second day after apheresis (+2), the platelet count dropped to 47,000/mL, procalcitonine level was elevated at 1.10 μg/L, C-reactive protein level was elevated at 10.5 mg/L, and a slight skin rash developed. Because of the clinical course, on day +1, physicians suspected a possible DENV infection. A serum sample test showed a weak positive result for DENV by using IgM and IgG antibody tests (in-house indirect immunofluorescence assay), as used by Tappe et al. (*5*), and a strong positive result for DENV nonstructural protein-1 (NS1) antigen, demonstrating acute DENV infection. Quantitative real-time reverse transcription PCR for DENV RNA (*6*) was positive and showed a DENV RNA load of 2.6×10^{10} copies/mL. Testing of the sample containing the progenitor cells showed a DENV RNA load of 4.8×10^8 copies/mL.

After being informed about possible infection of the donor, the transplant physicians administered immunoglobulin to the recipient intravenously (0.5 g/kg/y for 4 days). At posttransplantation day +3, antibiotic drug therapy was switched from piperacillin/tazobactam to meropenem. On the same day, physical examination revealed painful hepatomegaly and increased total bilirubin, diagnosed as hepatic veno-occlusive disease (Table). Therefore, defibrotide prophylaxis, which had been initiated on day –8, was increased to treatment dose. On day +7, empiric antifungal therapy was added. On the same day, *Staphylococcus epidermidis* was detected in blood cultures and vancomycin was given. On day +8, the recipient experienced severe abdominal pain accompanied by hematochezia, hypoxia, and metabolic acidosis. Bacteriologic culture of a tracheal aspirate grew *Acinetobacter baumannii,* which was only susceptible to colomycin and tigecyclin. The recipient was transferred to the intensive care unit and died from cardiopulmonary arrest 9 days posttransplant. A blood sample from the recipient on day +3 was retrospectively analyzed and tested negative for DENV IgM and IgG but positive for DENV NS1 antigen and DENV RNA with a DENV RNA load of 8.6×10^7 copies/mL. Sequencing of the

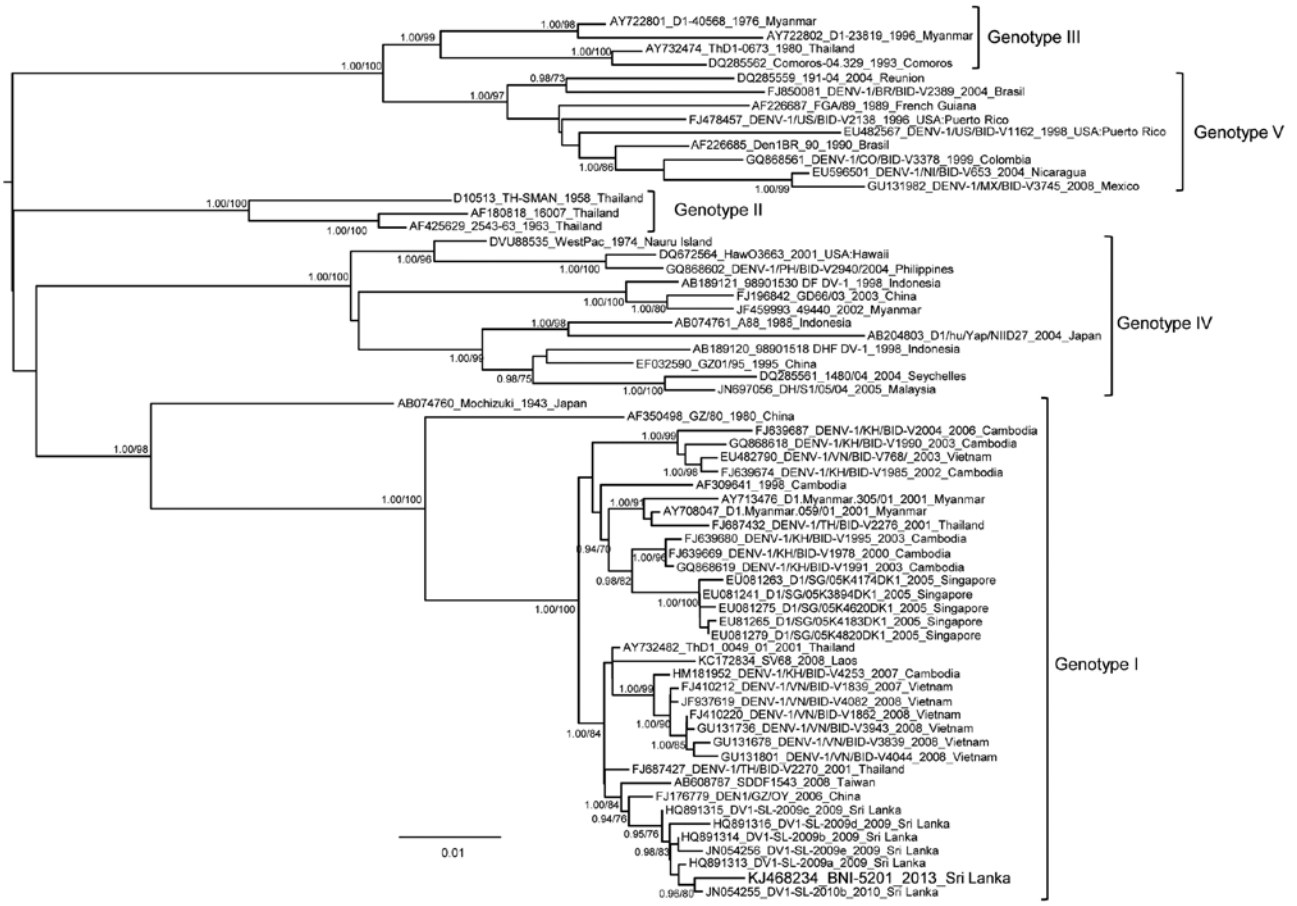


Figure. Bayesian phylogenetic tree based on complete envelope protein coding gene of dengue virus 1 (DENV-1) serotype. The tree was constructed by using the Bayesian Markov Chain Monte Carlo sampling method and BEAST software (http://beast.bio.ed.ac.uk). The general time reversible model of sequence evolution with gamma-distributed rate variation among sites and a proportion of invariable sites and a relaxed (uncorrelated log-normal) molecular clock model were used. Bayesian posterior probabilities and percentages of replicate trees in which the associated taxa clustered in the bootstrap test (1,000 replicates) obtained by using a maximum-likelihood analysis are shown at the branches. Strains are denoted by GenBank accession number, name, year of isolation, and country of origin. The strain BNI-5201 from this study is highlighted. Scale bar indicates mean number of nucleotide substitutions per site.

DENV amplicons from all samples demonstrated a DENV serotype 1 (DENV 1) genotype 1 infection of the donor and recipient. Phylogenetic analysis of the complete envelope protein coding gene of DENV 1 strains revealed that the DENV 1 genotype 1 strains detected in the donor were closely related to currently circulating DENV 1 genotype 1 strains in Sri Lanka (Figure).

Conclusions

This case demonstrates the transmission of DENV by allogeneic blood stem cell transplantation. However, although the transmission of DENV was demonstrated, the patient's death was probably caused by hepatic veno-occlusive disease and toxic enterocolitis related to the conditioning regimen.

To avoid transmission of tropical viruses such as DENV, under German Federal Ministry of Health rules, blood and stem cell donors are excluded from donation 4 weeks after returning from areas to which such disease agents are endemic (*7*). DENV has an incubation period of 3–14 days, and the risk for transmission of such viruses under this exclusion is very low. Few cases of DENV transmission by blood transfusion or organ transplantation have been published or reported (*8–11*). This case represents a difficult situation: a patient in urgent need of a lifesaving transplant that must be performed without delay, and the only matched donor scheduled for travel to a region to which DENV is endemic. The physician decided to proceed with the scheduled transplantation date because of the urgent need of his patient, although he was aware of the risk for transmission of tropical diseases.

In such situations it is difficult to estimate the risk/benefit ratio, so it will require a case-by-case decision between

donor interests and recipient needs. All diagnostic tools should be used to minimize the risk for viral transmission before transplantation. This could have been easily accomplished in this case, because preprocedure samples from the donor tested positive for DENV NS1 antigen. Thus, we recommend highly sensitive and specific testing for DENV NS1 antigen (*12*) of every donor returning from regions to which DENV is endemic for DENV NS1 antigen if transplantation cannot be postponed because of urgent medical need.

Acknowledgments

We thank Alexandra Bialonski, Insa Bonow, Corinna Thomé, Birgit Hüsing, Ihsan Durmaz, and Sabine Köhler for technical assistance.

This dispatch is dedicated to the late Ursula Herrmann (1927–2014), for making this study possible.

Dr Punzel is a lecturer at Technical University of Dresden Medical School. His research interest is hematopoietic stem cell transplantation.

References

1. Bhatt S, Gething PW, Brady OJ, Messina JP, Farlow AW, Moyes CL, et al. The global distribution and burden of dengue. Nature. 2013;496:504–7. http://dx.doi.org/10.1038/nature12060
2. Worldwide Network for Blood and Marrow Transplantation. 1 millionth blood stem cell transplant marks major medical milestone; press release 2013. [cited 2014 18 Mar]. http://www.wbmt.org/fileadmin/pdf/01_General/Press_release_final.pdf
3. Abdelkefi A, Ben Othman T, Torjman L, Ladeb S, Lakhal A, Belhadj S, et al. *Plasmodium falciparum* causing hemophagocytic syndrome after allogeneic blood stem cell transplantation. Hematol J. 2004;5:449–50. http://dx.doi.org/10.1038/sj.thj.6200531
4. Mejia R, Booth GS, Fedorko DP, Hsieh MM, Khuu HM, Klein HG, et al. Peripheral blood stem cell transplant-related *Plasmodium falciparum* infection in a patient with sickle cell disease. Transfusion. 2012;52:2677–82. http://dx.doi.org/10.1111/j.1537-2995.2012.03673.x
5. Tappe D, Schmidt-Chanasit J, Ries A, Ziegler U, Müller A, Stich A. Ross River virus infection in a traveller returning from northern Australia. Med Microbiol Immunol (Berl). 2009;198:271–3. http://dx.doi.org/10.1007/s00430-009-0122-9
6. Johnson BW, Russell BJ, Lanciotti RS. Serotype-specific detection of dengue viruses in a fourplex real-time reverse transcriptase PCR assay. J Clin Microbiol. 2005;43:4977–83. http://dx.doi.org/10.1128/JCM.43.10.4977-4983.2005
7. Mitteilungen des Arbeitskreises Blut des Bundesministeriums für Gesundheit. Dengue fieber virus (DENV); stellungnahmen des arbeitskreises blut des bundesministeriums für gesundheit Bundesgesundheitsbl. 2011 54:892–904. http://dx.doi.org/10.1007/s00103-011-1297-y
8. Tan FL, Loh DL, Prabhakaran K, Tambyah PA, Yap HK. Dengue haemorrhagic fever after living donor renal transplantation. Nephrol Dial Transplant. 2005;20:447–8. http://dx.doi.org/10.1093/ndt/gfh601
9. Bianco C. Dengue and Chikungunya viruses in blood donations: risks to blood supply? Transfusion. 2008;48:1279–81. http://dx.doi.org/10.1111/j.1537-2995.2008.01806.x
10. Tambyah PA, Koay ES, Poon ML, Lin RV, Ong BK; Transfusion-Transmitted Dengue Infection Study Group. Dengue hemorrhagic fever transmitted by blood transfusion. N Engl J Med. 2008;359:1526–7. http://dx.doi.org/10.1056/NEJMc0708673
11. Stramer SL, Linnen JM, Carrick JM, Foster GA, Krysztof DE, Zou S, et al. Dengue viremia in blood donors identified by RNA and detection of dengue transfusion transmission during the 2007 dengue outbreak in Puerto Rico. Transfusion. 2012;52:1657–66. http://dx.doi.org/10.1111/j.1537-2995.2012.03566.x.
12. Blacksell SD, Jarman RG, Bailey MS, Tanganuchitcharnchai A, Jenjaroen K, Gibbons RV, et al. Evaluation of six commercial point-of-care tests for diagnosis of acute dengue infections: the need for combining NS1 antigen and IgM/IgG antibody detection to achieve acceptable levels of accuracy. Clin Vaccine Immunol. 2011;18:2095–101. http://dx.doi.org/10.1128/CVI.05285-11.

Address for correspondence: Jonas Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany; email: jonassi@gmx.de

Geographic Distribution of MERS Coronavirus among Dromedary Camels, Africa

Chantal B.E.M. Reusken,[1] Lilia Messadi,[1] Ashenafi Feyisa,[1] Hussaini Ularamu,[1] Gert-Jan Godeke, Agom Danmarwa, Fufa Dawo, Mohamed Jemli, Simenew Melaku, David Shamaki, Yusuf Woma, Yiltawe Wungak, Endrias Zewdu Gebremedhin, Ilse Zutt, Berend-Jan Bosch, Bart L. Haagmans, and Marion P.G. Koopmans

We found serologic evidence for the circulation of Middle East respiratory syndrome coronavirus among dromedary camels in Nigeria, Tunisia, and Ethiopia. Circulation of the virus among dromedaries across broad areas of Africa may indicate that this disease is currently underdiagnosed in humans outside the Arabian Peninsula.

A novel betacoronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), was identified as the cause of severe respiratory disease in humans during 2012 (*1*). In August 2013, dromedary camels (*Camelus dromedarius*) were implicated for the first time as a possible source for human infection on the basis of the presence of MERS-CoV neutralizing antibodies in dromedaries from Oman and the Canary Islands of Spain (*2*). Since then, the presence of MERS-CoV antibodies in dromedaries has been reported in Jordan (*3*), Egypt (*4*,*5*), the United Arab Emirates (*6*,*7*), and Saudi Arabia (*8*,*9*). In October 2013, analysis of an outbreak associated with 1 barn in Qatar (*10*) found dromedaries and humans to be infected with nearly identical strains of MERS-CoV. Further proof of widespread circulation of MERS-CoV among dromedaries was provided by studies from Egypt and Saudi Arabia (*5*,*9*). These findings have raised questions about the geographic distribution of MERS-CoV among camel populations elsewhere. Here, we report our assessment of the geographic distribution of MERS-CoV circulation among dromedaries in Africa by serologic investigation of convenience samples from these animals in Nigeria, Tunisia, and Ethiopia.

The Study

In Nigeria, serum samples from 358 dromedaries that were raised for meat production were collected at abattoirs in 4 provinces (Kano, n = 245; Sokoto, n = 51; Borno, n = 51; and Adamawa, n = 11; Figure 1, panel A) during 2010–2011 for testing for peste des petits ruminants virus. The ages of the animals ranged from 4 to 15 years. The abattoirs also served the neighboring countries of Chad, Niger, and the Central African Republic. In Tunisia, serum samples from 204 dromedaries that were 1 to 16 years of age were collected in 3 provinces in 2009 and 2013 (Figure 1, panel B). Samples were collected from 155 dromedaries in Sidi Bouzid Province from 27 herds that were kept for meat production and from 39 dromedaries in Kebili Province from 16 herds that were kept for tourist rides; samples from both provinces had originally been collected for a study investigating the presence of *Anaplasma phagocytophilum.* Samples were collected from 10 dromedaries from Sousse Province that were kept for meat production because they were suspected of being infected with *Trypanosoma evansi*. In Ethiopia, samples from 188 dromedaries, 1 to 13 years of age, were collected as part of a study evaluating the presence of toxoplasmosis and respiratory tract diseases in 3 provinces (Afar, n = 118; Somalia, n = 11; and Oromia, n = 59; Figure 1, panel C) during 2011–2013. All samples were taken by jugular vein puncture according to local laws, and serum samples were stored at –20°C until testing. All serum samples were shipped to the Erasmus MC laboratory in the Netherlands in agreement with Dutch import regulations.

The serum samples were tested for the presence of IgG antibodies reactive with S1 antigens against MERS-CoV (residues 1–747), severe acute respiratory syndrome CoV (residues 1–676), and human CoV OC43 (residues 1–760) by using extensively validated protein-microarray technology, as described (*2*,*3*,*6*,*11*). Results were expressed as relative mean fluorescent intensity (RFU) for each set of quadruplicate spots of antigen, with a cutoff of 4,000 RFU as used by Meyer et al. (*6*). Human CoV OC43 S1 was used as a proxy for bovine coronavirus (BCoV), the latter of which is known to circulate commonly in dromedaries (*7*,*12*). High percentages of animals seropositive

Author affiliations: Netherlands Centre for Infectious Disease Control, Bilthoven, the Netherlands (C.B.E.M. Reusken, G.-J. Godeke, I. Zutt, M.P.G. Koopmans); Erasmus Medical Center, Rotterdam, the Netherlands (C.B.E.M. Reusken, B.L. Haagmans, M.P.G. Koopmans); National Veterinary Medicine School, University of La Manouba, Sidi Thabet, Tunisia (L. Messadi, M. Jemli); Addis Ababa University College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia (A. Feyisa, F. Dawo, S. Melaku, E. Z. Gebremedhin); National Veterinary Research Institute, Vom, Nigeria (H. Ularamu, A. Danmarwa, D. Shamaki, Y. Woma, Y. Wungak) and Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands (B.-J. Bosch)

DOI: http://dx.doi.org/10.3201/eid2008.140590

[1]These authors contributed equally to this article.

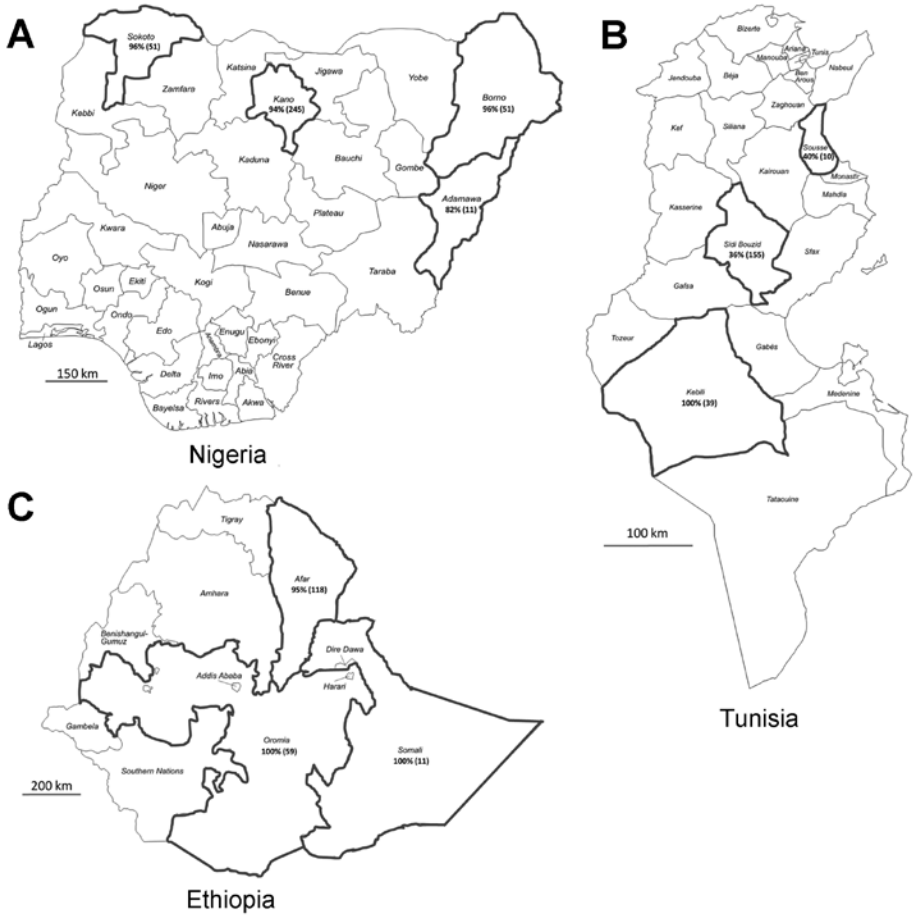


Figure 1. Countries and provinces sampled in this study: A) Nigeria, B)Tunisia, and C) Ethiopia. Black outline indicates provinces in which samples were collected. Serologic results are indicated in each province as percentage seropositive for Middle East respiratory syndrome coronavirus (total no. dromedaries tested). Maps adapted from http://d-maps.com/index.php

for MERS-CoV were observed in Nigeria and Ethiopia; the overall seropositivity was 94% in adult dromedaries in Nigeria and 93% and 97% for juvenile and adult animals, respectively, in Ethiopia (Table 1). All provinces in which dromedaries were sampled in both countries showed high rates of seropositivity (Figure 1). The overall seropositivity in dromedaries in Tunisia was 30% for animals ≤2 years of age and 54% for adult animals. Seropositivity of 36% and 40% was observed in Sidi Bouzid and Sousse Provinces, respectively, and 100% of the dromedaries in the southern province of Kebili were seropositive. Array results were confirmed on a selection of positive and negative serum samples (n = 14 per country) in MERS-CoV neutralization tests performed as described (*2*) (Table 2). Serum samples from 72%, 82%, and 67% of the dromedaries from Nigeria, Ethiopia, and Tunisia, respectively, reacted with the OC43 antigen, confirming common circulation of BCoV in camelids (*7*,*12*). All samples tested negative for severe acute respiratory syndrome CoV (data not shown).

Conclusions

Since the discovery of MERS-CoV in 2012, accumulating serologic and molecular evidence demonstrates that the virus in dromedaries is genetically very similar to MERS-CoV in humans and points to the conclusion that dromedary camels are reservoirs for human infection. MERS-CoV genomic fragments have been detected in dromedaries in Qatar (*10*) and Saudi Arabia (*9*); near full-genome sequences have been generated from dromedaries in Egypt (*5*) and full-genome sequences have been generated from dromedaries in Saudi Arabia (*13*). Here, we show serologic evidence for circulation of MERS-CoV or MERS-like CoV in dromedaries in countries in East, West, and North Africa, with possible herd-specific differences in prevalence in Tunisia. The lower seropositivity observed in herds raised for meat production in Tunisia might reflect a high turnover of camels with a continuous introduction of animals unexposed to the MERS-CoV into these herds. No camels imported from neighboring countries were found at the meat-producing farms in Sidi Bouzid and Sousse, only camels purchased from other farms in the same area or other areas in Tunisia. However, animals are frequently moved between Libya and Kebili for trade.

Samples in this study were collected during 2009–2011, confirming observations by us and others (*6*) that the virus circulated well before March 2012, which is the

Table 1. Overview of serologic evidence for Middle East respiratory syndrome coronavirus among dromedary camels, Africa and the Arabian Peninsula

Country	Year	No. camels*	% Middle East respiratory syndrome coronavirus antibodies	Reference
United Arab Emirates	2013	500 (A,J)	96†,‡,§,¶	(*6*)
	2013	59 (A)	97#, 100**, 98§	(*7*)
	2003	151 (A)	100‡,§	(*6*)
Egypt	2013	110 (A)	94§, 98††	(*4*)
	2013	17 (A)	82††	(*5*)
Spain (Canary Islands)	2012–2013	97 (A)	14§,¶	(*2*)
	2012–2013	8 (J)	13§,¶	(*2*)
Ethiopia	2010–2011	31 (J)	93¶	This study
	2010–2011	157 (A)	97¶	This study
Ethiopia, Sudan	2013	35 (A)	97††	(*5*)
Jordan	2013	11 (J)	100§,¶	(*3*)
Nigeria	2010–2011	358 (A)	94¶	This study
Oman	2013	50 (A)	100§,¶	(*2*)
Qatar	2013	14 (A)	100§,¶	(*10*)
Saudi Arabia	2010–2013	65 (J)	72††	(*8*)
	2010–2013	245 (A)	95††	(*8*)
	2013	104 (J)	55‡‡	(*9*)
	2013	98 (A)	95‡‡	(*9*)
	2010	21 (J)	76‡‡	(*9*)
	2010	23 (A)	91‡‡	(*9*)
	2009	56 (J)	72‡‡	(*9*)
	2009	26 (A)	92‡‡	(*9*)
	2004	6 (A)	100‡‡	(*9*)
	1996	6 (A)	100‡‡	(*9*)
	1994	123 (A)	93‡‡	(*9*)
	1993	2 (A)	100‡‡	(*9*)
	1992	1 (A)	100‡‡	(*9*)
Tunisia	2009	46 (J)	30¶	This study
	2009	158 (A)	54¶	This study

*Camel age range indicated where known. J, juvenile ≤2 y of age; A, adult >2 y of age.
†Determined by recombinant spike immunofluorescence assay.
‡Determined by neutralization test.
§Determined by nucleocapsid western blot.
¶Determined by S1 micro-array.
#Determined by whole virus IFA.
**Determined by pseudoparticle neutralization test.
††Determined by complete virus infected cell ELISA.

estimated time of identification of the most common ancestor for the MERS-CoV strains found in humans to date (*14*). The earliest serologic indication for circulation of MERS-CoV or MERS-like CoV in dromedaries was observed in 1992; however, this result was based on results of a whole-virus ELISA with undescribed specificity (*9*). On the basis of well-validated array and neutralization tests, the study of dromedaries in the United Arab Emirates showed the presence of MERS-CoV or MERS-Cov–like antibodies as early as 2003 (*6*). The accumulated data on MERS-CoV serology in dromedaries (Figure 2; Table 1) show circulation of MERS-CoV or MERS-like CoV in dromedaries in Africa and the Arabian Peninsula well before 2012, when the first cases in humans were identified, and show overall high levels of seropositivity, including in animals from countries without reported human cases.

A question raised by these findings is whether human cases occur outside the Arabian Peninsula and if such cases are currently underdiagnosed in Africa. In addition, for the whole region, the possibility exists that MERS-CoV illness occurred before its discovery in 2012 and that such infection has been overlooked in the areas with evidence for virus circulation among animals during the past 10 years. Retrospective studies of cohorts of humans with respiratory illnesses of unknown etiology should address this notion.

Alternative explanations for the lack of cases in Africa could be the following: a different risk profile, for instance, related to demographics and local practices; or subtle genetic differences in the circulating virus strain. Full-genome sequencing, virus isolation, and phenotypic characterization of viruses circulating outside the Arabian Peninsula will resolve this issue. Meanwhile, awareness of MERS-CoV infections should be raised among clinicians in Africa.

Acknowledgments

We thank Lotfi Sayahi from Tunisia for collecting samples within the framework of his dissertation of veterinary medicine within the research laboratory, funded by the Tunisian Ministry of Higher Education and Research. We also thank C. van Maanen and C. Gortazar-Schmidt for establishing contact between this research group and researchers in Nigeria and Tunisia.

Table 2. Background data and Middle East respiratory syndrome coronavirus serology results of selected camel serum samples from Nigeria, Ethiopia, and Tunisia*

Country, sample ID	Region	Age	Sex	MERS S1 (1:20)	MERS S1 (1:320)	MERS S1 (1:640)	VNT
Nigeria							
1	Kano	7	M	63410	52,254	NT	640
2	Kano	2	F	63,022	10,998	4,585	320
3	Adamawa	6	M	63,146	41,200	20,627	1,280
4	Kano	2	M	63,213	63,331	63,353	1,280
5	Sokoto	2	F	63,123	8,215	–	80
6	Borno	7	M	63,173	13,873	7,471	160
7	Borno	6	F	63,065	63,065	NT	2,560
8	Sokoto	7	F	64,118	63,285	54,669	640
9	Borno	6	M	63,592	28,033	NT	80
10	Sokoto	6	F	64,176	63,427	35,190	640
11	Sokoto	2	F	–	NT	NT	<20
12	Adamawa	7	M	–	NT	NT	<20
13	Unknown	7	M	–	NT	NT	<20
14	Kano	7	M	–	NT	NT	<20
Ethiopia							
1	Somali	5	F	63,592	63,357	50,563	640
2	Afar	6	F	63,341	63,005	NT	2,560
3	Afar	13	F	63,366	63,205	63,467	1,280
4	Afar	10	F	63,206	63,299	NT	640
5	Afar	5	F	63,466	10,583	5,911	160
6	Fentale	<4	M	63,408	63,480	60,135	1,280
7	Afar	4	F	63,476	33,909	19,161	80
8	Afar	4	F	–	NT	NT	<20
9	Afar	2	M	–	NT	NT	<20
10	Afar	1	F	10,937	NT	NT	<20
11	Afar	3	F	18,269	NT	NT	<20
12	Fentale	>8	F	63,486	23,654	10,246	1,280
13	Afar	6	F	63,496	63,380	53,030	1,280
14	Afar	1	F	63,401	19,087	9,834	80
Tunisia							
1	Sidi Bouzid	8	F	–	NT	NT	<20
2	Sidi Bouzid	8	F	63,217	20,620	NT	80
3	Sidi Bouzid	6	F	–	NT	NT	<20
4	Sidi Bouzid	1	M	–	NT	NT	<20
5	Kebili	7	M	63,139	–	–	320
6	Kebili	4	M	63,113	–	–	160
7	Sidi Bouzid	1	M	–	NT	NT	<20
8	Sidi Bouzid	9	F	63,005	17,821	9,652	80
9	Sidi Bouzid	6	F	–	NT	NT	<20
10	Kebili	4	M	63,120	18,320	9,732	160
11	Sidi Bouzid	<1	M	–	NT	NT	<20
12	Sidi Bouzid	2	F	63,060	63,236	63,366	2,560
13	Sousse	13	F	63,220	50,510	26,575	320
14	Sidi Bouzid	5	F	–	NT	NT	<20

*MERS S1, Middle East respiratory syndrome coronavirus S1 micro-array. Serum dilutions tested 1:20, 1:320 or 1:640. Results expressed as relative mean fluorescent intensity (RFU) for each set of quadruplicate spots of antigen, with a cutoff of 4,000 RFU and >63.000 RFU as saturation signal; VNT, virus neutralization test (highest neutralizing serum dilution indicated); NT, not tested; –, negative.

Sample collection in Ethiopia was funded by a thematic research grant for Animal Health Improvement by Addis Ababa University, the Office of the Vice President for Research and Technology Transfer. B.H. was funded by the European Union FP7 project EMPERIE (contract number 223498), and M.K. was funded by the European Union FP7 project ANTIGONE (contract number 278976).

Dr Reusken is a public health virologist at the Viroscience Department of Erasmus Medical Center, Rotterdam, the Netherlands. Her research interests include viruses operating at the animal-human interface.

References

1. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med. 2012;367:1814–20. http://dx.doi.org/10.1056/NEJMoa1211721
2. Reusken CB, Haagmans BL, Muller MA, Gutierrez C, Godeke GJ, Meyer B, et al. Middle East respiratory syndrome coronavirus neutralising serum antibodies in dromedary camels: a comparative serological study. Lancet Infect Dis. 2013;13:859–66. http://dx.doi.org/10.1016/S1473-3099(13)70164-6
3. Reusken CB, Ababneh M, Raj VS, Meyer B, Eljarah A, Abutarbush S, et al. Middle East Respiratory Syndrome coronavirus (MERS-CoV) serology in major livestock species in an affected region in Jordan, June to September 2013. Euro Surveill. 2013;18:20662.

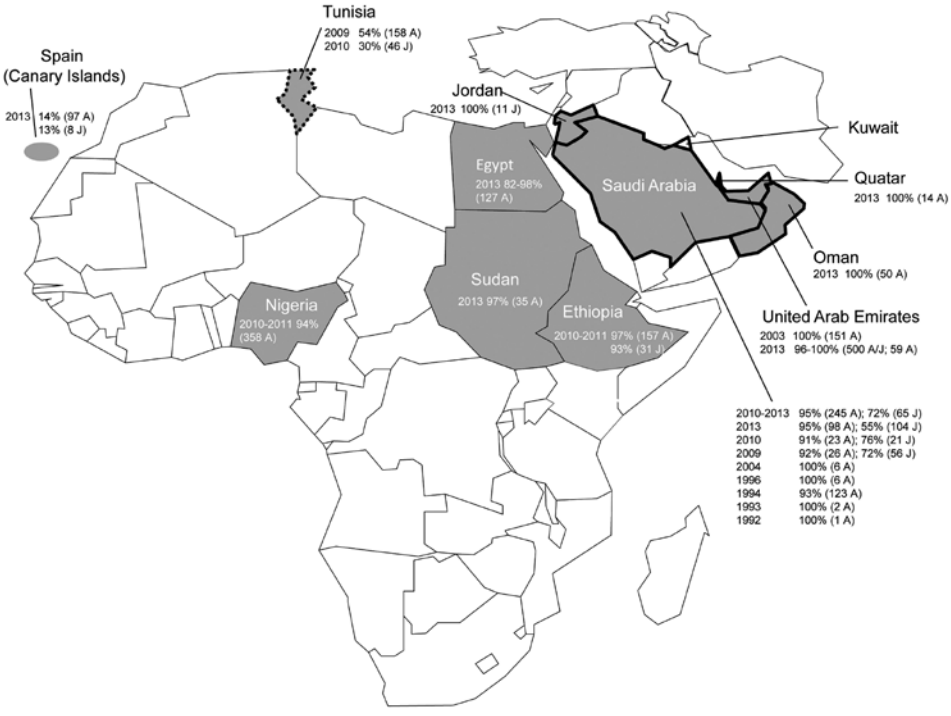


Figure 2. Geographic distribution of serologic evidence for Middle East respiratory syndrome coronavirus (MERS-CoV) or MERS-like CoV circulation in dromedaries in Africa and the Arabian Peninsula. Gray shading indicates countries with seropositive dromedaries; solid black outline indicates countries with primary human cases; dotted outline indicates countries with secondary human cases. For each country with affected dromedaries, the year of sampling, % seropositive, total number tested, and age group are indicated. A, adult, >2 years of age; J, juvenile, ≤2 years of age. Details on serologic tests used and references are in Table 1.

4. Perera RA, Wang P, Gomaa MR, El-Shesheny R, Kandeil A, Bagato O, et al. Seroepidemiology for MERS coronavirus using microneutralisation and pseudoparticle virus neutralisation assays reveal a high prevalence of antibody in dromedary camels in Egypt, June 2013. Euro Surveill. 2013;18:20574.
5. Chu DKW, Poon LLM, Gomaa MM, Shehata MM, Perera RAPM, Zeid DA, et al. MERS coronaviruses in dromedary camels, Egypt. 2014 [cited 2014 1 March]; http://dx.doi.org/10.3201/eid2006.140299
6. Meyer B, Müller MA, Corman VM, Reusken CBEM, Ritz D, Godeke GJ, et al. Antibodies against MERS coronavirus in dromedary camels, United Arab Emirates, 2003 and 2013. Emerg Infect Dis. 2014;20:552–9. http://dx.doi.org/10.3201/eid2004.131746
7. Woo PCY, Lau SKP, Wernery U, Wong EYM, Tsang AKL, Johnson B, et al. Novel betacoronavirus in dromedaries of the Middle East. Emerg Infect Dis. 2014;20:560–72. http://dx.doi.org/10.3201/eid2004.131769
8. Hemida MG, Perera RA, Wang P, Alhammadi MA, Siu LY, Li M, et al. Middle East Respiratory Syndrome (MERS) coronavirus seroprevalence in domestic livestock in Saudi Arabia, 2010 to 2013. Euro Surveill. 2013;18:20659.
9. Alagaili AN, Briese T, Mishra N, Kapoor V, Sameroff SC, de Wit E, et al. Middle East respiratory syndrome coronavirus infection in dromedary camels in Saudi Arabia. MBio. 2014;5(2). http://dx.doi.org/10.1128/mBio.00884-14
10. Haagmans BL, Al Dhahiry SH, Reusken CB, Raj VS, Galiano M, Myers R, et al. Middle East respiratory syndrome coronavirus in dromedary camels: an outbreak investigation. Lancet Infect Dis. 2014;14:140–5. http://dx.doi.org/10.1016/S1473-3099(13)70690-X
11. Reusken C, Mou H, Godeke GJ, van der Hoek L, Meyer B, Muller MA, et al. Specific serology for emerging human coronaviruses by protein microarray. Euro Surveill. 2013;18:20441.
12. Wünschmann A, Frank R, Pomeroy K, Kapil S. Enteric coronavirus infection in a juvenile dromedary (*Camelus dromedarius*). J Vet Diagn Invest. 2002;14:441–4. http://dx.doi.org/10.1177/104063870201400518
13. Hemida MG, Chu DKW, Poon LLM, Perera RAPM, Alhammadi MA, Ng H-Y, et al. MERS coronavirus in dromedary camel herd, Saudi Arabia. Emerg Infect Dis [Internet]. 2014 Jul [date cited]. http://dx.doi.org/10.3201/eid2007.140571
14. Cotten M, Watson SJ, Zumla AI, Makhdoom HQ, Palser AL, Ong SH, et al. Spread, circulation, and evolution of the Middle East respiratory syndrome coronavirus. MBio. 2014;5. http://dx.doi.org/10.1128/mBio.01062-13

Address for correspondence: Chantal B.E.M. Reusken, Erasmus MC, Viroscience; PO Box 2040, Rotterdam 3000 CA, Netherlands; email: c.reusken@erasmusmc.nl

Severe Murine Typhus with Pulmonary System Involvement

Thomas W. van der Vaart, Pieter P.A.M. van Thiel, Nicole P. Juffermans, Michèle van Vugt, Suzanne E. Geerlings, Martin P. Grobusch, and Abraham Goorhuis

We encountered a case of severe murine typhus complicated by acute respiratory distress syndrome. To determine worldwide prevalence of such cases, we reviewed the literature and found that respiratory symptoms occur in ≈30% of murine typhus patients. In disease-endemic areas, murine typhus should be considered for patients with respiratory symptoms and fever.

Murine typhus (endemic typhus) is a febrile illness caused by fleaborne *Rickettsia typhi*; it occurs mainly in environments where rats and humans live in close proximity. Murine typhus is found worldwide, but most reported cases originate from Southeast Asia, the Mediterranean region, and the United States. Among travelers, murine typhus is most frequently associated with travel to Southeast Asia (*1*). Recently, 2 cases of severe murine typhus with pulmonary manifestations have been reported (*2*,*3*). Near the same time, the Academic Medical Center (Amsterdam, the Netherlands) admitted a patient with severe murine typhus and respiratory failure. On the basis of these 3 cases, we hypothesized that pulmonary system involvement of murine typhus might be more common than previously assumed. We conducted this study because data on prevalence of pulmonary involvement in murine typhus are rarely reported. We therefore describe a clinical case and summarize the published literature on the pulmonary aspects of murine typhus.

The Study

In February 2012, a previously healthy 40-year-old man visited the Academic Medical Center outpatient department, reporting fever, headache, sweating, and nausea. The signs and symptoms had started 1 day earlier, on the day of his return from a 1-month holiday in Borneo. He reported frequent insect bites and exposure to fresh water. He had taken malaria chemoprophylaxis as recommended, and his vaccinations were up to date. Physical examination indicated that he was afebrile, was hemodynamically stable, and had a discrete macular rash on the trunk but no eschar. Laboratory results showed a hemoglobin concentration (16.8 g/dL) within reference range, a leukocyte count of 4,700 cells/mm^3 with lymphopenia (1,090 cells/mm^3), and thrombocytopenia (116,000 cells/mm^3). C-reactive protein (42 mg/L) and serum creatinine (1.32 mg/dL) concentrations were moderately elevated. A thick smear showed no plasmodia, and a dengue antigen test result was negative. By the next day, the patient's condition had deteriorated; he was experiencing chills, his temperature was 39°C, and the rash had become more pronounced. He was admitted to the hospital and given doxycycline (200 mg twice a day) for suspected rickettsiosis or leptospirosis. After admission, his condition deteriorated further; increasing dyspnea progressed to respiratory failure, necessitating intubation and admission to the intensive care unit on the second day after admission.

Chest radiographs revealed bilateral interstitial abnormalities (Figure). His condition fit a diagnosis of acute respiratory distress syndrome (ARDS). Empirical treatment was expanded to include broad-spectrum antimicrobial drugs and oseltamivir.

On the fourth day of intubation, the patient's condition improved; he was extubated 1 day later. Cultured blood, urine, and bronchial fluid remained sterile, and test results for *Leptospira*, *Legionella*, influenza virus, and HIV were negative. All antimicrobial drugs except doxycycline were discontinued; doxycycline was continued for a total of 14 days.

Serum collected 1 day after admission showed weakly positive IgG against *R. typhi;* after 7 days, the immunofluorescent antibody titer had increased 4-fold (from 1:64 to >1:256). The patient recovered completely and was doing well at his last follow-up visit.

To determine prevalence of such cases, we conducted a search of published studies mentioning pulmonary manifestations of murine typhus (details in the online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/13-1421-Techapp1.pdf). From 779 records, we selected 22 cohort studies and 18 case studies that, according to title and abstract, were relevant to our research question. We differentiated between studies with individual patient data (case reports and case series) and studies without individual patient data (cohort studies). For each study, we recorded year of publication, study design, and country of infection. We also recorded the presence of pulmonary involvement, defined as cough and any mention of an abnormal finding on chest radiograph, without further distinction.

An overview of study characteristics detailing prevalence of cough and chest radiograph abnormalities is provided in the online Technical Appendix Table 1. Two

Author affiliation: Academic Medical Center, Amsterdam, the Netherlands

DOI: http://dx.doi.org/10.3201/eid2008.131421

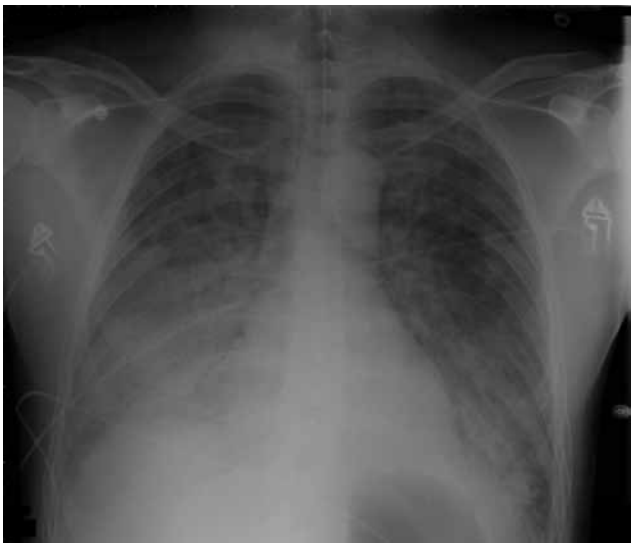


Figure. Chest radiograph of 40-year-old man with acute respiratory distress syndrome as a complication of murine typhus.

studies were prospective population-based studies of the causative agent of fever of unknown origin. The remaining 20 studies were all *Rickettsia* spp. specific; in 17 of these studies, patients had been recruited retrospectively from hospital databases or chart reviews.

The 22 study reports that contained data on the presence or absence of cough together accounted for 1,060 patients with murine typhus. The prevalence of cough among these patients ranged from 0 to 66%. Mean prevalence (all patients from all studies combined) of cough was 30.1% (95% CI 23.3–36.9).

Data on presence or absence of radiographic abnormalities were mentioned in 9 study reports (*4*–*12*). Taken together, these studies evaluated 621 patients and 104 chest radiographs showing abnormalities, leading to a prevalence rate of chest radiograph abnormalities of 16.7% (95% CI 8.21–25.5). The cohort studies reported 2 cases of ARDS, 1 with a fatal outcome. The Table shows the full-text descriptions of chest radiograph abnormalities.

Pulmonary manifestations were also documented by the case studies. Among these studies, 2 patients had ARDS, 7 had bilateral pulmonary infiltrates, 5 had unilateral pulmonary infiltrates, 1 had pulmonary embolism, and 1 had respiratory failure (no chest radiograph was reported) (online Technical Appendix Table 2). We also found that cough occurred more commonly among patients in studies conducted in Asia (99 [38.2%] of 259 patients) than among those in studies conducted in the Mediterranean region (118 [25.8%] of 457) and North America (56 [23.3%] of 240).

In the literature, we found 7 reported cases of respiratory distress associated with murine typhus (including the case reported here), 2 of which described respiratory distress not classified as ARDS (*2*,*13*). Of these 7 case-patients with ARDS/respiratory distress, 5 (71%) patients were from Asia (*2*,*3*,*11*,*13*), 1 was from the Mediterranean region (*4*), and 1 was from the United States (*14*).

Conclusions

Cough and chest radiograph abnormalities were frequent manifestations of murine typhus. For cough associated with murine typhus, we found a prevalence rate of ≈30%. The prevalence of chest radiograph abnormalities was more difficult to ascertain because this result was less often reported and more influenced by bias. The pulmonary aspects of *R. typhi* infection are probably the result of damaged pulmonary microcirculation, leading to pulmonary edema.

Severe pulmonary manifestations of murine typhus are thought to be rare. The case reported here is unusual in that the symptoms progressed rapidly and the response to doxycycline was relatively slow. It is worth noting that we found no more than 2 reported cases of fatal murine typhus associated with pulmonary system disease; both were the result of severe disease complicated by ARDS.

Table. Pulmonary manifestations of murine typhus reported from cohort studies*

Reference	Year	Region	No. cases	No. chest radiographs	No. chest radiographs showing abnormalities	Details
(*5*)	2001	USA	97	81	10	Radiographic evidence of pneumonitis in 10/81 cases
(*6*)	2004	Mediterranean	87	NM	6	4 cases of pulmonary infiltrates, 2 cases of pleural effusion
(*7*)	2008	Asia	50	16	6	6 cases of pulmonary infiltrates
(*8*)	2009	Mediterranean	41	NM	22	Abnormal chest radiographs for 22 patients
(*9*)	2009	Asia	28	15	9	9 bilateral reticulonodular infiltrates
(*10*)	2012	Mediterranean	90	NM	15	13 cases of interstitial pneumonia, 2 cases of pleural effusion
(*11*)	2012	Asia	81	49	16	15 cases of pulmonary infiltrates, 1 case of ARDS
(*12*)	2013	Mediterranean	43	39	12	2 cases of alveolar infiltrates, 10 cases of interstitial infiltrates

*NM, not mentioned; ARDS, acute respiratory distress syndrome.

In addition, we noted possible geographic variation in pulmonary manifestations. Most cases of severe murine typhus with pulmonary manifestations originated in Asia, and cough was more frequently a symptom among patients in Asia.

The primary strength of our study is the extensive literature search, which covered studies from different parts of the world and included cohort studies and case series. The main limitation of this study is the retrospective nature of the data collection for most studies, which is prone to bias and renders meaningful statistical analysis of results impossible. Therefore, prospective studies evaluating pulmonary manifestations of murine typhus and possible geographic variation are needed.

Although murine typhus usually follows a benign course, severe disease with pulmonary manifestations, including ARDS, can occur, as described for the patient reported here. We suggest that murine typhus should be included in the differential diagnosis for any patient who has a fever and respiratory signs and who has been in a typhus-endemic area within the incubation period.

Mr van der Vaart is a sixth-year medical student at the Academic Medical Center of the University of Amsterdam and is currently enrolled in his clinical rotations. His main research focus is infectious diseases.

References

1. Jensenius M, Han PV, Schlagenhauf P, Schwartz E, Parola P, Castelli F, et al. Acute and potentially life-threatening tropical diseases in western travelers—a GeoSentinel multicenter study, 1996–2011. Am J Trop Med Hyg. 2013;88:397–404. http://dx.doi.org/10.4269/ajtmh.12-0551
2. Stockdale AJ, Weekes MP, Kiely B, Lever AM. Case report: severe typhus group rickettsiosis complicated by pulmonary edema in a returning traveler from Indonesia. Am J Trop Med Hyg. 2011;85:1121–3. http://dx.doi.org/10.4269/ajtmh.2011.11-0340
3. Sakamoto N, Nakamura-Uchiyama F, Kobayashi K, Takasaki T, Ogasawara Y, Ando S, et al. Severe murine typhus with shock and acute respiratory failure in a Japanese traveler after returning from Thailand. J Travel Med. 2013;20:50–3. http://dx.doi.org/10.1111/j.1708-8305.2012.00678.x
4. Bernabeu-Wittel M, Pachon J, Alarcon A, Lopez-Cortes LF, Viciana P, Jimenez-Mejias ME, et al. Murine typhus as a common cause of fever of intermediate duration: a 17-year study in the south of Spain. Arch Intern Med. 1999;159:872–6. http://dx.doi.org/10.1001/archinte.159.8.872
5. Whiteford SF, Taylor JP, Dumler JS. Clinical, laboratory, and epidemiologic features of murine typhus in 97 Texas children. Arch Pediatr Adolesc Med. 2001;155:396–400. http://dx.doi.org/10.1001/archpedi.155.3.396
6. Gikas A, Doukakis S, Pediaditis J, Kastanakis S, Manios A, Tselentis Y. Comparison of the effectiveness of five different antibiotic regimens on infection with *Rickettsia typhi*: therapeutic data from 87 cases. Am J Trop Med Hyg. 2004;70:576–9.
7. Zimmerman MD, Murdoch DR, Rozmajzl PJ, Basnyat B, Woods CW, Richards AL, et al. Murine typhus and febrile illness, Nepal. Emerg Infect Dis. 2008;14:1656–9. http://dx.doi.org/10.3201/eid1410.080236
8. Gikas A, Kokkini S, Tsioutis C, Athenessopoulos D, Balomenaki E, Blasak S, et al. Murine typhus in children: clinical and laboratory features from 41 cases in Crete, Greece. Clin Microbiol Infect. 2009;15(Suppl 2):211–2. doi: 10.1111/j.1469-0691.2008.02133.x
9. Suputtamongkol Y, Suttinont C, Niwatayakul K, Hoontrakul S, Limpaiboon R, Chierakul W, et al. Epidemiology and clinical aspects of rickettsioses in Thailand. Ann N Y Acad Sci. 2009;1166:172–9. http://dx.doi.org/10.1111/j.1749-6632.2009.04514.x
10. Chaliotis G, Kritsotakis EI, Psaroulaki A, Tselentis Y, Gikas A. Murine typhus in central Greece: epidemiological, clinical, laboratory, and therapeutic-response features of 90 cases. Int J Infect Dis. 2012;16:e591-6. doi: 10.1016/j.ijid.2012.03.010
11. Chang K, Chen YH, Lee NY, Lee HC, Lin CY, Tsai JJ, et al. Murine typhus in southern Taiwan during 1992–2009. Am J Trop Med Hyg. 2012;87:141–7. http://dx.doi.org/10.4269/ajtmh.2012.11-0465
12. Znazen A, Hammami B, Mustapha AB, Chaari S, Lahiani D, Maaloul I, et al. Murine typhus in Tunisia: a neglected cause of fever as a single symptom. Med Mal Infect. 2013;43:226–9. http://dx.doi.org/10.1016/j.medmal.2013.02.007
13. Tan L, Beersma TM, van Beek Y, van Genderen PJ. Two travellers suffering from typhus [in Dutch]. Ned Tijdschr Geneeskd. 2011;155:A3845.
14. Walker DH, Parks FM, Betz TG, Taylor JP, Muehlberger JW. Histopathology and immunohistologic demonstration of the distribution of *Rickettsia typhi* in fatal murine typhus. Am J Clin Pathol. 1989;91:720–4.

Address for correspondence: Abraham Goorhuis, Center for Tropical and Travel Medicine, Division of Infectious Diseases, Department of Internal Medicine, Academic Medical Center, University of Amsterdam, Meibergdreef 9, Room F4-217, 1105 AZ Amsterdam, the Netherlands; email: a.goorhuis@amc.uva.nl

Detection of East/Central/South African Genotype of Chikungunya Virus in Myanmar, 2010

Mya Myat Ngwe Tun, Kyaw Zin Thant, Shingo Inoue, Takeshi Nabeshima, Kotaro Aoki, Aung Kyaw Kyaw, Tin Myint, Thi Tar, Kay Thwe Thwe Maung, Daisuke Hayasaka, and Kouichi Morita

In 2010, chikungunya virus of the East Central South African genotype was isolated from 4 children in Myanmyar who had dengue-like symptoms. Phylogenetic analysis of the E1 gene revealed that the isolates were closely related to isolates from China, Thailand, and Malaysia that harbor the A226V mutation in this gene.

Chikungunya virus (CHIKV; family *Togaviridae,* genus *Alphavirus*) is transmitted to humans by the bite of infected mosquitoes of the genus *Aedes*, particularly *Ae. aegypti* and *Ae. albopictus* (*1*). Dengue viruses (DENVs; family *Flaviviridae*, genus *Flavivirus*) are transmitted by the same mosquito vectors. CHIKV and DENV are often found co-circulating (*2*,*3*) and cause similar clinical signs and symptoms, including fever, arthralgia, headache, rash, and, in some cases, hemorrhage. Hemorrhage, however, is rare in CHIKV infection. Because of the similarity in signs and symptoms, misdiagnosis and underreporting of chikungunya infection in dengue-endemic areas are common (*3*).

Chikungunya is endemic to the tropical areas of Africa and Asia (*5*) and generally manifests as mild illness, but outbreaks in the Indian Ocean region have shown that the disease can lead to severe and life-threatening complications (*4*). CHIKV strains are clustered into 3 distinct genotypes: West African, East/Central/South African (ECSA), and Asian (*5*). Reemergence of CHIKV of the ECSA genotype began in Kenya in 2004, and during 2005–2010, the genotype spread to Comoros, Madagascar, Reunion Island, India, Sri Lanka, Singapore, Thailand, Malaysia, China, and Italy (*6*).

In Myanmar, CHIKV infection was serologically confirmed in 1973, and the virus was indicated to be of the Asian genotype (no sequence data available) (*5*,*7*). A few serologic studies in this country have found CHIKV-specific antibodies in patients who were suspected of having dengue virus infection (*7*–*9*), but no reports have been published on the molecular epidemiology of CHIKV. We conducted a serologic and molecular epidemiologic surveillance study of possible CHIKV infection among children who had a clinical diagnosis of dengue fever in Myanmar during 2010.

The Study

During July–October 2010, serum samples were collected from 116 children (≤12 years of age) who were

Figure 1. Location of Mandalay Children Hospital (triangle) in Myanmar.

Author affiliations: Nagasaki University, Nagasaki, Japan (M.M. Ngwe Tun, S. Inoue, T. Nabeshima, K. Aoki, A.K. Kyaw, D. Hayasaka, K. Morita); Upper Myanmar Medical Research, Pyin Oo Lwin, Myanmar (K.Z. Thant, A.K. Kyaw, K.T.T. Maung); and Mandalay Children Hospital, Mandalay, Myanmar (T. Myint, T. Tar)

DOI: http://dx.doi.org/10.3201/eid2008.131431

admitted to Mandalay Children Hospital in Mandalay, Myanmar (Figure 1). These children had received a clinical diagnosis of dengue, signs and symptoms of which included fever, hemorrhage, shock, and thrombocytopenia. A blood sample was collected from each child, 107 during the acute phase of illness (1–7 days after onset of fever) and 9 during the convalescent phase (8–16 days after onset of fever). Serum samples were stored at -70°C until further analysis. Informed consent was obtained from the accompanying parents or legal guardians before blood samples were collected by following the research protocol (approval no. 1/2010 by the Institutional Ethical Committee on Medical Research Involving Human Subjects in Myanmar).

All 116 serum samples were tested for IgM against CHIKV and DENV by using in-house IgM capture ELISAs and for IgG against CHIKV and flaviviruses by using in-house IgG indirect ELISAs (*10*). Results of IgM capture ELISAs showed 6 (5.2%) samples were positive for IgM against CHIKV only, 47 (40.5%) for IgM against DENV only, and 7 (6.0%) for IgM against CHIKV and DENV (Table 1). Results of IgG indirect ELISA showed 8 (6.9%) samples were positive for IgG against CHIKV only, 47 (40.5%) for IgG against flavivirus only, and 9 (7.8%) for IgG against CHIKV and flavivirus (Table 1).

For PCR testing, 10 μL of serum from each acute-phase sample was inoculated onto *Ae. albopictus* clone C6/36 mosquito cells, which were then incubated at 28°C for 7 days. RNA was extracted from infected culture fluid by using a QIAamp Viral RNA Mini Kit (QIAGEN, Hilden, Germany), and reverse transcription PCR was performed by using Superscript III Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). PCR was conducted by using Takara Tks Gflex DNA polymerase (Takara Bio, Shiga, Japan). All kits were used according to the manufacturer's instructions.

For the 107 acute-phase serum samples tested, 4 CHIKV strains were isolated from infected culture fluid. Detailed information about the children from which these samples were collected and the test results are shown in Table 2. The complete E1 gene nucleotide sequences (positions 9952–11271) for the 4 CHIKV isolates were determined and deposited into GenBank (accession nos. KF590564–7), and nucleotide sequences were aligned by using MAFFT version 7.058b (http://mafft.cbrc.jp/alignment/software). A Bayesian maximum clade credibility tree was constructed by using MrBayes version 3.1.2 (http://mrbayes.sourceforge.net), and a phylogenetic tree was drawn by using FigTree version 1.4.0 software (http://beast.bio.ed.ac.uk/FigTree). The phylogenetic tree of CHIKV was inferred for the 4 strains from Myanmar and 30 reference strains available from GenBank; results showed that the 4 CHIKV isolates from Myanmar belonged to the ECSA genotype (Figure 2).

Table 1. Antibody profiles of samples from 116 children who had suspected dengue virus infection, Myanmar, 2010*

Profile	CHIKV IgM	CHIKV IgG	DENV IgM	Flavivirus IgG	Total no. patients
A	+	+	–	+	2
B	+	+	–	–	4
C	+	–	–	+	0
D	+	–	–	–	0
E	–	+	+	+	4
F	–	+	+	–	2
G	–	–	+	+	32
H	–	–	+	–	9
I	+	+	+	+	3
J	+	+	+	–	0
K	+	–	+	+	2
L	+	–	+	–	2
M	–	+	–	+	0
N	–	+	–	–	2
O	–	–	–	+	13
P	–	–	–	–	41

*CHIKV, chikungunya virus; DENV, dengue virus; +, positive; –, negative.

Conclusions

Serologic results from this study confirmed that 47 (40.5%) of the 116 children who had received a clinical diagnosis of dengue had DENV infection only, but 6 (5.2%) had IgM capture ELISA results showing infection with CHIKV; CHIKV was isolated from 4 of these patients. Cross-reactivity in the IgM response against other alphaviruses in the same antigenic complex to which CHIKV belongs is possible (*11*), but other alphaviruses have not been reported in Myanmar. In addition, 7 (6.0%) of the children had positive test results for IgM against CHIKV and DENV. This finding could be the result of sequential infection by the 2 viruses in a short period or by concurrent infection with both viruses. Concurrent CHIKV and DENV infections have been reported in Gabon and Singapore (*3*,*12*). A total of 6.9%, 40.5%, and 7.8% of

Table 2. Characteristics of and testing results for 4 patients who had suspected dengue virus infection and from whom chikungunya virus was isolated, Myanmar. 2010*

Patient no.	Age, y	Days from illness onset to blood extraction*	Diagnosis at admission	CHIKV IgM	CHIKV IgG	CHIKV RT-PCR	DENV IgM	DENV RT-PCR	Flavivirus IgG
7	6.5	1	DHF I	Neg	Neg	Pos	Pos	Neg	Pos
20	11	2	DHF II	Neg	Neg	Pos	Neg	Neg	Neg
30	6	4	DF	Neg	Neg	Pos	Neg	Neg	Neg
105	7	3	DHF II	Neg	Neg	Pos	Neg	Neg	Pos

*DHF grades I and II classified according to World Health Organization criteria, 1997. CHIKV, chikungunya virus; DENV, dengue virus; RT-PCR, reverse transcription PCR; DHF, dengue hemorrhagic fever; Pos, positive; Neg, Negative; DF, dengue fever.

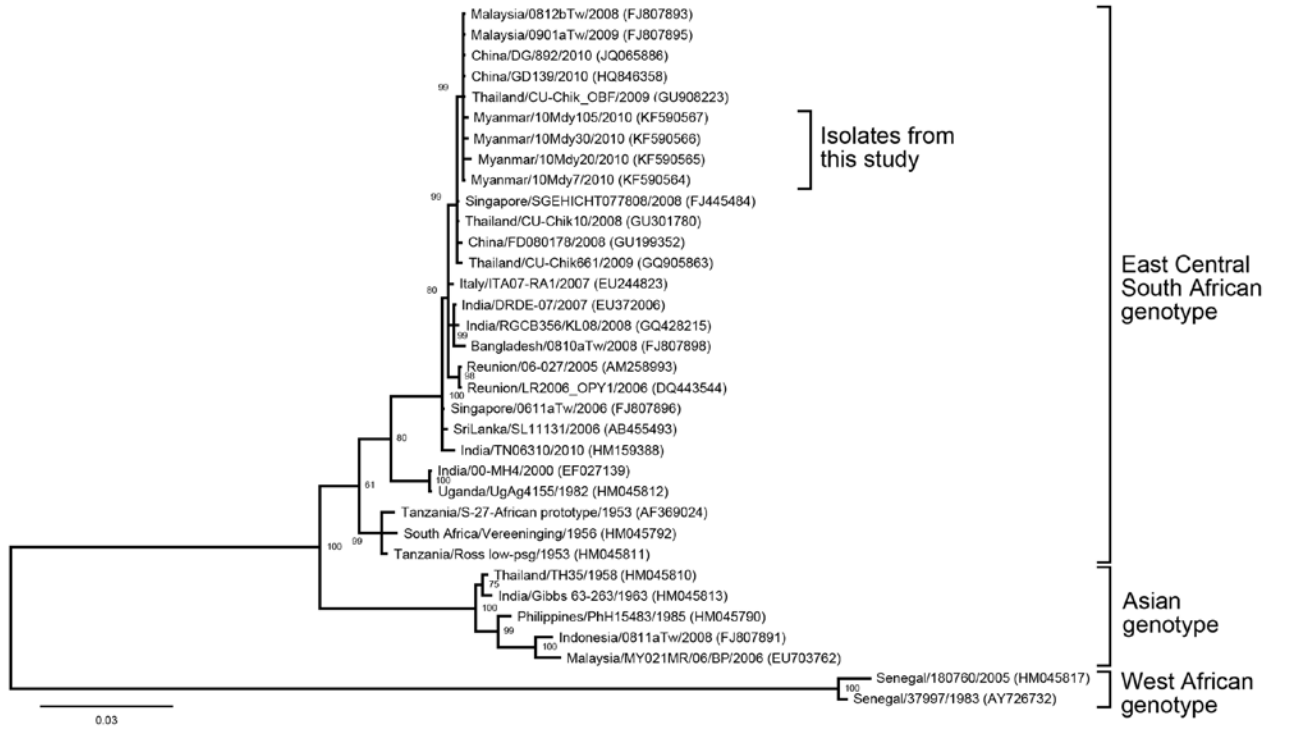


Figure 2. Phylogenetic tree constructed on the basis of whole E1 gene sequences of chikungunya viruses, showing location of 4 isolates obtained from children in Myanmar within the East Central South African genotype. Representative strains of each genotype obtained from GenBank are named by country of origin, strain name, year of isolation, and accession number (in parentheses). Bootstrap values are indicated at branch nodes. Scale bar indicates nucleotide substitutions per site.

children tested showed positive results for IgG against CHIKV, flavivirus, and CHIKV/flavivirus, respectively. This result suggests that arbovirus infections are common in Myanmar.

The 4 CHIKV isolates that we obtained belonged to an ECSA genotype subgroup that has the A226V mutation in the E1 gene. One of the 4 isolates (Myanmar/10Mdy20/2010) had an additional amino acid change (E1-E209V). All isolates had 99% nucleotide sequence similarity to isolates from China, Thailand, and Malaysia. Outbreaks caused by ECSA genotype CHIKV were reported in Malaysia in 2008–2009, in Thailand in 2008–2009, and in China in 2010 (*7*,*13*,*14*). The timing of these findings suggests that the strains of CHIKV we found in Myanmar were introduced from neighboring countries. Human migratory movements between the borders of Myanmar and neighboring countries such as India, Thailand, and China for trade and travel purposes are increasing and could be a contributing factor to the movement of the virus and the outbreaks. In Southeast Asia, CHIKV strains have been isolated not only from humans and mosquitoes but also from monkeys (*15*). We recommend surveillance for chikungunya and its vectors to prevent future outbreaks of CHIKV infection among the human population in Myanmar.

Acknowledgments

We thank Corazon Cerilla Buerano for scientific discussion. We also appreciate the support of the members of the Department of Virology, Institute of Tropical Medicine, Nagasaki University.

This work was supported by the Grant-in-Aid for Scientific Research (KAKENHI); Global Center of Excellence Program, Ministry of Education, Culture, Sports, Science and Technology, Japan; Japan Initiative for Global Research Network on Infectious Diseases; Research on Emerging and Re-emerging Infectious Diseases, JST, Japan (H23-shinkou-ippan-010); and grant(s) in-aid for scientific research by the Ministry of Health, Labour and Welfare, Japan.

Dr Ngwe Tun is a research scientist at the Department of Virology, Institute of Tropical Medicine, Nagasaki University. Her research interests focus on the tropical infectious diseases, with special emphasis on arboviruses.

References

1. Schwartz O, Albert ML. Biology and pathogenesis of chikungunya virus. Nat Rev Microbiol. 2010;8:491–500. http://dx.doi.org/10.1038/nrmicro2368
2. Caron M, Paupy C, Grard G, Becquart P, Mombo I, Nso BB, et al. Recent introduction and rapid dissemination of chikungunya virus

and dengue virus serotype 2 associated with human and mosquito coinfections in Gabon, central Africa. Clin Infect Dis. 2012;55:e45–53. http://dx.doi.org/10.1093/cid/cis530
3. Chahar HS, Bharai P, Dar L, Guleria R, Kabra SK, Broor S. Co-infection with chikungunya virus and dengue virus in Delhi, India. Emerg Infect Dis. 2009;15:1077–80. http://dx.doi.org/10.3201/eid1507.080638
4. Economopoulou A, Dominguez M, Helynck B, Sissoko D, Wichmann O, Quenel P, et al. Atypical Chikungunya virus infections: clinical manifesations, mortality and risk factors for severe disease during the 2005–2006 outbreak on Réunion. Epidemiol Infect. 2009;137:534–41. http://dx.doi.org/10.1017/S0950268808001167
5. Powers AM, Logue CH. Changing patterns of chikungunya virus: re-emergence of a zoonotic arbovirus. J Gen Virol. 2007;88:2363–77. http://dx.doi.org/10.1099/vir.0.82858-0
6. Wu D, Zhang Y, Zhouhui Q, Kou J, Liang W, Zhang H, et al. Chikungunya virus with E1-A226V mutation causing two outbreaks in 2010, Guangdong, China. Virol J. 2013;10:174. http://dx.doi.org/10.1186/1743-422X-10-174
7. Thaung U, Ming CK, Swe T, Thein S. Epidemiology feactures of dengue and chikungunya infection in Burma. Southeast Asian J Trop Med Public Health. 1975;6:276–83.
8. Khai Ming C, Thein S, Thaung U, Tin U, Myint KS, Swe T, et al. Clinical and laboratory studies on haemorrhagic fever in Burma, 1970–72. Bull World Health Organ. 1974;51:227–35.
9. Thein S, La Linn M, Aaskov J, Aung MM, Aye M, Zaw A, et al. Development of a simple indirect enzyme-linked immunosorbent assay for the detection of immunoglobulin M antibody in serum from patients following an outbreak of chikungunya virus infection in Yangon, Myanmar. Trans R Soc Trop Med Hyg. 1992;86:438–42. http://dx.doi.org/10.1016/0035-9203(92)90260-J
10. Inoue S, Alonzo MT, Kurosawa Y, Mapua CA, Reyes JD, Dimaano EM, et al. Evaluation of a dengue IgG indirect enzyme-linked immunosorbent assay and a Japanese encephalitis IgG indirect enzyme-linked immunosorbent assay for diagnosis of secondary dengue virus infection. Vector Borne Zoonotic Dis. 2010;10:143–50. http://dx.doi.org/10.1089/vbz.2008.0153
11. Calisher CH, el-Kafrawi AO, Al-Deen Mahmud MI, Travassos da Rosa AP, Bartz CR, Brummer-Korvenkontio M, et al. Complex-specific immunoglobulin M antibody patterns in humans infected with alphaviruses. J Clin Microbiol. 1986;23:155–9.
12. Chang SF, Su C, Shu PY, Yang CF, Liao TL, Cheng CH, et al. Concurrent isolation of chikungunya virus and dengue virus from a patient with coinfection resulting from a trip to Singapore. J Clin Microbiol. 2010;48:4586–9. http://dx.doi.org/10.1128/JCM.01228-10
13. Huang JH, Yang CF, Su CL, Chang SF, Cheng CH, Yu SK, et al. Imported chikungunya virus strains, Taiwan, 2006–2009. Emerg Infect Dis. 2009;15:1854–6. http://dx.doi.org/10.3201/eid1511.090398
14. Rianthavorn P, Prianantathavorn K, Wuttirattanakowit N, Theamboonlers A, Poovorawan Y. An outbreak of chikungunya in southern Thailand from 2008 to 2009 caused by African strains with A226V mutation. Int J Infect Dis. 2010;14:e161–5. http://dx.doi.org/10.1016/j.ijid.2010.01.001
15. Apandi Y, Nazni WA, Noor Azleen ZA, Vythilingam I, Noorazian MY, Azahari AH, et al. The first isolation of chikungunya virus from non-human primates in Malysia. J Gen Mol Virol. 2009;1:35–9.

Address for correspondence: Kouichi Morita, Department of Virology, Institute of Tropical Medicine, Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan; email: moritak@nagasaki-u.ac.jp

Pulmonary Infection and Colonization with Nontuberculous Mycobacteria, Taiwan, 2000–2012

Jung-Yien Chien, Chih-Cheng Lai, Wang-Huei Sheng, Chong-Jen Yu, and Po-Ren Hsueh

We analyzed samples from 13,652 patients who had respiratory cultures positive for mycobacteria in Taiwan during 2000–2012 and found that 56.9% were positive for nontuberculous mycobacteria (NTM). Whereas annual prevalence of tuberculosis decreased during the study period, prevalence of NTM disease and colonization increased, particularly among older patients and male patients.

In countries to which tuberculosis (TB) is endemic, the isolation of nontuberculous mycobacteria (NTM) from clinical specimens, especially respiratory specimens, is not uncommon (*1*–*3*). In 2011, Taiwan recorded 12,634 new TB cases (55 cases/100,000 population) and 638 TB-related deaths (2.8 deaths/100,000 population); the overall incidence of TB fell 1.7% from 2010 (http://www.cdc.gov.tw/uploads/files/201308/9590e86d-875f-4d65-8435-dbcc95d9fe6d.pdf). However, in the clinical setting of patients with suspected TB, clinicians rely on local epidemiologic knowledge to evaluate whether cultures positive for mycobacteria are *Mycobacterium tuberculosis* (MTB) or NTM. Therefore, the prevalence of isolation of *M. tuberculosis* and NTM must be investigated so that the epidemiologic features of these pathogens can be better understood. Many studies have shown that there is great geographic diversity in the distribution of NTM species (*1,2,4,5*). However, few studies have investigated the distribution of NTM species, including colonizers and causative pathogens, with respect to patient gender and age (*6,7*).

Author affiliations: National Taiwan University College of Medicine Graduate Institute of Clinical Medicine, Taipei, Taiwan (J.-Y. Chien); Ministry of Health and Welfare Chest Hospital, Tainan (J.-Y. Chien); National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei (J.-Y. Chien, W.-H. Sheng, C.-J. Yu, P.-R. Hsueh); and C.-M. Medical Center, Liouying, Taiwan (C.-C. Lai)

DOI: http://dx.doi.org/10.3201/eid2008.131673

The Study

This study was conducted at the National Taiwan University Hospital (NTUH), a 2,500-bed tertiary medical center in northern Taiwan where 8,000 clinical visits occur daily. We evaluated all patients registered in the hospital's Mycobacterial Laboratory database with cultures positive for NTM or *M. tuberculosis* during 2000–2012. The techniques used in the preparation of different clinical specimens for cultures of mycobacteria have been described in a previous study (*2*). We used the definition of pulmonary NTM infection from the 2007 American Thoracic Society/Infections Disease Society of America NTM guideline, which encompassed 3 major components: clinical signs and symptoms, radiologic findings, and microbiologic evidence (*1*). If patients had positive cultures for both NTM and *M. tuberculosis*, we defined the patients as having *M. tuberculosis* infection only. The annual prevalence rates of NTM colonization and disease were calculated as the annual number of patients with NTM colonization and disease divided by the total number of patients who visited the NTUH, including outpatients and inpatients in each indicated year.

During January 2000–December 2012, a total of 13,652 nonduplicate isolates obtained from respiratory specimens had positive test results for mycobacteria; *M. tuberculosis* was isolated from 5,878 (43.1%) patients and NTM from 7,774 (56.9%) patients. In addition, cultures of extrapulmonary specimens from 823 patients were positive for NTM. We found a significant decreasing trend in *M. tuberculosis* isolation among positive mycobacteria cultures and a significant increasing trend in NTM isolation during the study period ($p<0.01$ for both trends).

Among the 3,317 patients who had NTM infections (Table 1, http://wwwnc.cdc.gov/EID/article/20/8/13-1673-T1.htm), the most prevalent species were *M. avium-intracellulare* complex (MAC) (n = 1,377, 41.5%) and *M. abscessus* (n = 710, 21.4%). Of the 4,457 patients who had with evidence of NTM colonization, the most prevalent species were MAC (n = 1,304, 29.3%) and *M. fortuitum* (n = 1,019, 22.9%) (Table 1).

In contrast to the decreasing trend in prevalence of TB during the study period, the annual incidence of pulmonary NTM disease and colonization increased significantly over time ($p<0.01$) (Figure 1). In addition, the clinical significance of mycobacterial isolates differed according to patient sex and age (Figure 2); the rate of NTM isolation increased with age, whereas the rate for *M. tuberculosis* isolation decreased with age (Figure 2, panel A; $p<0.01$). Most cases of NTM disease and colonization were found in patients 65–84 years of age (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/8/13-1673-Techapp1.pdf), and the risk of developing NTM disease significantly increased with age ($p<0.01$). We also found that the male:female ratio was significantly lower among

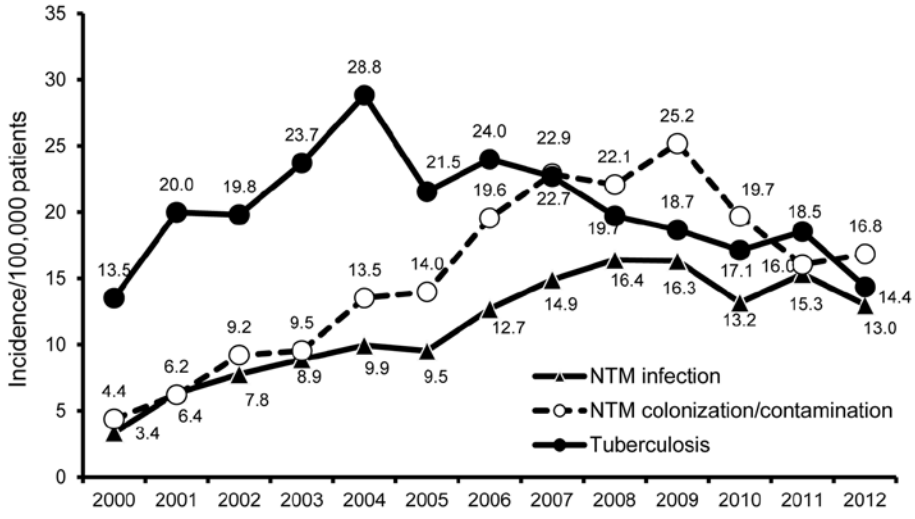


Figure 1. Annual incidence of tuberculosis, pulmonary nontuberculous mycobacteria (NTM) infection, and NTM colonization among patients registered in the National Taiwan University Hospital Mycobacterial Laboratory database with cultures positive for *Mycobacterium tuberculosis* or NTM, 2000–2012.

patients ≥45 years of age who had NTM infection or colonization than among patients in the same age group who had *M. tuberculosis* infection (p<0.01; online Technical Appendix Table).

Infections caused by MAC, *M. abscessus,* and *M. chelonae* were more common in female than in male patients. In contrast, diseases caused by *M. fortuitum* and *M. kansasii* were more common in male patients than in female patients. We also found that the prevalence of infection caused by *M. tuberculosis*, MAC, *M. abscessus* (p<0.01)*, M. fortuitum* (p<0.01)*, M. chelonae* (p = 0.04)*, M. kansasii* (p = 0.04)*,* and *M. gordonae* (p<0.01) in each age group differed significantly and that the prevalence of colonization by MAC (p<0.01), *M. fortuitum* (p = 0.01)*, M. gordonae* (p<0.01)*,* and *M. kansasii* (p<0.01)in each age group differed significantly (Table 2).

Conclusions

Our cross-sectional analysis of data from mycobacterial isolates collected over 13 years in northern Taiwan resulted in several notable findings. First, the prevalence of NTM pulmonary infection and colonization significantly increased during the period, whereas prevalence of *M. tuberculosis* infection significantly decreased. This phenomenon has been reported previously in Taiwan (*8*) and in other countries (*9*,*10*). We also found that the annual prevalence of NTM pulmonary infections and colonization significantly increased (p<0.01). The increasing trend in NTM infections has also been noted in South Korea, Canada, Denmark, Australia, the United States, and the Netherlands (*9*–*15*). These findings suggest that, although the rates of NTM pulmonary infection have gradually increased in Taiwan, more than half of NTM isolates caused colonization only.

Second, we found the frequency of isolation of *M. tuberculosis* and NTM from patients ≥85 years of age was 40.7% and 59.3%, respectively. Furthermore, half of NTM infections (54.1%) and colonizations (55.5%) occurred in patients ≥65 years of age. Our findings are consistent with those in a recent study conducted in South Korea (*7*) and

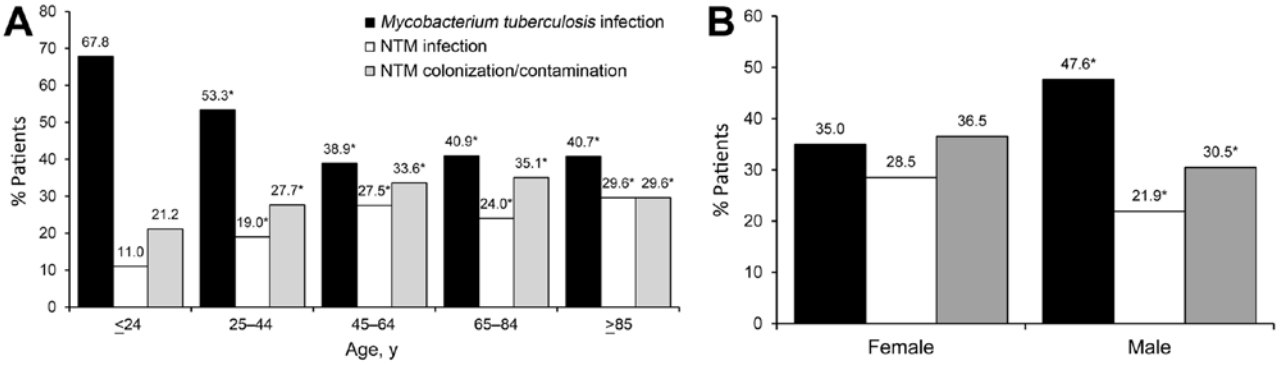


Figure 2. Rates of *Mycobacterium tuberculosis* infection, nontuberculous mycobacteria (NTM) infection, and NTM colonization/contamination, by age (A) and sex (B), among patients registered in the National Taiwan University Hospital Mycobacterial Laboratory database with cultures positive for *Mycobacterium tuberculosis* or NTM, 2000–2012. *p<0.01 compared with first group.

Table 2. Bacterial species implicated in pulmonary mycobacterial infections and colonization/contamination, by patient sex and age, Taiwan, 2000–2012*

Bacterial species	Sex, % (no.) patients			Age, y, % (no.) patients					
	Female, n = 4,931	Male, n = 8,721	p value	≤24, n = 572	25–44, n = 1,792	45–64, n = 4,062	65–84, n = 6,156	≥85, n = 1,070	p value
Mycobacterium tuberculosis infection	35.0 (1,725)	47.6 (4,153)	<0.01	67.8 (388)	53.3 (956)	38.9 (1,579)	40.9 (2,519)	40.7 (436)	<0.01
NTM probable infection									
M. avium–intracellulare complex	13.1 (645)	8.4 (732)	<0.01	3.7 (21)	6.3 (113)	11.1 (449)	10.3 (637)	14.7 (157)	<0.01
M. abscessus	6.8 (334)	4.3 (376)	<0.01	2.3 (13)	5.7 (102)	5.9 (241)	4.5 (275)	7.4 (79)	<0.01
M. fortuitum	2.7 (132)	3.6 (311)	<0.01	1.2 (7)	1.8 (33)	3.8 (155)	3.5 (218)	2.8 (30)	<0.01
M. chelonae	3.0 (148)	1.9 (164)	<0.01	2.6 (15)	1.9 (34)	2.9 (116)	2.0 (122)	2.3 (25)	0.04
M. kansasii	1.2 (61)	2.0 (173)	<0.01	0.7 (4)	2.1 (38)	1.9 (77)	1.7 (105)	0.9 (10)	0.04
M. gordonae	1.1 (55)	1.3 (110)	0.5	0.5 (3)	0.4 (8)	1.3 (52)	1.5 (91)	1.0 (11)	<0.01
Other	0.6 (31)	0.5 (45)	0.4	0	0.7 (12)	0.7 (28)	0.5 (31)	0.5 (5)	0.22
M. terrae complex	0.2 (8)	0.1 (9)	–	0	0.2 (4)	0.1 (4)	0.1 (8)	0.1 (1)	–
M. scrofulaceum	0.2 (8)	0.1 (7)	–	0	0	0.2 (9)	0.1 (6)	0	–
M. phlei	0.1 (5)	0.1 (9)	–	0	0.3 (5)	0.1 (3)	0.1 (5)	0.1 (1)	–
M. smegmatis	0	0.1 (10)	–	0	0.1 (1)	0.0 (2)	0.1 (5)	0.2 (2)	–
M. xenopi	0.1 (4)	0.1 (6)	–	0	0.1 (1)	0.0 (2)	0.1 (6)	0.1 (1)	–
M. flavescens	0.1 (4)	0	–	0	0.1 (1)	0.1 (3)	0	0	–
M. celatum	0	0.0 (2)	–	0	0	0.0 (2)	0	0	–
M. asiaticum	0	0.0 (1)	–	0	0	0	0.0 (1)	0	–
M. mageritense	0	0.0 (1)	–	0	0	0.0 (1)	0	0	–
M. szulgai	0.0 (1)	0	–	0	0	0.0 (1)	0	0	–
M. vaccae	0.0 (1)	0	–	0	0	0.0 (1)	0	0	–
NTM colonization/contamination									
M. avium–intracellulare complex	10.6 (525)	8.9 (779)	<0.01	4.4 (25)	6.7 (120)	9.6 (388)	10.7 (659)	10.5 (112)	<0.01
M. fortuitum	7.9 (389)	7.2 (630)	0.17	5.8 (33)	6.4 (114)	7.6 (310)	8.1 (499)	5.9 (63)	0.01
M. gordonae	6.0 (296)	4.7 (409)	<0.01	2.6 (15)	4.1 (73)	5.9 (241)	5.6 (343)	3.1 (33)	<0.01
M. abscessus	5.5 (273)	4.2 (365)	<0.01	4.5 (26)	4.1 (74)	5.0 (205)	4.6 (284)	4.6 (49)	0.64
M. kansasii	2.0 (101)	2.2 (191)	0.63	0.5 (3)	2.1 (38)	1.8 (72)	2.5 (153)	2.4 (26)	<0.01
M. chelonae	2.4 (120)	1.7 (149)	<0.01	2.4 (14)	2.3 (42)	1.8 (72)	2.0 (126)	1.4 (15)	0.31
Other	1.9 (96)	1.5 (134)	0.07	0.9 (5)	2.0 (35)	1.9 (77)	1.5 (94)	1.8 (19)	0.27
M. terrae complex	0.6 (30)	0.5 (45)	–	0.3 (2)	0.7 (13)	0.6 (25)	0.5 (30)	0.5 (5)	–
M. scrofulaceum	0.3 (15)	0.3 (22)	–	0	0.3 (6)	0.3 (11)	0.3 (16)	0.4 (4)	–
M. smegmatis	0.3 (14)	0.2 (17)	–	0	0.3 (5)	0.2 (9)	0.2 (13)	0.4 (4)	–
M. xenopi	0.2 (9)	0.2 (17)	–	0	0.3 (5)	0.3 (11)	0.1 (8)	0.2 (2)	–
M. flavescens	0.2 (11)	0.1 (9)	–	0.5 (3)	0.1 (1)	0.1 (4)	0.2 (10)	0.2 (2)	–
M. phlei	0.1 (6)	0.2 (14)	–	0	0.1 (2)	0.2 (9)	0.1 (9)	0	–
M. szulgai	0.1 (5)	0.0 (3)	–	0	0	0.1 (4)	0.0 (2)	0.2 (2)	–
M. vaccae	0.1 (6)	0.0 (2)	–	0	0.1 (1)	0.1 (3)	0.1 (4)	0	–
M. simiae	0	0.0 (3)	–	0	0.1 (1)	0	0.0 (2)	0	–
M. marinum	0	0.0 (1)	–	0	0	0.0 (1)	0	0	–
M. neoaurum	0	0.0 (1)	–	0	0.1 (1)	0	0	0	–

*NTM, nontuberculous mycobacteria ; –, not applicable (insufficient data).

suggest that most cases of NTM infection occur in patients of advanced age and that its associated clinical significance may be as important as TB among elderly patients.

Third, we found that gender may be associated with the acquisition of diseases caused by mycobacterial species. We found that *M. tuberculosis* infection, NTM pulmonary infection, and NTM colonization were more common among men than among women (70.1% vs. 29.3% for TB, 57.6% vs. 42.4% for NTM infections, and 59.6% vs. 40.4% for NTM colonization). In addition, the male:female ratio among patients ≥45 years of age was significantly higher among patients with TB than among patients with NTM infections or colonization. This finding might indicate that older women are more resistant to TB than to NTM infection/colonization.

This study has several limitations. First, because the study was conducted in a single medical center in Taiwan, we cannot safely generalize our findings to other parts of Taiwan. However, this hospital is the only medical center that can fully identify the species of NTM in Taiwan, which suggests that our results still provide useful information about the current status in this country. Second, we did not evaluate the effects of co-existing conditions such as HIV infection. Third, the American Thoracic Society/Infections Disease Society of America diagnostic criteria for NTM infections were determined on the basis of experience with MAC, *M. kansasii*, and *M. abscessus* but not for other NTM species (*1*). Therefore, we might have overestimated the incidence of lung infections caused by other NTM species (e.g., *M. gordonae* and *M. fortuitum*). These

species need further investigation. Fourth, in this retrospective analysis, we did not try to identify factors associated with the changing epidemiology of these diseases.

In summary, in Taiwan, the rate of NTM isolation among cultures positive for mycobacteria increased significantly during the period 2000–2012, whereas the rate of *M. tuberculosis* isolation decreased. Moreover, the prevalence of NTM pulmonary infection and colonization rapidly increased with time. This phenomenon was more evident among patients of advanced age and among male patients.

Dr Chien is an attending physician at Chest Hospital, Ministry of Health and Welfare, Taiwan. His primary research interest is clinical infectious disease, especially tuberculosis, and diseases caused by NTM.

References

1. Griffith DE, Aksamit T, Brow-Elliott BA, Catanzaro A, Daley C, Gordin F, et al. An official ATS/IDSA statement: diagnosis, treatment, and prevention of nontuberculous mycobacterial diseases. Am J Respir Crit Care Med. 2007;175:367–416. http://dx.doi.org/10.1164/rccm.200604-571ST
2. Lai CC, Tan CK, Chou CH, Hsu HL, Liao CH, Huang YT, et al. Increasing incidence of nontuberculous mycobacteria, Taiwan, 2000–2008. Emerg Infect Dis. 2010;16:294–6. http://dx.doi.org/10.3201/eid1602.090675
3. de Lima CA, Gomes HM, Oelemann MA, Ramos JP, Caldas PC, Campos CE, et al. Nontuberculous mycobacteria in respiratory samples from patients with pulmonary tuberculosis in the state of Rondonia, Brazil. Mem Inst Oswaldo Cruz. 2013;108:457–62.
4. Hoefsloot W, van Ingen J, Andrejak C, Angeby K, Bauriaud R, Bemer P, et al. The geographic diversity of nontuberculous mycobacteria isolated from pulmonary samples. A NTM-NET collaborative study. [Epub ahead of print]. Eur Respir J. 2013. http://dx.doi.org/10.1183/09031936.00149212
5. Simons S, van Ingen J, Hsueh PR, Van Hung N, Dekhuijzen PN, Boeree MJ, et al. Nontuberculous mycobacteria in respiratory tract infections, Eastern Asia. Emerg Infect Dis. 2011;17:343–9. http://dx.doi.org/10.3201/eid170310060
6. Lai CC, Tan CK, Lin SH, Liu WL, Liao CH, Hung YT, et al. Clinical significance of nontuberculous mycobacteria isolates in elderly Taiwanese patients. Eur J Clin Microbiol Infect Dis. 2011;30:779–83. http://dx.doi.org/10.1007/s10096-011-1155-8
7. Kim JK, Rheem I. Identification and distribution of nontuberculous mycobacteria from 2005 to 2001 in Cheonan, Korea. Tuberc Respir Dis (Seoul). 2013;74:215–21. http://dx.doi.org/10.4046/trd.2013.74.5.215
8. Tsai CF, Shiau MY, Chang YH, Wang YL, Huang TL, Liaw YC, et al. Trends of mycobacterial clinical isolates in Taiwan. Trans R Soc Trop Med Hyg. 2011;105:148–52. http://dx.doi.org/10.1016/j.trstmh.2010.11.005
9. Yoo JW, Jo KW, Kim MN, Lee SD, Kim WS, Kim DS, et al. Increasing trend of isolation of non-tuberculous mycobacteria in a tertiary university hospital in South Korea. Tuberc Respir Dis (Seoul). 2012;72:409–15. http://dx.doi.org/10.4046/trd.2012.72.5.409
10. Marras TK, Chedore P, Ying AM, Jamieson F. Isolation prevalence of pulmonary non-tuberculous mycobacteria in Ontario, 1997–2003. Thorax. 2007;62:661–6. http://dx.doi.org/10.1136/thx.2006.070797
11. van Ingen J, Bendien SA, de Lange WC, Hoefsloot W, Dekhuijzen PN, Boeree MJ, et al. Clinical relevance of non-tuberculous mycobacteria isolated in Nijmegen-Arnhem region, The Netherlands. Thorax. 2009;64:502–6. http://dx.doi.org/10.1136/thx.2008.110957
12. Andréjak C, Thomsen VØ, Johansen IS, Riis A, Benfield TL, Duhaut P, et al. Nontuberculous pulmonary mycobacteriosis in Denmark: incidence and prognostic factors. Am J Respir Crit Care Med. 2010;181:514–21. http://dx.doi.org/10.1164/rccm.200905-0778OC
13. Park YS, Lee CH, Lee SM, Yang SC, Yoo CG, Kim YW, et al. Rapid increase of non-tuberculous mycobacterial lung diseases at a tertiary referral hospital in South Korea. Int J Tuberc Lung Dis. 2010;14:1069–71.
14. Thomson RM; NTM working group at Queensland TB Control Centre and Queensland Mycobacterial Reference Laboratory. Changing epidemiology of pulmonary nontuberculous mycobacteria infections. Emerg Infect Dis. 2010;16:1576–83. http://dx.doi.org/10.3201/eid1610.091201
15. Satyanarayana G, Heysell SK, Scully KW, Houp ER. Mycobacterial infections in a large Virginia hospital, 2001–2009. BMC Infect Dis. 2011;11:113. http://dx.doi.org/10.1186/1471-2334-11-113

Address for correspondence: Po-Ren Hsueh, Departments of Laboratory Medicine and Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Rd, Taipei, 100, Taiwan; email: hsporen@ntu.edu.tw

Levofloxacin-Resistant *Haemophilus influenzae*, Taiwan, 2004–2010

Shu-Chen Kuo, Pei-Chen Chen, Yih-Ru Shiau, Hui-Ying Wang, Jui-Fen Lai, I-Wen Huang, and Tsai-Ling Yang Lauderdale

Levofloxacin resistance in *Haemophilus influenzae* has increased significantly in Taiwan, from 2.0% in 2004 to 24.3% in 2010 (p<0.001). Clinical and molecular investigations of 182 levofloxacin-resistant isolates revealed that the increase was mainly the result of the spread of several clones in the elderly population in different regions.

Since their first introduction in 1980s, fluoroquinolones have been used extensively (*1*). The wide use of these antimicrobial drugs has been attributed to the emergence of resistance in several bacterial species (*1*,*2*). Respiratory tract infection (RTI) is one of the most common conditions for which fluoroquinolones are used (*1*,*3*). *Haemophilus influenzae* is a major bacterial pathogen causing RTIs. Globally, fluoroquinolone resistance in *H. influenzae* has remained sporadic and uncommon, and international surveillance data showed the resistance rate to be <1% (*4*–*6*). However, emergence of fluoroquinolone-resistant *H. influenzae* strains has been reported in elderly patients and in those in long-term care facilities (*7*–*9*).

The Taiwan Surveillance of Antimicrobial Resistance (TSAR) is a biennial nationwide surveillance program of inpatient and outpatient clinical isolates (*10*). Levofloxacin-resistant *H. influenzae* isolates were not detected in the TSAR collection before 2002 but emerged in 2004, and prevalence increased rapidly. We conducted a study to delineate the clinical and molecular characteristics of emerging levofloxacin-resistant *H. influenzae* in Taiwan.

The Study

H. influenzae isolates were collected from 26 hospitals during 2004–2010 by the TSAR program, following previously described protocols (*10*). After species identification was confirmed, MICs were determined by reference broth microdilution method according to Clinical and Laboratory Standards Institute guidelines (*11*). Sensititre Standard HPB panels (ThermoFisher Scientific, East Grinstead, UK) were used. The MICs of levofloxacin, ciprofloxacin, moxifloxacin, gatifloxacin, and gemifloxacin of the levofloxacin-resistant isolates identified by broth microdilution were further determined by Etest (bioMérieux, Marcy l'Etoile, France). Pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) were performed on levofloxacin-resistant isolates following published protocols (*12*,*13*). Mutations in the quinolone resistance–determining regions (QRDR) of the drug targets *gyr*A and *par*C were determined by PCR and sequencing (*14*).

A total of 1,462 nonduplicate *H. influenzae* isolates were collected during the study period. Hospitals in the northern (32.1%), central (47.7%), and southern (18.7%) regions of Taiwan provided nearly all of the isolates. Specimens of respiratory origin (88.8%) comprised the largest proportion; few isolates (1.4%) came from blood specimens. Most (77.1%) patients were adults, and half (50.0%) were ≥65 years of age.

Among the 16 antibacterial agents tested (Table 1), nonsusceptibility to levofloxacin, sparfloxacin, and trimethoprim/sulfamethoxazole increased steadily and significantly over the study period (p<0.05). Significant increases in levofloxacin and sparfloxacin resistance were the most prominent (p<0.001). The overall levofloxacin resistance rate increased from 2.0% (7/344) in 2004 to 10.6% (52/490) in 2006, 15.2% (49/323) in 2008, and 24.3% (74/305) in 2010 (p<0.001) (Table 1). In 2004, levofloxacin-resistant isolates were detected in 6 hospitals (2 in the south and 4 in the central region), but by 2010, isolates were detected in 19 hospitals in all 4 regions of Taiwan. The MIC_{90} of the 5 fluoroquinolones tested by Etest was >32 μg/mL for the 182 levofloxacin-resistant isolates detected by broth microdilution.

Levofloxacin-resistant *H. influenzae* isolates were more commonly found in elderly patients, from respiratory origins, from regional hospitals and inpatient settings, and from central and southern Taiwan (Table 2). Multivariate analysis revealed that elderly patients (OR 3.601, 95% CI 2.435–5.325), regional hospitals (OR 2.054, 95% CI 1.379–3.059), geographic region (OR 3.656, 95% CI 2.214–6.038 for central; OR 5.428, 95% CI 3.050–9.611 for southern), and later study year (OR 2.13, 95% CI 1.692–2.395) were independent factors associated with levofloxacin resistance (Table 2).

Among the 182 levofloxacin-resistant isolates, 153 could be grouped into 19 distinct clusters (A to S) (Figure). MLST was performed on 160 isolates, and a total of 50 sequence types (STs; ST630, ST787–ST802,

Author affiliations: National Health Research Institutes, Zhunan, Taiwan (S.-C. Kuo, P.-C. Chen, Y.-R. Shiau, H.-Y. Wang, J.-F. Lai, I.-W. Huang, T.-L.Y. Lauderdale); National Yang-Ming University School of Medicine, Taipei, Taiwan (S.-C. Kuo); and Taipei Veterans General Hospital, Taipei (S.-C. Kuo)

DOI: http://dx.doi.org/10.3201/eid2008.140341

Table 1. Trends in antimicrobial nonsusceptibility in *Haemophilus influenzae* from the Taiwan Surveillance of Antimicrobial Resistance program, 2004–2010*

Antimicrobial agent	% Nonsusceptible 2004, n = 344	2006, n = 490	2008, n = 323	2010, n = 305	2004–2010, n = 1,462	p value†	Odds ratio (95% CI)
Amoxicillin/clavulanate	3.8	5.5	2.8	3.9	4.2	0.573	0.933 (0.731–1.189)
Ampicillin	61.3	56.5	49.2	59.3	56.6	0.242	0.943 (0.856–1.040)
Ampicillin/sulbactam	34.0	26.7	24.8	35.7	29.9	0.790	1.014 (0.913–1.127)
Cefaclor	54.4	48.2	53.6	57.0	52.7	0.241	1.060 (0.962–1.167)
Cefepime	2.6	1.0	0.0	2.0	1.4	0.287	0.790 (0.513–1.218)
Cefixime	4.4	1.8	0.6	2.3	2.3	0.044	0.698 (0.462–0.991)
Ceftriaxone	1.5	0.4	0.0	1.0	0.7	0.370	0.754 (0.406–1.398)
Cefuroxime	13.7	14.3	25.1	16.1	16.9	0.033	1.150 (1.011–1.307)
Chloramphenicol	39.8	37.8	28.8	33.1	35.3	0.01	0.875 (0.791–0.968)
Clarithromycin	40.7	50.4	58.5	43.6	48.5	1.148	1.704 (0.975–1.183)
Imipenem	3.8	3.3	1.9	3.0	3.0	0.333	0.867 (0.650–1.157)
Levofloxacin	2.0	10.6	15.2	24.3	12.5	<0.001	1.964 (1.675–2.302)
Meropenem	2.0	0.6	0.0	1.0	0.9	0.110	0.625 (0.351–1.112)
Sparfloxacin	4.9	15.1	19.5	26.9	16.1	<0.001	1.688 (1.472–1.936)
Tetracycline	40.7	38.6	30.7	33.4	36.3	0.010	0.875 (0.790–0.969)
TMP/SMX	67.4	66.5	71.8	74.1	69.5	0.023	1.131 (1.017–1.257)

*TMP/SMX, trimethoprim/sulfamethoxazole.
†For the trend test calculation, a continuous variable was used as previously described (*10*).

ST1079–ST1095, and ST1097–ST1112) were identified. All but 1 ST, ST630, were new STs that had not been reported previously. However, most of the identified STs were single-locus variants (SLVs) of 7 ST types: ST788, ST790, ST792, ST793, ST795, ST799, and ST802. Some isolates of the same ST belonged to different PFGE clusters (Figure). PFGE and MLST results revealed emergence and regional predominance of some clones (Figure). For example, in the predominant STs (ST788 and its SLVs) in clusters A to F (n = 54, 29.7%), cluster A isolates were mostly from southern Taiwan and were found in all study years, whereas cluster B isolates were mostly from central Taiwan and found in later years (2008–2010). All but 1 of the isolates of ST799 and its SLV (n = 19) in clusters G–I were from central Taiwan, and most were from 2008–2010. Isolates of ST795 and its SLVs (n = 17) belonged to 3 clusters, were from central and northern Taiwan, and were recovered in later years (2008–2010).

All levofloxacin-resistant *H. influenzae* isolates had ≥2 mutations in the QRDR of *gyr*A and *par*C. A total of 25 QRDR mutation patterns were found (Table 3, http://wwwnc.cdc.gov/EID/article/20/8/14-0341-T3.htm). The amino acid changes at residues 84 and 88 in GyrA and ParC that we found are the same as those found in previous reports (*1*,*14*,*15*). Most isolates of the same PFGE cluster had similar QRDR mutation patterns. However, isolates within the same PFGE cluster that had the same ST or its SLV but different QRDR mutation patterns were also found (Figure).

Conclusions

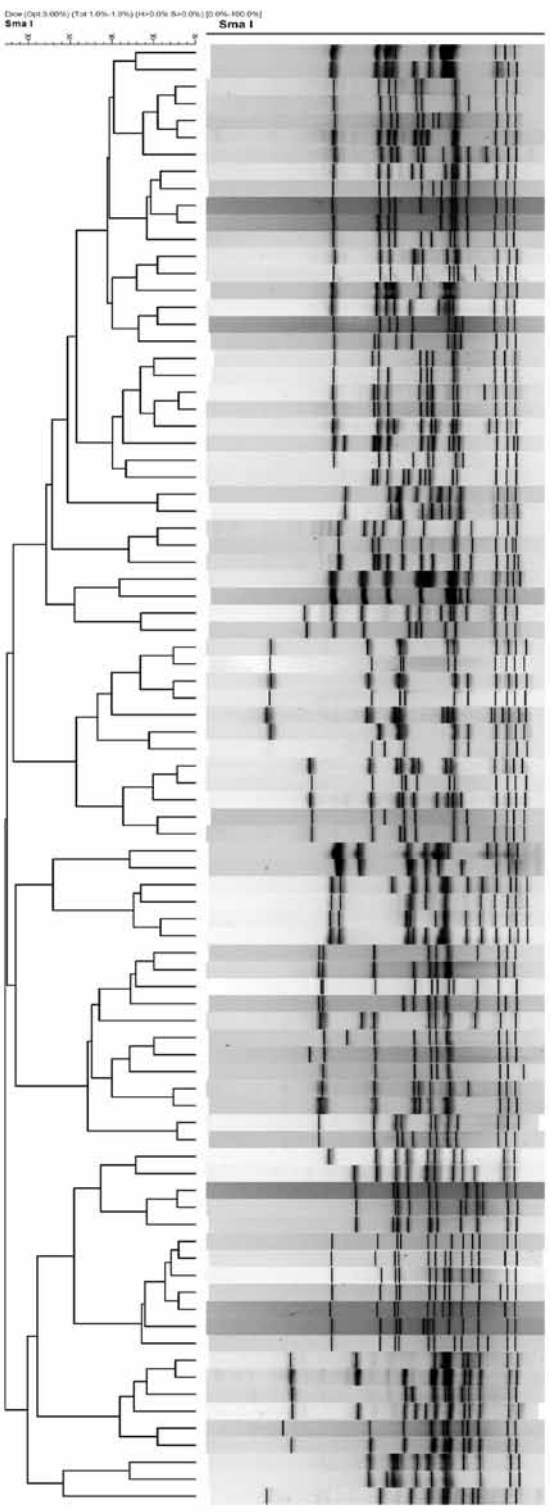
Our study indicates that levofloxacin-resistant *H. influenzae* emerged in Taiwan around 2004 and increased over the next 6 years, especially in elderly patients, regional hospitals, and central and southern Taiwan. We found age and regional differences in this resistance phenotype, which might have resulted from differences in fluoroquinolone use and patient populations.

Longitudinal and international surveillance data from North America, Europe, and other regions have found low fluoroquinolone resistance in *H. influenzae* (<1%) (*4*–*6*). However, emergence and spread of fluoroquinolone-resistant *H. influenzae* have been reported. In 2001,

Table 2. Factors associated with isolation of levofloxacin-resistant *Haemophilus influenzae*, Taiwan, 2004–2010

Factor	No. (%) isolates Susceptible	Resistant	p value*	Odds ratio (95% CI)	p value†
Total	1,280 (87.5)	182 (12.5)			
Patient age ≥65 y	591 (80.8)	140 (19.2)	<0.001	3.601 (2.435–5.325)	<0.001
Respiratory tract specimen	1,123 (86.5)	175 (13.5)	<0.001		NS
Regional hospital	766 (85.0)	135 (15.0)	<0.001	2.054 (1.379–3.059)	<0.001
Inpatient hospital stay	849 (85.1)	149 (14.9)	<0.001		NS
Geographic region					
Northern	448 (95.5)	21 (4.5)	<0.001	Reference	
Central	585 (83.9)	112 (16.1)	<0.001	3.656 (2.214–6.038)	<0.001
Southern	225 (82.4)	48 (17.6)	0.006	5.428 (3.050–9.611)	<0.001
Eastern	22 (95.7)	1 (4.3)	0.346		
Study year				2.013 (1.692–2.395)	<0.001

*By χ^2 test.
†By multivariate logistic regression analysis. NS, not significant.



PFGE cluster (n)*	Year isolated 2004–2006	2008–2010	Region†	MLST (N)	QRDR profile
A(1)	-	1	S	1085(788-SLV)(1)	11(1)
A(1)	-	1	S	788(1)	11(1)
A(1)	1	-	S	1089(788-SLV)(1)	11(1)
A(1)	1	-	S	1081(788-SLV)(1)	10(1)
A(1)	1	-	S	788(1)	11(1)
A(2)	2	-	S	788(1)	11(1)
A(1)	1	-	S	1089(788-SLV)(1)	11(1)
A(1)	1	-	S	788(1)	18(1)
A(1)	-	1	C	788(1)	11(1)
A(1)	-	1	S	788(1)	11(1)
A(1)	-	1	S	788(1)	11(1)
A(2)	1	1	S	788(2)	11(2)
A(1)	1	-	S	788(1)	18(1)
A(1)	-	1	S	788(1)	11(1)
A(1)	-	1	S	1090(1)	18(1)
A(1)	-	1	S	788(1)	18(1)
A(1)	1	-	C	788(1)	8(1)
A(2)	2	-	C	788(1)	8(1)
B(1)	-	1	C	787(788-DLV)(1)	18(1)
B(1)	-	1	C	788(1)	18(1)
B(1)	-	1	C	1082(788-SLV)(1)	18(1)
B(6)	1	5	C	788(4),1086(788-SLV)(1)	18(6)
B(1)	-	1	S	788(1)	11(1)
B(1)	1	-	C	788(1)	21(1)
B(1)	-	1	S	788(1)	18(1)
B(2)	-	2	C	788(2)	18(2)
C(1)	-	1	N	1094(1)	20(1)
C(2)	-	2	C	1094(2)	20(2)
D(1)	1	-	C	788(1)	16(1)
D(1)	1	-	C	788(1)	16(1)
D(1)	1	-	C	788(1)	16(1)
E(1)	-	1	N	788(1)	18(1)
E(3)	-	3	C	788(2)	18(3)
F(2)	-	2	C	788(2)	14(2)
F(7)	-	7	C	788(4)	14(7)
G(1)	-	1	C	800(799-SLV)(1)	6(1)
G(1)	-	1	C	800(799-SLV)(1)	6(1)
G(1)	-	1	C	800(799-SLV)(1)	6(1)
G(1)	-	1	C	798(799-SLV)(1)	21(1)
G(1)	-	1	C	800(799-SLV)(1)	6(1)
H(1)	-	1	C	800(799-SLV)(1)	6(1)
H(2)	-	2	C	800(799-SLV)(2)	14(2)
I(1)	-	1	C	1111(799-SLV)(1)	8(1)
I(2)	-	2	C	799(1)	8(2)
I(1)	-	1	S	800(799-SLV)(1)	11(1)
I(1)	-	1	C	801(799-SLV)(1)	8(1)
I(6)	2	4	C	799(3),800(799-SLV)(1)	8(6)
J(1)	-	1	C	1108(1)	14(1)
J(2)	-	2	C	1106(1),1107(1)	21(2)
K(2)	-	2	C	1104(802-DLV)(2)	14(2)
K(4)	-	4	C	1105(802-SLV)(3),1104(802-DLV)(1)	14(4)
K(1)	-	1	C	802(1)	14(1)
K(2)	-	1	C	802(1),1105(802-SLV)(1)	14(2)
L(1)	1	-	C	792(1)	16(1)
L(7)	7	-	C	792(3),1095(792-SLV)(1)	16(7)
L(1)	-	1	C	792(1)	16(1)
L(1)	-	1	C	792(1)	16(1)
L(3)	-	3	C	792(1),1109(2)	16(3)
M(1)	1	-	C	1095(792-SLV)(1)	16(1)
M(1)	-	1	C	792(1)	16(1)
M(1)	1	-	C	1095(792-SLV)(1)	16(1)
M(1)	1	-	C	792(1)	16(1)
M(1)	-	1	C	1097(792-DLV)(1)	16(1)
N(1)	-	1	C	792(1)	16(1)
N(3)	-	3	C	792(2)	16(3)
O(2)	-	2	S	1079(790-DLV)(1),1080(1)	23(2)
O(3)	-	3	S	1093(790-DLV)(1),1091(1),1092(1)	23(3)
O(1)	-	1	N	790(1)	22(1)
O(2)	-	2	N	790(1)	21(1)
O(4)	3	1	N	790(2)	4(2),21(2)
P(1)	1	-	S	793(1)	18(1)
P(5)	5	-	S	793(1)	18(4),19(1)
P(5)	3	2	S	793(2),796(793-SLV)(1),1099(793-SLV)(1)	18(4),9(1)
P(1)	1	-	S	1099(793-SLV)(1)	18(1)
P(2)	2	-	S	1099(793-SLV)(1)	18(2)
P(1)	-	1	S	793(1)	15(1)
P(2)	1	1	S	793(2)	18(2)
Q(1)	-	1	C	1102(795-SLV)(1)	25(1)
Q(2)	1	1	N	794(795-SLV)(2)	16(2)
Q(1)	1	-	N	795(1)	16(1)
Q(4)	-	4	C	794(795-SLV)(4)	16(4)
Q(1)	-	1	N	795(1)	16(1)
Q(2)	1	1	N	795(2)	16(2)
R(1)	-	1	N	1101(795-SLV)(1)	20(1)
R(2)	-	2	N	1101(795-SLV)(1),1100(795-SLV)(1)	20(2)
S(3)	-	3	C	795(3)	18(3)

Figure. Dendrogram showing pulsed-field gel electrophoresis (PFGE) results for levofloxacin-resistant *Haemophilus influenzae* isolates digested by SmaI. *Salmonella enterica* serovar Braenderup strain H9812 (ATCC BAA664) was used as standard for DNA pattern normalization. PFGE patterns were analyzed by using BioNumerics software (Applied Maths NV, Sint-Martens-Latem, Belgium). For mutation profiles of the quinolone-resistance determining regions (QRDR) in GyrA and ParC, see Table 3 (http://wwwnc.cdc.gov/EID/article/20/8/14-0341-T3.htm). *Isolates having ≥80% similarity or <6 band differences were assigned a PFGE cluster if there were ≥3 isolates within the cluster; †region of hospital location: C, central; N, north; S, south. –, no isolates found. (n), number of isolates having the same PFGE pattern; MLST, multilocus sequence typing; (N), number of isolates on which MLST was performed; SLV, single locus variant, DLV, double locus variant.

fluoroquinolone-resistant *H. influenzae* spread in a hospital-affiliated long-term care facility in New York, NY, USA, and almost all resistant isolates belonged to 1 clone (*8*). From 2002 to 2004, the rate of fluoroquinolone resistance in *H. influenzae* in hospitals in the Hokkaido Prefecture in Japan increased from 0.5% to 2.6%, and the resistant isolates were found only in elderly patients (*9*). In 2007, a high rate of levofloxacin resistance (41.7%, 20/48 isolates) was found in *H. influenzae* that was colonizing 4 nursing home residents in southern Taiwan, and 2 major clones accounted for 90% (18/20) of the resistant isolates (*7*).

In our study, isolates of the same PFGE cluster mostly had similar mutation patterns. Therefore, the emergence of fluoroquinolone-resistant isolates may be the results of several clones spreading in the same region. However, isolates within the same PFGE cluster having the same ST but different QRDR mutation patterns were also found. In addition, nearly all the levofloxacin-resistant *H. influenzae* isolates belong to 7 previously unreported STs or their SLVs. This finding indicates that the emergence of levofloxacin-resistant isolates likely occurred through spontaneous mutation of hypermutable clones under selective pressure (*8*,*14*), and these clones then disseminated locally in each region.

In summary, our study revealed the emergence and spread of levofloxacin-resistant *H. influenzae* in different regions of Taiwan, with regional predominance of certain STs. We studied a large number of levofloxacin-resistant *H. influenzae* from clinical diagnostic samples of multiple hospitals in different regions of Taiwan, so physicians should take into account the high rate of fluoroquinolone resistance when they prescribe empirical therapy for *H. influenzae* infections. Surveillance and intervention measures should be directed at the risk groups identified in this study to halt the increase in fluoroquinolone resistance in *H. influenzae*.

Acknowledgments

We thank the following 26 hospitals for their participation in the 2004–2010 Taiwan Surveillance of Antimicrobial Resistance program: Buddhist Tzu Chi General Hospital, Cathay General Hospital, Changhua Christian Hospital, Cheng-Ching Hospital, Chung Shan Medical University Hospital, Ditmanson Medical Foundation Chia-Yi Christian Hospital, Far Eastern Memorial Hospital, Hua-Lien Hospital, Jen-Ai Hospital, Kaohsiung Armed Forces General Hospital, Kaohsiung Chang Gung Memorial Hospital of the C. G. M. F, Kaohsiung Medical University Chung-Ho Memorial Hospital, Kaohsiung Veterans General Hospital, Kuang Tien General Hospital, Lo-Hsu Foundation, Inc., Lotung Poh-Ai Hospital, Mennonite Christian Hospital, Min-Sheng Healthcare, National Cheng Kung University Hospital, Saint Mary's Hospital Luodong, Show Chwan Memorial Hospital, Tungs' Taichung MetroHarbor Hospital, Taichung Veterans General Hospital, Tainan Sin-Lau Hospital–the Presbyterian Church in Taiwan, Taipei City Hospital Heping Fuyou Branch, Taipei City Hospital Zhongxiao Branch, Tri-Service General Hospital.

This project was supported by intramural grants from the National Health Research Institutes (IV-101-PP-01 and IV-102-PP-01).

Dr Kuo is an attending physician at the National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, Taiwan. His primary research interest involves resistance mechanisms and pathophysiology of antimicrobial drug–resistant pathogens.

References

1. Hooper DC. Emerging mechanisms of fluoroquinolone resistance. Emerg Infect Dis. 2001;7:337–41. http://dx.doi.org/10.3201/eid0702.010239
2. Cuevas O, Oteo J, Lazaro E, Aracil B, de Abajo F, Garcia-Cobos S, et al. Significant ecological impact on the progression of fluoroquinolone resistance in *Escherichia coli* with increased community use of moxifloxacin, levofloxacin and amoxicillin/clavulanic acid. J Antimicrob Chemother. 2011;66:664–9. http://dx.doi.org/10.1093/jac/dkq471
3. Mandell LA, Wunderink RG, Anzueto A, Bartlett JG, Campbell GD, Dean NC, et al. Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. Clin Infect Dis. 2007;44:S27–72. http://dx.doi.org/10.1086/511159
4. Darabi A, Hocquet D, Dowzicky MJ. Antimicrobial activity against *Streptococcus pneumoniae* and *Haemophilus influenzae* collected globally between 2004 and 2008 as part of the Tigecycline Evaluation and Surveillance Trial. Diagn Microbiol Infect Dis. 2010;67:78–86. http://dx.doi.org/10.1016/j.diagmicrobio.2009.12.009
5. Jones RN, Farrell DJ, Mendes RE, Sader HS. Comparative ceftaroline activity tested against pathogens associated with community-acquired pneumonia: results from an international surveillance study. J Antimicrob Chemother. 2011;66:iii69–80. http://dx.doi.org/10.1093/jac/dkr101
6. Pfaller MA, Farrell DJ, Sader HS, Jones RN. AWARE Ceftaroline Surveillance Program (2008–2010): trends in resistance patterns among *Streptococcus pneumoniae, Haemophilus influenzae,* and *Moraxella catarrhalis* in the United States. Clin Infect Dis. 2012;55:S187–93. http://dx.doi.org/10.1093/cid/cis561
7. Chang CM, Lauderdale TL, Lee HC, Lee NY, Wu CJ, Chen PL, et al. Colonisation of fluoroquinolone-resistant *Haemophilus influenzae* among nursing home residents in southern Taiwan. J Hosp Infect. 2010;75:304–8. http://dx.doi.org/10.1016/j.jhin.2009.12.020
8. Nazir J, Urban C, Mariano N, Burns J, Tommasulo B, Rosenberg C, et al. Quinolone-resistant *Haemophilus influenzae* in a long-term care facility: clinical and molecular epidemiology. Clin Infect Dis. 2004;38:1564–9. http://dx.doi.org/10.1086/420820
9. Yokota S, Ohkoshi Y, Sato K, Fujii N. Emergence of fluoroquinolone-resistant *Haemophilus influenzae* strains among elderly patients but not among children. J Clin Microbiol. 2008;46:361–5. http://dx.doi.org/10.1128/JCM.01561-07
10. Kuo S-C, Chang S-C, Wang H-Y, Lai J-F, Chen P-C, Shiau Y-R, et al. Emergence of extensively drug-resistant *Acinetobacter baumannii* complex over 10 years: nationwide data from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) program. BMC Infect Dis. 2012;12:200. http://dx.doi.org/10.1186/1471-2334-12-200

11. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing: 22nd informational spplement. M100–S22. Wayne (PA); The Institute; 2012.
12. Hunter SB, Vauterin P, Lambert-Fair MA, Van Duyne MS, Kubota K, Graves L, et al. Establishment of a universal size standard strain for use with the PulseNet standardized pulsed-field gel electrophoresis protocols: converting the national databases to the new size standard. J Clin Microbiol. 2005;43:1045–50. http://dx.doi.org/10.1128/JCM.43.3.1045-1050.2005
13. Meats E, Feil EJ, Stringer S, Cody AJ, Goldstein R, Kroll JS, et al. Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. J Clin Microbiol. 2003;41:1623–36. http://dx.doi.org/10.1128/JCM.41.4.1623-1636.2003
14. Pérez-Vázquez M, Roman F, Garcia-Cobos S, Campos J. Fluoroquinolone resistance in *Haemophilus influenzae* is associated with hypermutability. Antimicrob Agents Chemother. 2007;51:1566–9. http://dx.doi.org/10.1128/AAC.01437-06
15. Georgiou M, Munoz R, Roman F, Canton R, Gomez-Lus R, Campos J, et al. Ciprofloxacin-resistant *Haemophilus influenzae* strains possess mutations in analogous positions of GyrA and ParC. Antimicrob Agents Chemother. 1996;40:1741–4.

Address for correspondence: Tsai-Ling Yang Lauderdale, National Institute of Infectious Diseases and Vaccinology, National Health Research Institutes, No. 35 Keyan Road, Zhunan 35053, Taiwan; email: lauderdale@nhri.org.tw

etymologia

Borrelia miyamotoi [bə-rel′ e-ə mi″ a-mo-to′ e]

A genus of gram-negative, anaerobic spirochete bacteria, *Borrelia* was named after French biologist Amédée Borrel. In 1995, Masahito Fukunaga et al. isolated a novel *Borrelia* species and named it *Borrelia miyamotoi*, in honor of Kenji Miyamoto, who first isolated spirochetes from ixodid ticks in Hokkaido, Japan. Human cases of *B. miyamotoi* infection were subsequently found in Russia in 2011 and North America in 2013.

Sources

1. Branda JA, Rosenberg ES. *Borrelia miyamotoi*: a lesson in disease discovery. Ann Intern Med. 2013;159:61–2. http://dx.doi.org/10.7326/0003-4819-159-1-201307020-00009
2. Dorland's Illustrated Medical Dictionary. 32nd ed. Philadelphia: Elsevier Saunders; 2012.
3. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, McCelland M, et al. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol. 1995;45:804–10. http://dx.doi.org/10.1099/00207713-45-4-804

Address for correspondence: Ronnie Henry, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E03, Atlanta, GA 30333, USA; email: boq3@cdc.gov

DOI: http://dx.doi.org/10.3201/eid2008.ET2008

Human Infections with *Borrelia miyamotoi*, Japan

Kozue Sato, Ai Takano, Satoru Konnai, Minoru Nakao, Takuya Ito, Kojiro Koyama, Minoru Kaneko, Makoto Ohnishi, and Hiroki Kawabata

We confirmed infection of 2 patients with *Borrelia miyamotoi* in Japan by retrospective surveillance of Lyme disease patients and detection of *B. miyamotoi* DNA in serum samples. One patient also showed seroconversion for antibody against recombinant glycerophosphodiester phosphodiesterase of *B. miyamotoi*. Indigenous relapsing fever should be considered a health concern in Japan.

Borrelia miyamotoi, which is genetically grouped with relapsing fever borreliae, was recently identified as a human pathogen in Russia (*1*), the United States (*2–4*), and Europe (*5*). Ticks of the *Ixodes persulcatus* species complex are transmission vectors. Pathogenic borreliae were discovered in *I. persulcatus* ticks in Japan (*6*).

In areas of Japan to which Lyme disease is endemic, wild rodents have been found to be infected with *B. miyamotoi* (*7*), although no human infections have been confirmed. *B. miyamotoi* isolates from Japan are potential human pathogens because they form a monophyletic lineage with isolates from patients in Russia (*1*). We conducted a retrospective investigation to identify occult cases of human infections with *B. miyamotoi* in Japan.

The Study

A total of 615 serum samples were obtained from 408 persons in Japan who had confirmed Lyme disease or unconfirmed, clinically suspected Lyme disease and used to detect *B. miyamotoi* DNA. The serum archive was established during 2008–2013 at the National Institute of Infectious Diseases (Tokyo, Japan). Use of human samples was approved by the ethical committee of the National Institute of Infectious Diseases for medical research with humans (approval no. 360; July 2, 2012).

All serum samples were centrifuged (15,000 × *g* for 10 min), and sediments were used for DNA extraction. DNA extraction was performed by using the DNeasy Blood and Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions with minor modification (the extraction column was incubated for 10 min at 70°C before DNA was collected).

For detection of *B. miyamotoi* DNA, real-time PCR was performed with primers and probes described by Barbour et al. (*8*). The reaction was performed in a 25-μL volume in single tubes with 1 μmol/L of each primer and 0.25 μmol/L of each probe. The PCR was conducted on a 7000 Real Time PCR Apparatus (Applied Biosystems, Foster City, CA, USA), and conditions were 42 cycles at 95°C for 5 s and 60°C for 31 s. For confirmation of positive samples, a *flaB* gene nested PCR and sequencing of amplicons were performed as described (*9*).

The number of copies of DNA in patient serum samples was estimated by quantitative PCR (qPCR). Plasmid pBMrrs1 that contained part of the 16S rRNA gene for *B. miyamotoi* strain HT31 was prepared as described (*9*) and was used as a quantitative control.

A recombinant *B. miyamotoi* glycerophosphoryl diester phosphodiesterase (GlpQ) was used for serologic testing. The GlpQ gene (*glpQ)* of *B. miyamotoi* strain HT31 was cloned into plasmid vector pET-19b (Merck KGaA, Darmstadt, Germany). His-tagged GlpQ was subsequently expressed in *Escherichia coli* Rossetta strain (Merck KGaA) by using MagicMedia (Life Technologies, Carlsbad, CA, USA). Western blotting for detection of antibodies against GlpQ in patient serum was performed as described (*10*). Our retrospective investigation identified 2 cases of *B. miyamotoi* infection.

Case-patient 1 was a previously healthy, 72-year-old woman who lived in Hokkaido, Japan. The patient reported no history of foreign travel. Myalgia and anorexia developed on July 23, 2011, and she was hospitalized on July 25, at which time she had a fever of 39°C. Physical examination showed erythema migrans, and the patient confirmed that she had been bitten by a tick 10 days earlier.

Laboratory tests on July 25 showed increased levels of C-reactive protein (44 mg/L), alanine aminotransferase (94 IU/mL), and aspartate aminotransferase (90 IU/ml). The procalcitonin level was ≥0.05 ng/mL, as determined by using a PCT-Q Test (Brahms GmbH, Hennigsdorf, Germany). The leukocyte count was 3,900 cells/mL (87% neutrophils), and a left shift was observed. Erythrocyte and platelet counts were within reference ranges. The patient was given a clinical diagnosis of acute Lyme disease because of typical erythema migrans and a history of a tick bite. The patient was treated with minocycline (100

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (K. Sato, M. Ohnishi, H. Kawabata); Yamaguchi University, Yamaguchi, Japan (A. Takano); Hokkaido University, Sapporo, Japan (S. Konnai); Asahikawa Medical College, Asahikawa, Japan (M. Nakao); Hokkaido Institute of Public Health, Sapparo (T. Ito); Oumu National Health Insurance Hospital, Oumu, Japan (K. Koyama); Kamifurano Hospital, Kamifurano, Japan (M. Kaneko, Japan); and Gifu University, Gifu, Japan (H. Kawabata)

DOI: http://dx.doi.org/10.3201/eid2008.131761

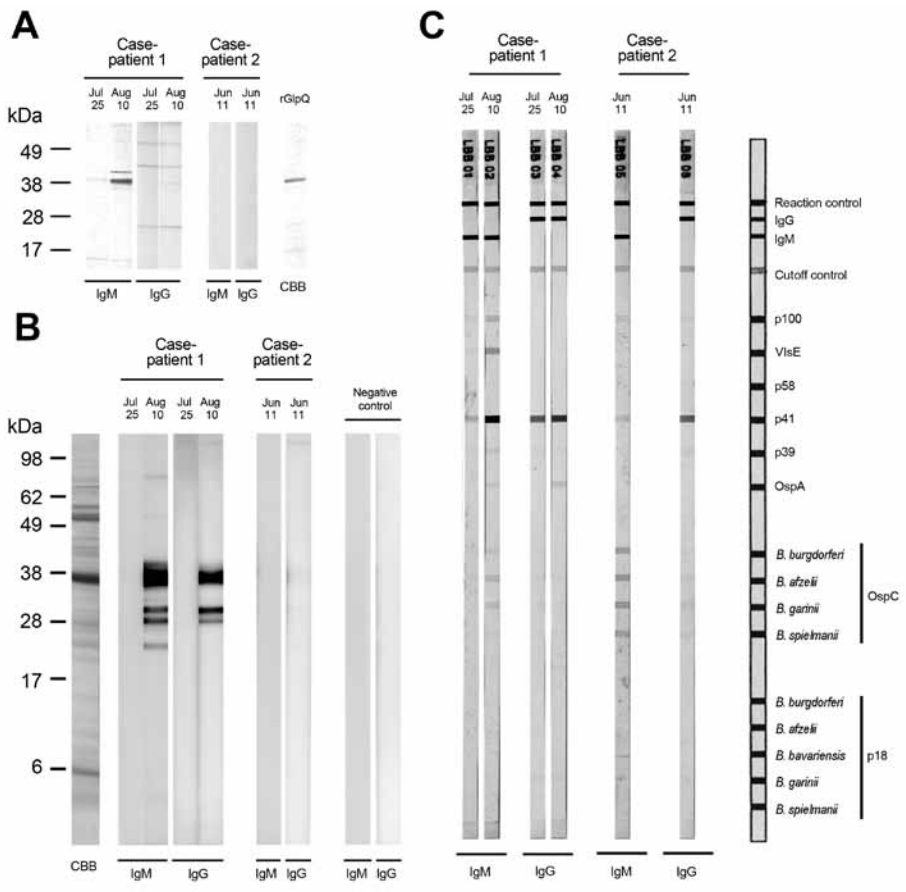


Figure. Immunoblot analysis of serum reactivity to antigens of *Borrelia miyamotoi* and Lyme disease borreliae, Japan. Serum samples obtained from 2 patients were examined. For case-patient 1, acute-phase serum obtained on July 25, 2011, and convalescent-phase serum obtained on August 10 were used. For case-patient 2, acute-phase serum obtained on June 11 was used. A) Reactivity to recombinant glycerophosphodiester phosphodiesterase (GlpQ) antigen. Crude rGlpQ were used for immunoblot analysis (*10*). Recombinant GlpQ was separated by electrophoresis on a 5%–20% polyacrylamide gradient gel (Wako Pure Chemical Industries Inc., Osaka, Japan), and antigen was stained with Coomassie brilliant blue. CBB, protein profile. Molecular mass markers are shown on the left. B) Reactivity of patient serum samples to whole cell lysate of *B. miyamotoi* antigens. A low-passage strain of *B. miyamotoi* (strain MYK3) was used for immunoblot analysis (*10*). A negative control was serum obtained from a healthy human (resident of an area to which Lyme disease was not endemic). Molecular mass markers are shown on the left. C) Serodiagnosis of Lyme disease by immunoblot analysis of serum samples from the 2 patients. OspC, outer surface protein C.

mg/day for 5 days). Symptoms improved rapidly, and she was discharged from the hospital on July 30.

Borrelial DNA was detected in a serum sample obtained on July 25 by 16S rRNA gene–based-real time qPCR and a *flaB* gene–specific nested PCR. Sequencing of a *flaB* PCR amplicon (294 bp) indicated that the infectious borreliae was *B. miyamotoi* (GenBank accession no. AB921566) because the sequence was identical to that of *B. miyamotoi* HT31 (GenBank accession no. D43777). The number of copies of the *Borrelia* genome in serum was estimated to be 7.2×10^3 copies/mL by qPCR.

The level of IgM against GlpQ was increased in a convalescent-phase serum sample obtained on August 10 (Figure, panel A). We also found that antibody levels were increased in convalescent-phase serum by conducting immunoblot analysis with a whole cell lysate of *B. miyamotoi* strain MYK3 (Figure, panel B).

Case-patient 2 was a previously healthy 37-year-old man who lived in Hokkaido, Japan. The patient reported no history of foreign travel. The patient was bitten by a tick on May 28, 2013, and was subsequently hospitalized on June 11, at which time he had a fever of 39.8°C and erythema migrans at the site of the tick bite. The patient was given a clinical diagnosis of acute Lyme disease and erythema migrans. The patient was treated with ceftriaxone (1 g/day for 7 days). Symptoms improved rapidly, and treatment was administrated until July 17 in the outpatient setting.

Borrelial DNA was detected from a serum sample obtained on June 11 by qPCR and nested *flaB* PCR. Sequencing of the *flaB* PCR amplicon (294 bp) confirmed *B. miyamotoi* (GenBank accession no. AB921567) infection in the patient because the sequence was identical to *B. miyamotoi* HT31 (GenBank accession no. D43777). The number of copies of the borrelia genome in serum was estimated to 2.8×10^4 copies/mL by qPCR. Antibodies against GlpQ were not detected in serum obtained on June 11 (Figure, panel A).

Serologic analysis with a commercial kit for IgM (RecomLine Borrelia IgG/IgM; Mikrogen, Neuried, Germany) (Figure, panel C) showed that serum from these 2 patients reacted with several antigens of Lyme disease borreliae (Figure, panel C). Convalescent-phase serum from case-patient 1 reacted to P100 from *B. afzelii*, VlsE

from various *Borrelia* species, P41 from *B. burgdorferi* sensu stricto, and OspC from *B. afzelii* and *B. garinii*. Acute-phase serum from case-patient 2 reacted with OspC for all *Borrelia* species included in the kit. However, the commercial serologic test used does not provide enough evidence to determine whether these 2 patients were co-infected with Lyme disease borreliae because antigenic difference between *B. miyamotoi* and *B. burgdorferi* sensu lato have not been investigated.

Conclusions

Platonov et al. (*1*) reported that *I. persulcatus* ticks are a transmission vector for *B. miyamotoi* and Lyme disease borreliae in Russia. This tick species is also ubiquitous in Hokkaido, Japan, and host-seeking behavior of adult ticks is active during spring–late summer. Humans in Hokkaido are bitten most often by *I. persulcatus* ticks, and Lyme disease borrelia is transmitted to humans mainly through the bite of the adult tick (*11*). Although the causative tick species was not identified for the 2 case-patients, circumstantial evidence suggests that *I. persulcatus* ticks are a main transmission vector for *B. miyamotoi* in Hokkaido, as shown in Russia (*1*).

Emerging relapsing fever caused by *B. miyamotoi* has been identified in Russia, North America, and Europe, and *B. miyamotoi*-related meningoencephalitis has been reported in the United States and the Netherlands. Our study indicates that a human health threat from emerging relapsing fever is present in Japan. For risk analysis of this emerging relapsing fever, epidemiologic surveys (e.g., determining infection rates of host-seeking ticks of the *I. persulcatus* species complex in various locations in Japan) and improvement of serologic diagnostic systems (especially early diagnosis) should be considered.

Acknowledgment

We thank Kyle R. Taylor for critically reading and editing this manuscript.

This study was supported in part by a grant for research on emerging and reemerging infectious diseases from the Japanese Ministry of Health, Labor and Welfare.

Dr Sato is a research resident at the National Institute of Infectious Diseases, Tokyo, Japan. Her primary research interests are epidemiology and vector-borne infectious diseases, especially Lyme disease and relapsing fever.

References

1. Platonov AE, Karan LS, Kolyasnikova NM, Makhneva NA, Toporkova MG, Maleev VV, et al. Humans infected with relapsing fever spirochete *Borrelia miyamotoi*, Russia. Emerg Infect Dis. 2011;17:1816–23. http://dx.doi.org/10.3201/eid1710.101474
2. Gugliotta JL, Goethert HK, Berardi VP, Telford SR III. Meningoencephalitis from *Borrelia miyamotoi* in an immunocompromised patient. N Engl J Med. 2013;368:240–5. http://dx.doi.org/10.1056/NEJMoa1209039
3. Krause PJ, Narasimhan S, Wormser GP, Rollend L, Fikrig E, Lepore T, et al. Human *Borrelia miyamotoi* infection in the United States. N Engl J Med. 2013;368:291–3. http://dx.doi.org/10.1056/NEJMc1215469
4. Chowdri HR, Gugliotta JL, Berardi VP, Goethert HK, Molloy PJ, Sterling SL, et al. *Borrelia miyamotoi* infection presenting as human granulocytic anaplasmosis: a case report. Ann Intern Med. 2013;159:21–7. http://dx.doi.org/10.7326/0003-4819-159-1-201307020-00005
5. Hovius JW, de Wever B, Sohne M, Brouwer MC, Coumou J, Wagemakers A, et al. A case of meningoencephalitis by the relapsing fever spirochaete *Borrelia miyamotoi* in Europe. Lancet. 2013;382:658. http://dx.doi.org/10.1016/S0140-6736(13)61644-X
6. Fukunaga M, Takahashi Y, Tsuruta Y, Matsushita O, Ralph D, McClelland M, et al. Genetic and phenotypic analysis of *Borrelia miyamotoi* sp. nov., isolated from the ixodid tick *Ixodes persulcatus*, the vector for Lyme disease in Japan. Int J Syst Bacteriol. 1995;45:804–10. http://dx.doi.org/10.1099/00207713-45-4-804
7. Taylor KR, Takano A, Konnai S, Shimozuru M, Kawabata H, Tsubota T. *Borrelia miyamotoi* infections among wild rodents show age and month independence and correlation with *Ixodes persulcatus* larval attachment in Hokkaido, Japan. Vector Borne Zoonotic Dis. 2013;13:92–7. http://dx.doi.org/10.1089/vbz.2012.1027
8. Barbour AG, Bunikis J, Travinsky B, Hoen AG, Diuk-Wasser MA, Fish D, et al. Niche partitioning of *Borrelia burgdorferi* and *Borrelia miyamotoi* in the same tick vector and mammalian reservoir species. Am J Trop Med Hyg. 2009;81:1120–31. http://dx.doi.org/10.4269/ajtmh.2009.09-0208
9. Takano A, Fujita H, Kadosaka T, Konnai S, Tajima T, Watanabe H, et al. Characterization of reptile-associated *Borrelia* sp. in the vector tick, *Amblyomma geoemydae*, and its association with Lyme disease and relapsing fever *Borrelia* spp. Environ Microbiol Rep. 2011;3:632–7. http://dx.doi.org/10.1111/j.1758-2229.2011.00280.x
10. Saito K, Ito T, Asashima N, Ohno M, Nagai R, Fujita H, et al. Case report: *Borrelia valaisiana* infection in a Japanese man associated with traveling to foreign countries. Am J Trop Med Hyg. 2007;77:1124–7.
11. Hashimoto Y, Miyamoto K, Iizuka H. Tick bites and Lyme disease in Hokkaido, Japan [in Japanese]. Hihubyo Shinryo. 2003;25:926–9.

Address for correspondence: Hiroki Kawabata, Department of Bacteriology-I, National Institute of Infectious Diseases. Toyama 1-23-1, Shinjuku, Tokyo 162-8640, Japan; email: kbata@nih.go.jp

Movement of Chikungunya Virus into the Western Hemisphere

Roger S. Nasci

Chikungunya virus (CHIKV) is an alphavirus transmitted in an urban epidemic cycle by the mosquitoes *Aedes aegypti* and *Ae. albopictus*. CHIKV outbreaks are characterized by rapid spread and infection rates as high as 75%; 72%–93% of infected persons become symptomatic. The disease manifests as acute fever and potentially debilitating polyarthralgia. In a variable proportion of cases, polyarthritis and fatigue can persist for 2 years or longer (*1*). During outbreaks, the large percentage of symptomatic infections places a considerable strain on resources of local health care providers and hospitals. Fortunately, death from chikungunya is rare.

CHIKV was first identified in Tanganyika (now Tanzania) in 1952 (*2*). The virus was later found to be widely distributed and to cause sporadic, mostly small outbreaks in Africa and Asia through the 1960s and 1970s. Little activity was reported from the mid-1980s until June 2004, when an epidemic occurred on Lamu Island, Kenya, then spread during 2005 to Comoros, La Reunion, and to other Indian Ocean islands, causing ≈500,000 cases (*3*). This was followed in 2006–2009 by an epidemic in India that produced >1.5 million cases in 17 of the country's 28 states and subsequently spread through Southeast Asia to the islands of the Pacific Ocean (*4*). The public health community has come to recognize CHIKV as a major emerging, epidemic-prone pathogen.

The global expansion of CHIKV has been broadened by the movement of infected persons to areas with competent mosquito vectors and a susceptible human population (*5*). CHIKV-infected travelers have been documented in ≥22 countries throughout Asia, Europe, and North America (*1*,*6*,*7*); their travel led to outbreaks in northern Italy (*8*) and southern France (*9*). Until a few months ago, only travel-related cases had been detected in the Western Hemisphere (*7,10,11*) with no evidence of local transmission.

The first known autochthonous chikungunya cases in the Western Hemisphere occurred in October 2013 on the island of Saint Martin and were reported in December 2013 (*12*). During the next 4 months, >31,000 confirmed and probable autochthonous cases were reported from numerous other Caribbean islands (as of April 28, 2014: British territories Anguilla and British Virgin Islands; overseas departments of France consisting of Dominica, Guadeloupe, Martinique, Saint Barthélemy, and Saint Martin; constituent country of the Netherlands, Sint Maarten; the Federation of St. Kitts and Nevis; the Dominican Republic; and Saint Vincent and the Grenadines). Infected travelers originating from the island countries have carried the virus around the region, leading to authochthonous chikungunya cases occurring in mid-February 2014 in French Guiana on the mainland of South America. Virus spread to other island countries and expansion into mainland areas of South, Central, and North America are inevitable.

Three CHIKV genotypes (East-Central-South African [ECSA], West African, and Asian) have been described; apparently they evolved independently in the different regions (*13*). The ECSA genotype has primarily been associated with the current epidemics in the Indian Ocean region, and the Asian genotype has been associated with recent outbreaks in the Pacific region. A single-base mutation in 1 strain of the ECSA genotype enhances replication of the virus in *Ae. albopictus*, contributing to the explosive epidemic that was observed in the La Reunion outbreak (*14*). Enhanced *Ae. albopictus* competence is also produced by a different substitution in a CHIKV ESCA lineage that has been associated with an outbreak in Kerala, India, in 2009 (*15*). Sequence analysis demonstrated that an Asian genotype of CHIKV caused the current outbreak in the Caribbean (*12*). In this issue of Emerging Infectious Diseases (http://wwwnc.cdc.gov/eid/article/20/8/14-0333-article htm), Lanciotti and Valadere compare CHIKV strains circulating in the Caribbean to those obtained from human serum samples from locally transmitted cases on the British Virgin Islands in January 2014. Their findings indicate that the strain circulating in the Caribbean is most closely related to strains isolated in China during 2012 and from Yap, Federated States of Micronesia, during 2013–14 (*16*), confirming the extent and speed at which CHIKV strains move around the globe.

Such knowledge about the specific virus lineage circulating in the region is essential to understanding the potential disease burden that may result. *Ae. aegypti* and

Author affiliation: Centers for Disease Control and Prevention, Fort Collins, Colorado, USA

DOI: http://dx.doi.org/10.3201/eid2008.130333

Ae. albopictus are competent vectors of Asian genotype CHIKV (*17*), although there is little evidence supporting a substantive role of *Ae. albopictus* in epidemic transmission of the Asian CHIKV genotype. However, the capacity for *Ae. albopictus* to transmit Asian CHIKV provides the potential for introductions from the Caribbean islands, which will facilitate local transmission in areas of the continental United States and South America where *Ae. albopictus* is common, but *Ae. aegypti* is absent.

CHIKV has the same urban epidemic transmission ecology as dengue virus, with *Ae. aegypti* and *Ae. albopictus* serving as vectors (*6*). Like dengue, epidemic chikungunya is an anthroponosis that does not require a nonhuman vertebrate amplifier host. This means that the estimated 3.6 billion persons in 124 countries at risk for dengue (*18*) are at risk for chikungunya. In the Americas, dengue incidence has been increasing (*19*), indicating that the likelihood of CHIKV outbreaks is high in areas in the Americas where the population is prone to dengue. There are currently no CHIKV vaccines or specific treatments; the only public health intervention available is reduction of mosquito-to-human contact through personal protection measures and vector control efforts to reduce mosquito abundance.

The entry of CHIKV into the Americas was anticipated and prompted health agencies in the region to develop preparedness and response plans (*1*). Now that CHIKV is here, health agencies and health care providers in areas of the Americas where dengue is endemic, as well as in parts of temperate North and South America where *Ae. aegypti* and *Ae. albopictus* are present, should be aware of the potential for CHIKV introduction and establishment, particularly over the coming months as the rainy season starts and conditions that promote dengue transmission traditionally increase. Existing diagnostic and surveillance networks must be enhanced, and effective vector control activities must be intensified to address this new public health threat to the region.

Dr Nasci is chief of the Arboviral Diseases Branch in the Division of Vector-Borne Diseases, Centers for Disease Control and Prevention. His research interests include the ecology and control of mosquito-transmitted pathogens.

References

1. Pan American Health Organization (PAHO). Preparedness and response for chikungunya virus: introduction in the Americas. Washington (DC): PAHO, 2011 [cited 2014 Feb 21]. http://www.paho.org/hq/index.php?option=com_docman&task=doc_download&gid=16984&Itemid=&lang=en
2. Lumsden WHR. An epidemic of virus disease in Southern Province, Tanganyika Territory, in 1952–53: II. General description and epidemiology. Trans R Soc Trop Med Hyg. 1955;49:33–57. http://dx.doi.org/10.1016/0035-9203(55)90081-X
3. Powers AM. Chikungunya. Clin Lab Med. 2010;30:209–19. http://dx.doi.org/10.1016/j.cll.2009.10.003
4. Staples JE, Brieman RF, Powers AM. Chikungunya fever: an epidemiological review of a re-emerging infectious disease. Clin Infect Dis. 2009;49:942–8. http://dx.doi.org/10.1086/605496
5. Charrel RN, de Lamballerie X, Raoult D. Chikungunya outbreaks–the globalization of vectorborne diseases. N Engl J Med. 2007;356:769–71. http://dx.doi.org/10.1056/NEJMp078013
6. Powers AM, Logue CH. Changing patterns of chikungunya virus: reemergence of a zoonotic arbovirus. J Gen Virol. 2007;88:2363–77. http://dx.doi.org/10.1099/vir.0.82858-0
7. Chaves TS, Pellini AC, Mascheretti M, Jahnel MT, Ribeiro AF, Rodrigues SG, et al. Travelers as sentinels for chikungunya fever, Brazil. Emerg Infect Dis. 2012;18:529–30. http://dx.doi.org/10.3201/eid1803.110838
8. Angelini P, Macini P, Finarelli AC, Pol C, Venturelli C, Bellini R, et al. Chikungunya epidemic outbreak in Emilia-Romagna (Italy) during summer 2007. Parassitologia. 2008;50:97–8.
9. Grandadam M, Caro V, Plumet S, Thiberge JM, Souarès Y, Failloux AB, et al. Chikungunya virus, southeastern France. Emerg Infect Dis. 2011;17:910–3. http://dx.doi.org/10.3201/eid1705.101873
10. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis. 2007;13:764–7. http://dx.doi.org/10.3201/eid1305.070015
11. Gibney KB, Fischer M, Prince HE, Kramer LD, St George K, Kosoy OL, et al. Chikungunya fever in the United States: a fifteen year review of cases. Clin Infect Dis. 2011;52:e121–6. http://dx.doi.org/10.1093/cid/ciq214
12. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014;383:514. http://dx.doi.org/10.1016/S0140-6736(14)60185-9
13. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, et al. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. J Virol. 2010;84:6497–504. http://dx.doi.org/10.1128/JVI.01603-09
14. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007;3:e201. http://dx.doi.org/10.1371/journal.ppat.0030201
15. Tsetsarkin KA, Weaver SC. Sequential adaptive mutations enhance efficient vector switching by chikungunya virus and its epidemic emergence. PLoS Pathog. 2011;7:e1002412 http://dx.doi.org/10.1371/journal.ppat.1002412.
16. Lanciotti, RS, AM Valadere. Transcontinental movement of Asian genotype chikungunya virus. Emerg Infect Dis. 2014 Aug [cited 2014 Apr 29]. http://dx.doi.org/10.3201/eid2008.140268
17. Vega-Rúa A, Zouache K, Girod R, Failloux AB, Lourenço-de-Oliveira R. High vector competence of *Aedes aegypti* and *Aedes albopictus* from ten American countries as a crucial factor of the spread of Chikungunya. [Epub ahead of print]. J Virol. 2014;88:e00370-14 http://dx.doi.org/10.1128/JVI.00370-14.
18. Gubler DJ. The economic burden of dengue. Am J Trop Med Hyg. 2012;86:743-4. http://dx.doi.org/10.4269/ajtmh.2012.12-0157
19. Brathwaite Dick O, San Martín JL, Montoya RH, del Diego J, Zambrano B, Dayan GH. The history of dengue outbreaks in the Americas. Am J Trop Med Hyg. 2012;87:584–93. http://dx.doi.org/10.4269/ajtmh.2012.11-0770

Address for correspondence: Roger S. Nasci, Centers for Disease Control and Prevention, 3150 Rampart Rd, Mailstop P02, Fort Collins, Colorado 80521, USA; email: rsn0@cdc.gov

Diagnosis of *Bartonella henselae* Prosthetic Valve Endocarditis in Man, France

To the Editor: *Bartonella* spp. cause 2% of cases of blood culture–negative endocarditis (*1*). Early diagnosis of *Bartonella* spp. infectious endocarditis, is challenging, especially for patients with preexisting valvular heart disease. A diagnosis for these patients requires bacterial culture, serologic testing, or molecular detection in serum or tissue (*2*). The sensitivity and specificity of Duke modified criteria (*3*) for detecting endocardial involvement by echocardiography are not optimal, which results in decreased diagnostic accuracy (*4*).

^{18}Fluorodeoxyglucose–positron emission tomography/computed tomography (^{18}FDG-PET/CT), has been shown to be beneficial for diagnosis (*4*) and management of prosthetic valve endocarditis (*5*), particularly if echocardiographic findings are inconclusive (*6*). This procedure can be performed in patients of all ages by adjusting the dose of ^{18}FDG to the weight of the patient. We report a case that illustrates the usefulness of ^{18}FDG-PET/CT for diagnosis of *Bartonella henselae* infectious endocarditis in a patient with a prosthetic valve.

On October 18, 2012, a 56-year-old man was admitted to Timone Hospital (Marseille, France) with fatigue and weight loss (–6 kg) over the past 6 months. He had had an aortic valve replacement and a bioprosthesis was inserted in 2005 for rheumatic disease. The patient had owned a kitten for 6 months.

Laboratory findings showed moderate anemia (hemoglobin level 114 g/L), an elevated C-reactive protein level (34.5 mg/L), and polyclonal hypergammaglobulinemia. A test result was negative for rheumatoid factor. Transthoracic and transesophageal echocardiograms showed a thickened and partial aortic stenosis around the bioprosthesis.

Because infectious endocarditis was suspected, treatment with intravenous antimicrobial drugs (amoxicillin, 200 mg/kg/day for 6 weeks and gentamicin, 160 mg/day for 2 weeks) was initiated. On day 7, he was transferred to the cardiology department of Timone Hospital in Marseille, France. An endocarditis test was performed by using an endocarditis kit as described (*7*). Three routine blood cultures were negative. The patient was given a diagnosis of possible infectious endocarditis by using the Duke score (*3*).

An ^{18}FDG-PET/CT scan was performed and showed ^{18}FDG uptake in the aortic bioprosthesis area (Figure). Results of a whole body scan were normal. An immunofluorescence test for *Bartonella* spp. showed titers of 400 for IgG against *B. quintana* and *B. henselae* (*1*), and Western blot confirmed a reactivity pattern pathognomonic for *B. henselae* endocarditis. Results of a PCR performed with a blood sample stored in EDTA (*1*) were positive for *B. henselae*. On day 13, antimicrobial drug therapy was

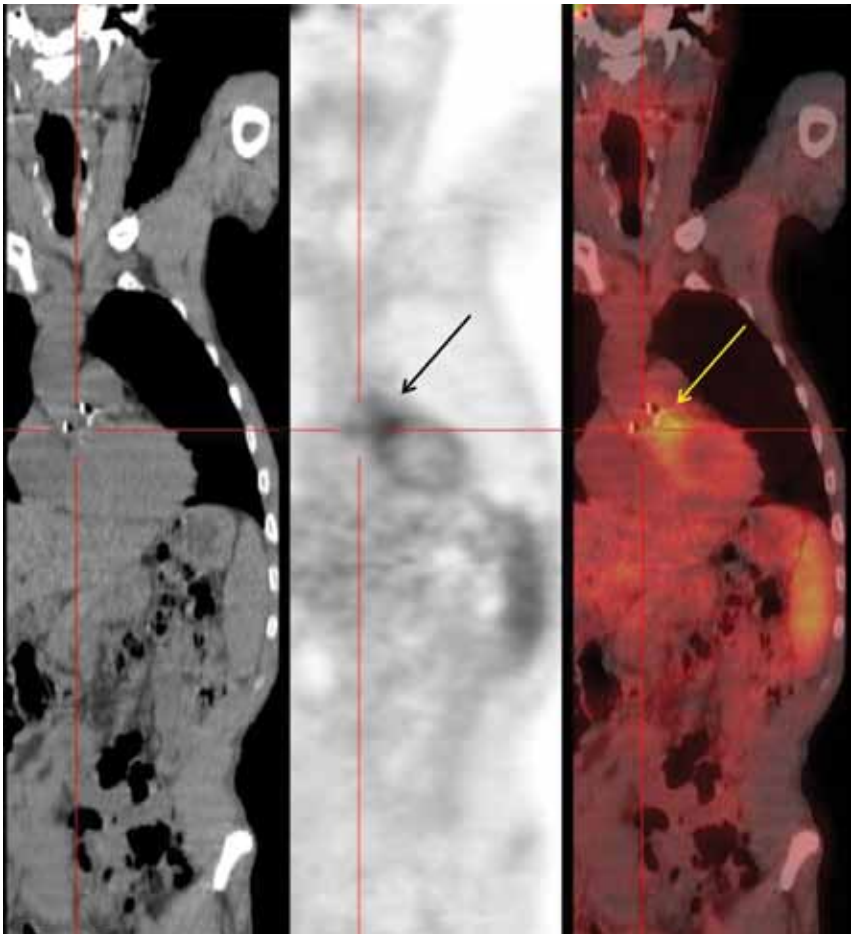


Figure. Positron emission tomography/computed tomography fusion imaging for a 56-year-old man in southern France with *Bartonella henselae* prosthetic valve endocarditis. Left panel, frontal computed tomography image showing morphologic findings. Middle panel, ^{18}F-fluorodeoxyglucose–positron emission tomography (^{18}FDG-PET) showing a cardiac hotspot (arrow) in relation to abnormal uptake of ^{18}FDG. Right panel, fusion image combining ^{18}FDG-PET and computed tomography showing localization of an aortic valve periprosthetic infection (arrow).

changed to oral doxycycline, 200 mg/day for 1 month and intravenous gentamicin, 160 mg/day for 15 days. On day 28, the patient was released from the hospital.

Two weeks later while still taking doxycycline, the patient was readmitted for a subarachnoid hemorrhage caused by a ruptured cerebral mycotic aneurysm. After arterial ligation, intravenous gentamicin was given with doxycycline for an additional 15 days. The patient recovered during this therapy.

On February 25, 2013, because of valvular stenosis, the patient underwent a new replacement with an aortic bioprosthesis. Valve culture remained sterile, but a PCR result was positive for *B. henselae*. Histologic analysis of the valve with Warthin-Starry stain showed no microorganisms. The patient remained asymptomatic for 2 months after surgery.

Diagnosis of possible infectious endocarditis in the patient was suspected after results of a transesophageal echocardiogram were used as a major criterion (thickened and partial aortic stenosis), and predisposing heart condition (aortic bioprosthesis) were used as a minor criterion (*3*) Because blood cultures were negative for the organism, the diagnosis of *B. henselae* infection was made by using serologic analysis and PCR (*7*). ^{18}FDG-PET/CT was especially valuable in early diagnosis of infectious endocarditis because echocardiography showed no vegetation or abscess, a common feature of *Bartonella* spp. endocarditis in which vegetations are often small or absent. In addition, a diagnosis of infectious endocarditis remains challenging, particularly in cases of prosthetic valve infections, in which results of initial echocardiography are not useful in 30% of cases (*5*). In such cases, the diagnostic accuracy of modified Duke criteria decreases.

Use of ^{18}FDG-PET/CT for diagnosis and monitoring of infectious endocarditis showed promising results, particularly for prosthetic valve infections (*5*), cardiac device–related infections (*6*), and when results of echocardiography are inconclusive or blood cultures are negative (*5*). ^{18}FDG-PET/CT with abnormal uptake of FDG was proposed as a diagnostic criterion of prosthetic valve infectious endocarditis (*4*). In contrast, a negative ^{18}FDG-PET/CT result does not rule out infectious endocarditis (*8*).

Use of ^{18}FDG-PET/CT has been discussed mainly because of problems of sensitivity of tracer uptake in heart tissue and small vegetations (*9*). Improvements, such as patient preparation with low carbohydrate–fat diet and technical advances in the newest ^{18}FDG-PET/CT scanners, may increase this sensitivity in future studies (*10*). Although ^{18}FDG-PET/CT will not replace clinical evaluation, laboratory tests, and echocardiography, this procedure might be helpful in diagnosis of *Bartonella* spp. infectious endocarditis.

Acknowledgments

We thank Clémence Bassez and Laurie-Anne Mansou for patient management.

Frédérique Gouriet, Pierre-Edouard Fournier, Caroline Zaratzian, Marion Sumian, Serge Cammilleri, Alberto Riberi, Jean-Paul Casalta, Gilbert Habib, and Didier Raoult

Author affiliations: Aix Marseille Université, Marseille, France (F. Gouriet, P.-E. Fournier, C. Zaratzian, J.-P. Casalta, D. Raoult); and Hôpital de La Timone, Marseille (F. Gouriet, M. Sumian, S. Cammilleri, A. Riberi, J.-P. Casalta, G. Habib)

DOI: http://dx.doi.org/10.3201/eid2008.130789

References

1. Brouqui P, Raoult D. Endocarditis due to rare and fastidious bacteria. Clin Microbiol Rev. 2001;14:177–207. http://dx.doi.org/10.1128/CMR.14.1.177-207.2001
2. Fournier PE, Lelievre H, Eykyn SJ, Mainardi JL, Marrie TJ, Bruneel F, et al. Epidemiologic and clinical characteristics of *Bartonella quintana* and *Bartonella henselae* endocarditis: a study of 48 patients. Medicine (Baltimore). 2001;80:245–51. http://dx.doi.org/10.1097/00005792-200107000-00003
3. Li JS, Sexton DJ, Mick N, Nettles R, Fowler VG Jr, Ryan T, et al. Proposed modifications to the Duke criteria for the diagnosis of infective endocarditis. Clin Infect Dis. 2000;30:633–8. http://dx.doi.org/10.1086/313753
4. Basu S, Chryssikos T, Moghadam-Kia S, Zhuang H, Torigian DA, Alavi A. Positron emission tomography as a diagnostic tool in infection: present role and future possibilities. Semin Nucl Med. 2009;39:36–51. http://dx.doi.org/10.1053/j.semnuclmed.2008.08.004
5. Saby L, Laas O, Habib G, Cammilleri S, Mancini J, Tessonnier L, et al. Positron emission tomography/computed tomography for diagnosis of prosthetic valve endocarditis: increased valvular ^{18}F-fluorodeoxyglucose uptake as a novel major criterion. J Am Coll Cardiol. 2013;61:2374–82. http://dx.doi.org/10.1016/j.jacc.2013.01.092
6. Millar BC, Prendergast BD, Alavi A, Moore JE. (18)FDG-positron emission tomography (PET) has a role to play in the diagnosis and therapy of infective endocarditis and cardiac device infection. Int J Cardiol. 2013;167:1724–36. http://dx.doi.org/10.1016/j.ijcard.2012.12.005
7. Raoult D, Casalta JP, Richet H, Khan M, Bernit E, Rovery C, et al. Contribution of systematic serological testing in diagnosis of infective endocarditis. J Clin Microbiol. 2005;43:5238–42. http://dx.doi.org/10.1128/JCM.43.10.5238-5242.2005
8. Sankatsing SU, Kolader ME, Bouma BJ, Bennink RJ, Verberne HJ, Ansink TM, et al. ^{18}F-fluoro-2-deoxyglucose positron emission tomography-negative endocarditis lenta caused by *Bartonella henselae.* J Heart Valve Dis. 2011;20:100–2.
9. Kouijzer IJ, Vos FJ, Janssen MJ, van Dijk AP, Oyen WJ, Bleeker-Rovers CP. The value of ^{18}FDG PET/CT in diagnosing infectious endocarditis. Eur J Nucl Med Mol Imaging. 2013;40:1102–7. http://dx.doi.org/10.1007/s00259-013-2376-0
10. Williams G, Kolodny GM. Suppression of myocardial ^{18}F-FDG uptake by preparing patients with a high-fat, low-carbohydrate diet. AJR Am J Roentgenol. 2008;190:W151–6. http://dx.doi.org/10.2214/AJR.07.2409

Address for correspondence: Didier Raoult, Aix Marseille Université, URMITE, UM 63, CNRS 7278, IRD 198, INSEM U1095, 13005 Marseille, France; email: didier.raoult@gmail.com

Sika Deer Carrying *Babesia* Parasites Closely Related to *B. divergens*, Japan

To the Editor: Human babesiosis caused by *Babesia divergens*, a zoonotic pathogen of bovines in Europe, is an emerging tickborne disease (*1*). In the United States, a closely related *Babesia* sp. was identified in persons in Missouri and Kentucky and in eastern cottontail rabbits (*Sylvilagus floridanus*) on Nantucket Island, Massachusetts (*2–5*). We report that sika deer (*Cervus nippon*) in Japan also carry parasites genetically closely related to *B. divergens*.

During November 2007–February 2008 and November 2008–February 2009, we collected blood samples from 96 wild sika deer throughout Japan. We purified DNA from the blood, which had been stored in a freezer, by phenol/chloroform extraction and performed nested PCR for *B. divergens 18S rRNA* (*rDNA*), chaperonin-containing T-complex protein 1 eta subunit (*CCT7*, formerly described as CCTη) (*6*), and β-*tubulin* (*7*) genes. Primers for *rDNA* were designed from the sequences of related *Babesia* spp. (GenBank accession nos. U16370, U16369, and AY046575): dv101F (5′-ACAACAGTTATAGTTTCTTTGGTATTCG-3′) and dv1353R (5′-GCCTTAAACTTCCTTGCGGCTTAGAGC-3′), and dv159F (5′-GCTAATGCAAGTTCGAGGCCTTTTGGCG-3′) and dv1296R (5′-CGGACGAACCTTTTTACGGACACTAG-3′) for the first and second rounds, respectively. *CCT7* primers were similarly designed (GenBank accession nos. AB367924 and AB367925): Bdiv/odoCCTF1 (5′-CAAAATGAGYCACCTMCTCAACCTACC-3′) and BdivCCTR1 (5′-ATCTCAGCAGCTCACTACAGTGACCACCTC-3′), and Bdiv/odoCCTF2 (5′- CAACCTACCRATTCTCCTYYTGAAGGAGGG-3′) and BdivCCTR2 (5′-GGCTAATAAGTCGATATTGCGGGGCTCACG-3′) for the first and second rounds, respectively. The β-*tubulin* PCR protocol has been described (*7*).

Of the 96 blood samples, 12 from 5 prefectures (Hokkaido, Iwate, Tochigi, Nagano, and Miyazaki) were positive for *Babesia rDNA* (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/13-0061-Techapp1.pdf). The sequence for sample 08–22 from Hokkaido (GenBank accession no. KC465978) was distinct from sequences of the other 11 samples (97.5%–97.6% identity in 1,041 bp), which consisted of 7 variant sequences (GenBank accession nos. KC465973–7 and AB857845–6) and 5 identical sequences (GenBank accession nos. KC465977 and AB861504–7) (99.7%–100% identity). The 5 identical sequences varied in only 1 of 909 bp from *B. divergens rDNA* from an *Ixodes persulcatus* tick in Russia [GenBank accession no. GU057385] (*8*).

β-*tubulin* (900 bp) was also amplified from the 12 *Babesia rDNA*–positive samples. Ten of the sequences consisted of 3 sequence variants (99.9% identity; GenBank accession nos. KC465969, KC465970, and KC465968/AB861508–14). The 2 divergent sequences (GenBank accession nos. KC465971 [08–22] and KC465989 [08–25]) were most similar to *B. odocoilei* (GenBank accession no. KC465972; 91% identity) and *Theileria orientalis* (GenBank accession no. AP011947; 79.9% identity), respectively. Thus, at least 1 deer likely had *Babesia* and *Theileria* spp. infections.

CCT7 was amplified from 10 of the 12 *Babesia*-positive blood samples. The sequences (GenBank accession nos. KC465979–88) were more heterogeneous (98.7%–99.9% identity) than those for *rDNA* and β-*tubulin*; this finding was expected because *CCT7* evolves more quickly (*6*).

We generated a neighbor-joining phylogenetic tree (ClustalW, http://clustalw.ddbj.nig.ac.jp/index.php?lang=ja) from the *Babesia rDNA* sequences from our study and from GenBank (Figure, panel A). The tree shows a distinct lineage (Asian) cluster for the deer parasites, except for 08–22 (GenBank accession no. KC465978), within a clade also holding the *B. divergens* strains (human and bovine) from Europe (European Union lineage). The tree also shows a cluster encompassing *Babesia* spp. (human and rabbit) from the United States; *B. divergens* (deer), *B. capreoli* (deer), and *Babesia* sp. (chamois) from Europe; and *B. divergens* (human) from Portugal. Sequence 08–22 branches with *Babesia* spp. in *Ix. ovatus* ticks from Japan (GenBank accession nos. AY190123 and AY190124) (*9*). The branch lengths indicate clear separation between the isolates from sika deer and ticks, suggesting that the clustering may be attributable to the limited number of available related sequences.

We also generated a phylogenetic tree of β-*tubulin* sequences (900 bp), which produced similar topology and high bootstrap support (Figure, panel B). However, the limited number of relevant β-*tubulin* sequences precludes conclusions regarding the phylogeny of *Babesia* parasites. A *CCT7* phylogenetic tree was not generated because of the paucity of sequences. Overall, the phylogenies suggest that *B. divergens*– and *B. capreoli*–related parasites are found worldwide in temperate zones of the Northern Hemisphere, including Europe (*1*), the United States (*2–5*), Russia (*8*), and Japan.

We showed the presence of *B. divergens*–like *rDNA,* β-*tubulin*, and *CCT7* genes in sika deer from different Japanese prefectures, confirming the presence of this parasite in Japan. *B. capreoli*, which is serologically indistinguishable from *B. divergens*, was previously reported in sika deer (*10*). However, no molecular data for the *B. capreoli* isolate exist, so no

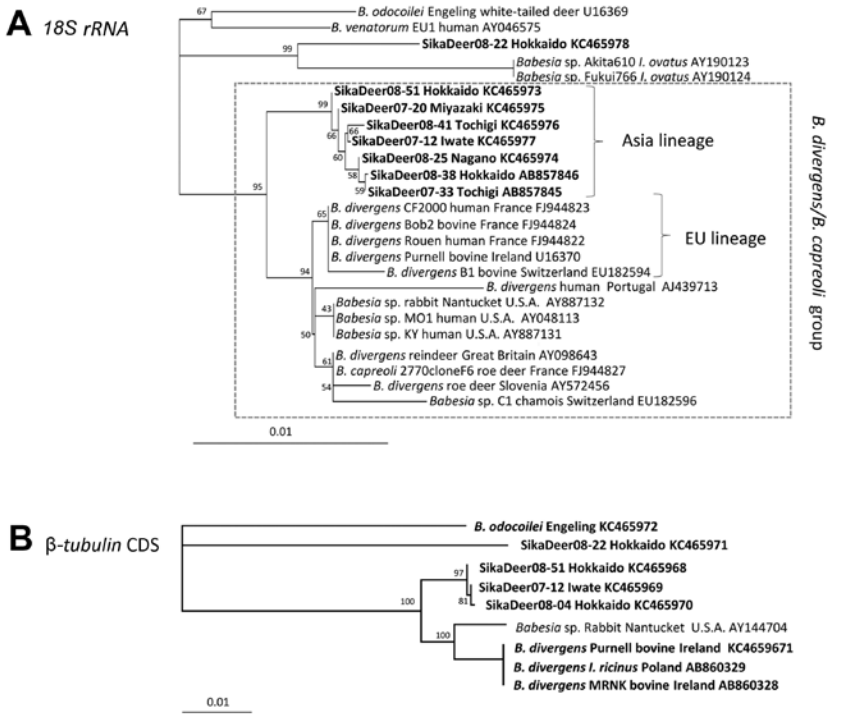


Figure. Neighbor-joining phylogenetic trees generated from *Babesia* sequences from GenBank and from our study in Japan, November 2007–February 2008 and November 2008–February 2009. Bootstrap support (1,000 repetitions) is indicated at the nodes. The trees are based on the partial (1,041 bp) *Babesia 18S rRNA* gene (A) and the partial (900 bp) β-*tubulin* gene (B). Sequences determined in this study are in boldface. Scale bar indicates the inferred number of substitutions per site. Lineages are indicated. CDS, coding DNA sequence.

conclusion may be drawn regarding its relationship to the *B. divergens*–like parasites from sika deer in our study.

There is an overabundance of wild sika deer in Japan because these animals easily adapt to a variety of climates, vegetation, and geography. Increased human exposure to deer habitats increases the risk of exposure to tickborne zoonoses, such as those caused by *Babesia* spp. In humans, infections caused by *B. divergens* and *B. divergens*–like parasites can be life threatening; fatality rates of 42% and 33% have been reported in infected asplenic patients in Europe and the United States, respectively (*1*–*3*). The findings from our study emphasize the need for increased clinical awareness of babesiosis in Japan and globally. They also emphasize the need for the swift diagnosis of suspected cases and prompt treatment of confirmed cases, especially in asplenic patients at high risk for the potentially deadly consequences of babesiosis.

Acknowledgments

We are grateful to Danny H.-K. Jokl for his critical reading of the manuscript. We thank Jeremy Gray for providing genomic DNA of *B. divergens* MRNK and Marek Sawczuk and Bogumiła Skotarczak for providing genomic DNA of *B. divergens* from *Ix. ricinus* tick collected in Poland.

Financial support for this study was provided in part by grants-in-aid from the Ministry of Education, Science and Culture of Japan and by the Ministry of Health, Labor and Welfare Science of Japan (H25-Shinko-Ippan-008 and H25-Shinko-Shitei-009). Support was also provided by a Grant-in-Aid for Cooperative Research from Rakuno-Gakuen University and by Gakujutsu Frontier Cooperative Research in Rakuno-Gakuen University.

Aya Zamoto-Niikura, Masayoshi Tsuji,[1] Koichi Imaoka, Masanobu Kimura, Shigeru Morikawa, Patricia J. Holman, Haruyuki Hirata, and Chiaki Ishihara

Author affiliations: National Institute of Infectious Diseases, Tokyo, Japan (A. Zamoto-Niikura, K. Imaoka, M. Kimura, S. Morikawa); Rakuno-Gakuen University, Hokkaido, Japan (A. Zamoto-Niikura, M. Tsuji, H. Hirata, C. Ishihara); and Texas A&M University, College Station, Texas, USA (P.J. Holman)

DOI: http://dx.doi.org/10.3208/eid2008.130061

References

1. Gorenflot A, Moubri K, Precigout E, Carcy B, Schetters TP. Human babesiosis. Ann Trop Med Parasitol. 1998;92:489–501. http://dx.doi.org/10.1080/00034989859465
2. Herwaldt B, Persing DH, Precigout EA, Goff WL, Mathiesen DA, Taylor PW, et al. A fatal case of babesiosis in Missouri: identification of another piroplasm that infects humans. Ann Intern Med. 1996;124:643–50. http://dx.doi.org/10.7326/0003-4819-124-7-199604010-00004
3. Beattie JF, Michelson ML, Holman PJ. Acute babesiosis caused by *Babesia divergens* in a resident of Kentucky. N Engl J Med. 2002;347:697–8. http://dx.doi.org/10.1056/NEJM200208293470921
4. Goethert HK, Telford SR III. Enzootic transmission of *Babesia divergens* among cottontail rabbits on Nantucket Island, Massachusetts. Am J Trop Med Hyg. 2003;69:455–60.
5. Spencer AM, Goethert HK, Telford SR III, Holman PJ. In vitro host erythrocyte specificity and differential morphology of *Babesia divergens* and a zoonotic *Babesia* sp. from eastern cottontail rabbits (*Sylvilagus floridanus*). J Parasitol. 2006;92:333–40. http://dx.doi.org/10.1645/GE-662R.1

[1]Deceased.

6. Nakajima R, Tsuji M, Oda K, Zamoto-Niikura A, Wei Q, Kawabuchi-Kurata T, et al. *Babesia microti*–group parasites compared phylogenetically by complete sequencing of the *CCTeta* gene in 36 isolates. J Vet Med Sci. 2009;71:55–68. http://dx.doi.org/10.1292/jvms.71.55
7. Hirata H, Kawai S, Maeda M, Jinnai M, Fujisawa K, Katakai Y, et al. Identification and phylogenetic analysis of Japanese macaque *Babesia*-1 (JM-1) detected from a Japanese macaque (*Macaca fuscata fuscata*). Am J Trop Med Hyg. 2011;85:635–8. http://dx.doi.org/10.4269/ajtmh.2011.11-0035
8. Rar VA, Epikhina TI, Livanova NN, Panov VV. Genetic diversity of *Babesia* in *Ixodes persulcatus* and small mammals from North Ural and West Siberia, Russia. Parasitology. 2011;138:175–82. http://dx.doi.org/10.1017/S0031182010001162
9. Inokuma H, Yoshizaki Y, Shimada Y, Sakata Y, Okuda M, Onishi T. Epidemiological survey of *Babesia* species in Japan performed with specimens from ticks collected from dogs and detection of new *Babesia* DNA closely related to *Babesia odocoilei* and *Babesia divergens* DNA. J Clin Microbiol. 2003;41:3494–8. http://dx.doi.org/10.1128/JCM.41.8.3494-3498.2003
10. Gray JS, Murphy TM, Waldrup KA, Wagner GG, Blewett DA, Harrington R. Comparative studies of *Babesia* spp. from white-tailed and sika deer. J Wildl Dis. 1991;27:86–91. http://dx.doi.org/10.7589/0090-3558-27.1.86

Address for correspondence: Aya Zamoto-Niikura, Division of Experimental Animal Research, National Institute of Infectious Diseases, 4-7-1 Gakuen, Musashimurayama, Tokyo 208-0011, Japan; email: zamoto@nih.go.jp

Transcontinental Movement of Asian Genotype Chikungunya Virus

To the Editor: Chikungunya virus (CHIKV), a mosquito–transmitted virus (family *Togaviridae,* genus *Alphavirus*), was first isolated >60 years ago in Africa and is responsible for epidemics of acute polyarthralgia. During CHIKV epidemics, the transmission cycle is from humans to mosquitoes, with no intervening amplifying host, and the virus can rapidly disseminate, infecting large numbers of persons. Epidemics have been described in Africa, the Middle East, Europe, India, and Southeast Asia. On the basis of detailed clinical descriptions of the disease, chikungunya fever, it appears that CHIKV caused epidemics in the Caribbean (St. Thomas, US Virgin Islands) and the southeastern coastal United States during the early 19th century (*1*).

Genetic studies show that the virus has evolved into 3 distinct genotypes: West African, East/Central/South African (ECSA), and Asian (*2*). The genotypes likely indicate independent evolution of the virus in historically isolated areas. Phenotypic differences have been described between genotypes and between individual strains, most notably an E1 mutation among some ECSA strains, which facilitates replication in *Aedes albopictus* mosquitoes (*3*). However, more recently, the movement of virus genotypes has increased dramatically, probably as a direct result of increased movement of humans and increased commercial trade. Beginning in 2005 and through 2006, the ECSA genotype virus was responsible for an explosive epidemic, during which the virus moved from coastal Kenya to islands adjacent to southeastern Africa and then to India, where >1 million cases were recorded (*2*). During this time, imported cases were reported worldwide, and in some instances, autochthonous transmission was detected in distal locations (*4*,*5*).

In October 2013, the arbovirus diagnostic laboratory at the Centers for Disease Control and Prevention (CDC; Fort Collins, CO, USA) detected CHIKV in human serum specimens from Yap State, Federated States of Micronesia; the specimens were collected during an epidemic of disease clinically compatible with chikungunya fever. In December 2013, the French National Reference Centre for arboviruses confirmed that CHIKV was responsible for an epidemic occurring on St. Martin Island, French West Indies, in the Caribbean (*6*). In January 2014, the Caribbean Public Health Agency detected CHIKV in 2 human serum specimens from the British Virgin Islands (BVI); the samples were subsequently confirmed by CDC to be positive for CHIKV.

By using next-generation sequencing, we determined the complete nucleotide sequence for 1 of the CHIKV specimens detected in BVI and for 2 of the CHIKV specimens detected in Yap. DNA libraries for next-generation sequencing were prepared directly from RNA extracted from serum, and the amplified libraries were sequenced by using the Ion Torrent Personal Genome Machine (Life Technologies, Grand Island, NY, USA). The CLC Genomics Workbench (CLC bio, Aarhus, Denmark) and Lasergene NextGen (DNASTAR, Madison, WI, USA) were used to analyze and assemble raw sequence reads. ClustalW (http://www.ebi.ac.uk/Tools/msa/clustalw2/) was used to align the complete genome sequences with a variety of CHIKV sequences, representing the 3 genotypes, from GenBank. Nearly identical phylogenetic trees were generated by several methods (i.e., minimum evolution, maximum likelihood, neighbor joining); a representative neighbor-joining tree generated and analyzed with 1,000 replicates for bootstrap testing is shown in the Figure.

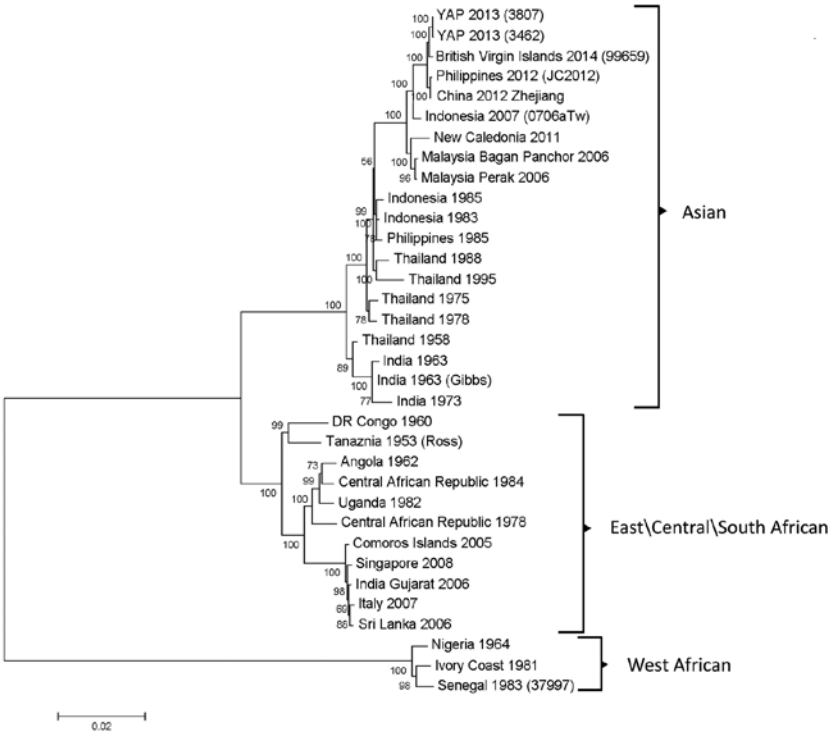


Figure. Phylogenetic tree derived by neighbor-joining methods (1,000 bootstrap replications) using complete genome sequences of chikungunya viruses obtained from GenBank. Scale bar represents the number of nucleotide substitutions per site. Genotypes are indicated on the right. GenBank accession numbers for viruses used for construction of the tree follow: Yap 2013–3807 (KJ451622), Yap 2013–3462 (KJ451623), British Virgin Islands-99659 (KJ451624), Philippines 2012-JC2012 (KC488650), China 2012 Zhejiang (KF318729), Indonesia 2007–0706aTw (FJ807897), New Caledonia 2011 (HE806461), Malaysia 2006 Bagan Panchor (EU703759), Malaysia 2006 Perak (EU703760), Indonesia 1985 (HM045797), Indonesia 1983 (HM045791), Philippines 1985 (HM045790), Thailand 1988 (HM045789), Thailand 1995 (HM045796),Thailand 1975 (HM045814),Thailand 1978 (HM045808),Thailand 1958 (HM045810), India 1963 (HM045803), India 1963 Gibbs (HM045813), India 1973 (HM045788), DR Congo 1960 (HM045809), Tanzania 1953 Ross (AF490259), Angola 1962 (HM045823), Central African Republic 1984 (HM045784), Uganda 1982 (HM045812),Central African Republic 1978 (HM045822), Comoros 2005 (HQ456251), Singapore 2008 (FJ445510), India 2006 Gujarat (JF274082), Italy 2007 (EU244823), Sri Lanka 2006 (GU189061), Nigeria 1964 (HM045786), Ivory Coast 1981 (HM045818), and Senegal 1983–37997 (AY726732).

In agreement with findings in a recent report characterizing the 2013 CHIKV detected on St. Martin Island (*6*), the phylogenetic tree generated from our sequence data showed that the 2014 CHIKV from BVI is within the Asian genotype and is closely related to strains recently isolated in China and the Philippines. This finding supports the idea that a single CHIKV strain of the Asian genotype was recently introduced into the Caribbean and is currently moving throughout the region. The 2 CHIKVs isolated in Yap in 2013 are most closely related to the CHIKV from BVI, differing by only 18–19 nt.

The tree also demonstrates that the CHIKV strains from Yap, BVI, China, and the Philippines form a strongly supported clade (bootstrap of 1,000) within the Asian genotype (Figure). Within this clade, the CHIKVs detected in 2012 in Zhejiang Province, China, and the Philippines are nearly identical, differing by only 4 nt in the entire genome. However, there is some ambiguity regarding the exact origins of these 2 strains. The 2012 CHIKV from the Philippines is described in GenBank (http://www.ncbi.nlm.nih.gov/nuccore/KC352904.1) as an "imported Chikungunya fever case in Ningbo port"; the virus was isolated and identified in Ningbo, China, but was detected in samples from a traveler from the Philippines (GenBank accession nos. KC352904.1 and KC488650.1). The 2012 Zhejiang CHIKV was detected and characterized in Zhejiang Province, but the virus was from a sailor who traveled around Southeast Asia; therefore, the exact origin of this virus is also unknown (*7*).

The striking similarity between the 2012 CHIKVs from the Philippines and Zhejiang Province suggests a common origin, perhaps the Philippines, where CHIKV transmission was documented in 2012 and 2013. Regardless of the exact origins of these 2 strains, it is clear that the CHIKV strain currently moving throughout the Caribbean originated from a CHIKV strain that was recently circulating between China, the Philippines, and Yap in Southeast Asia.

Robert S. Lanciotti and Anne Marie Valadere

Author affiliations: Centers for Disease Control and Prevention, Fort Collins, Colorado, USA (R.S. Lanciotti); and Caribbean Public Health Agency, Port of Spain, Trinidad and Tobago (A.M. Valadere)

DOI: http://dx.doi.org/10.3201/eid2008.140268

References

1. Carey DE. Chikungunya and dengue: a case of mistaken identity. J Hist Med Allied Sci. 1971;26:243–62. http://dx.doi.org/10.1093/jhmas/XXVI.3.243
2. Caglioti C, Lalle E, Castilletti C, Carletti F, Capobianchi MR, Bordi L. Chikungunya virus infection: an overview. New Microbiol. 2013;36:211–27.
3. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007;3:e201. http://dx.doi.org/10.1371/journal.ppat.0030201

4. Lanciotti RS, Kosoy OL, Laven JJ, Panella AJ, Velez JO, Lambert AJ, et al. Chikungunya virus in US travelers returning from India, 2006. Emerg Infect Dis. 2007;13:764–7. http://dx.doi.org/10.3201/eid1305.070015
5. Angelini R, Finarelli AC, Angelini P, Po C, Petropulacos K, Macini P, et al. An outbreak of chikungunya fever in the province of Ravenna, Italy. Euro Surveill. 2007;12:E070906.1.
6. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014;383:514. http://dx.doi.org/10.1016/S0140-6736(14)60185-9
7. Sun Y, Yan J, Mao H, Zhang L, Lyu Q, Wu Z, et al. Characterization of the complete genome of chikungunya in Zhejiang, China, using a modified virus discovery method based on cDNA-AFLP. PLoS ONE. 2013;8:e83014. http://dx.doi.org/10.1371/journal.pone.0083014

Address for correspondence: Robert S. Lanciotti, Centers for Disease Control and Prevention, 3150 Rampart Rd., Mailstop P02, Fort Collins, CO 80521, USA; email: rsl2@cdc.gov

Rickettsia felis Infections and Comorbid Conditions, Laos, 2003–2011

To the Editor: Fleaborne disease is highly prevalent in Laos, mainly attributed to murine typhus (*Rickettsia typhi* infection), transmitted by *Xenopsylla cheopis* fleas, but data on other fleaborne diseases are limited (*1*). We screened blood and cerebrospinal fluid (CSF) from participants in 2 large prospective studies in Laos for *Rickettsia* spp. using a genus-specific 17-kDa–based *Rickettsia* real-time quantitative PCR assay, and positive results were confirmed by DNA sequencing (*2,3*). In samples from >2,500 patients (2,540 blood and 1,112 CSF), we detected 3 cases of sequence-confirmed *R. felis* infections.

A 50-year-old man, an official in Vientiane City, was admitted to a hospital with fever and headache in October, 2008. HIV infection and cryptococcal meningitis were diagnosed. Treatment with intravenous amphotericin B, then oral fluconazole, was successful; antiretroviral treatment was initiated 1 month after diagnosis. Among a panel of diagnostic PCRs, the CSF sample specimen tested positive for genus-specific 17-kDa–*Rickettsia* quantitative PCR, but was negative for *Orientia tsutsugamushi* and *R. typhi*. DNA sequencing of 434 bp of the 17-kDa gene (Macrogen, Seoul, South Korea) revealed a 100% homology to the *R. felis* URRWXCal2 strain (Table).

R. felis positivity in CSF is rare; 4 cases have been reported (*3*). The combined findings of *R. felis* infection and severe immunodeficiency in this patient led to a reevaluation of the 2 reported *R. felis* infections in Laos (*2*). Before this study, *R. felis* DNA or culture had not been handled in our facility. The interval between processing positive samples, dedicated separate areas for samples before and after PCR, and the low positivity rate make DNA contamination highly unlikely.

A 39-year-old housewife from Luang Namtha in northern Laos had a history of diabetes mellitus, which had been treated with glibenclamide. On arrival at the hospital in November, 2008, she had fever, headache, myalgia, and an eschar. She was empirically treated with doxycycline (Table). An eschar biopsy specimen was PCR-positive for *Rickettsia* spp. and *O. tsutsugamushi*; PCR of buffy coat detected *O. tsutsugamushi* DNA only (*2*). Molecular characterization included 17-kDa and *sca4* gene sequencing, which both revealed amplicons of 100% identity to the *R. felis* URRWXCal2 strain. Serologic evidence for *O. tsutsugamushi* infection (scrub typhus) included a 4-fold rise in IgM and IgG titers, and IgM and IgG titers against typhus group rickettsiae, spotted fever group rickettsiae, and *R. felis* (isolate B377 in XTC-2 cells, Australian Rickettsial Reference Laboratory) were negative in admission and convalescent-phase samples (6-day interval) (Table).

A 13-year-old boy from Salavan, in southern Laos, had fever, headache, and nonspecific symptoms in July, 2009. *P. falciparum* malaria and dengue were diagnosed, both confirmed by PCR (Table). PCR results for the buffy coat specimen were positive for the 17-kDa gene; subsequent sequencing confirmed *R. felis* with 100% identity to the URRWXCal2 strain. The fever resolved after treatment with antimalarial drugs and ceftriaxone; neither would be expected to be efficacious for *R. felis* infection.

These data suggest that *R. felis* occurs in Laos, and is possibly emerging, but whether it results in clinical disease or commonly causes subclinical infection is unknown. The screened cohorts of consecutively enrolled patients with febrile illnesses across 3 diverse geographic regions are representative of etiologic agents of fever across Laos. PCR has previously been used for detection of *R. felis* and resulted in the discovery of a new *R. felis*–like organism in fleas in Kenya, Candidatus *Rickettsia asemboensis* (*4*). Reports from Southeast Asia suggest that *R. felis* is not a common cause of febrile illness (*1*,*2*), which contrasts with findings in Kenya, where *R. felis* was found in ≈7% of febrile patients (*4*,*5*), and also in ≈3% of afebrile patients (*5*).

The high *R. felis* carriage rate in fleas found in Laos (77% overall; 53% in *Ctenocephalides felis felis*, 89% in *C. f. orientis*) contrasts strongly with the apparent low incidence of *R. felis* human infections (*6*). Among febrile hospitalized patients in Vientiane, 1 case of *R. felis* infection was serologically diagnosed by using species-specific cross-absorption (*1*). Seroprevalence studies in the region could

Table: Clinical and laboratory findings of 3 patients with *Rickettsia felis* infections, Laos*

Patient characteristics	Signs and symptoms	Molecular findings	Serologic findings	Other laboratory findings
Male, 50 y, Vientiane City, central Laos	Fever, severe headache × 7 d; contact with cats and dogs 14 d before admission; HIV/AIDS (CD4-count: 34 cell/μL)	qPCR: *Orientia tsutsugamushi* (CSF, blood): negative; *Rickettsia* spp. (CSF): positive; *Rickettsia* spp. (blood): negative; *R. typhi* (CSF, blood): negative Conventional PCR and sequencing (CSF):*Rickettsia* spp. 17 kDa; GenBank accession no: KF489454	Scrub/murine typhus: IgM/IgG static titers (<1:100; negative) Spotted fever group: IgM/IgG static titers (1:200; negative) *R. felis:* IgM/IgG static titers (<1:128; negative)	Increased intracranial pressure (>40 cm H_20) CSF: clear CSF cellularity: 5 leukocytes/μL (100% lymphocytes) CSF glucose: 1.1 mmol/L; CSF/blood glucose ratio: 1:5 CSF protein: 80 mg/L; CSF *Cryptococcus* culture: positive/serotyping (PCR/RFLP) *C. neoformans* var. *grubii* HIV rapid tests:† positive
Female, 39 y, Luang Namtha, northern Laos	Fever × 7 d; diabetes mellitus, treated with glibenclamide; HIV status: unknown	qPCR: *O. tsutsugamushi* (eschar, blood): positive; *Rickettsia* spp. (eschar): positive; *R. typhi* (eschar): negative Conventional PCR and sequencing (eschar): *Rickettsia* spp. 17kDa and *sca4;* GenBank accession no: KF489455, KF489457	Scrub typhus: dynamic IgM/IgG 4-fold rise (1:3,200/1:12,800) Murine typhus: IgM/IgG static titers (<1:100; negative) Spotted fever group: IgM/IgG static titers (1:200; negative); *R. felis:* IgM/IgG static titers (<1:128; negative)	None
Male, 13 y, Salavan, southern Laos	Fever × 7 d, contact with cat, rat, and fleas 14 d before admission; HIV status: unknown	qPCR: *O. tsutsugamushi* (blood): negative; *Rickettsia* spp. (blood): positive *R. typhi* (blood): negative Conventional PCR and sequencing (blood): *Rickettsia* spp. 17 kDa GenBank accession no: KF489456	Data not available	Malaria microscopy: *P. falciparum;* Malaria rapid test: *P. falciparum* ICT Malaria Combo Cassette Test‡: *P. falciparum* PCR: positive; Dengue fever reverse transcription qPCR: positive Dengue genotyping PCR: serotype 4

*qPCR, quantitative PCR; CSF, cerebrospinal fluid; leukocyte; RFLP, restriction fragment length polymorphism; ICT, immunochromatographic test.
†Uni-GoldTM HIV, Trinity Biotech, Ireland; Alere Determine HIV-1/2 Ag/Ab Combo, Alere Medical, Japan.
‡ICT Diagnostics, Cape Town, South Africa.

elucidate exposure to this pathogen and unmask subclinical infections missed in fever etiology studies.

The 3 patients from Laos described herein had comorbidities associated with variable degrees of immunodeficiency (HIV infection and malaria with cellular and humoral deficiencies, diabetes with functional neutrophil/macrophage impairment) (*7*,*8*). *R. felis* infections have not been associated with immunosuppression, but few investigations of this possible association have been published. Of the 3 patients, the woman and the boy had other vectorborne infections: scrub typhus, transmitted by *Leptotrombidium* mites, and *P. falciparum* malaria and dengue, transmitted by *Anopheles* and *Aedes* mosquitoes, respectively. Recent reports described *R. felis* within a great diversity of vectors, including mites in South Korea and *Anopheles* and *Aedes* mosquitoes in Africa (*9*,*10*).

More work is needed on the role of non-flea vectors in transmission of *R. felis* and the consequences this may have in terms of mixed infections of diverse vectorborne pathogens. The rare detection of *R. felis* in patients, combined with high flea carriage rates, unusual signs and symptoms linked to immunodeficiencies or multiple infections, and reports from Africa describing *R. felis* in asymptomatic patients, underscore the need for further investigations into the organism's natural history and its uncertain role as a pathogen.

Acknowledgments

We thank the patients and the families of those who participated in the studies. We also thank Rattanaphone Phetsouvanh, Mayfong Mayxay, and the staff of Mahosot Hospital, with special appreciation to the Microbiology Laboratory and Luang Nam Tha. We thank the Salavan Hospital directors and staff, especially Phouvieng Douangdala, and Phoutthalavanh Souvannasing. We also thank Ampai Tanganuchitcharnchai and Suthatip Jintawon for performing the IFAs.

Funding for this study was provided by the Wellcome Trust of Great Britain.

Sabine Dittrich, Koukeo Phommasone, Tippawan Anantatat, Phonepasith Panyanivong, Günther Slesak, Stuart D. Blacksell, Audrey Dubot-Pérès, Josée Castonguay-Vanier, John Stenos, Paul N. Newton, and Daniel H. Paris

Author affiliations: Lao-Oxford-Mahosot Hospital-Wellcome Trust Research Unit, Mahosot Hospital, Vientiane, Laos (S. Dittrich, K. Phommasone, P. Panyanivong, S.D. Blacksell, A. Dubot-Pérès, J. Castonguay-Vanier, P.N. Newton, D.H. Paris); Nuffield Department of Medicine, University of Oxford, Oxford, UK (S. Dittrich, S.D. Blacksell, A. Dubot-Pérès, J. Castonguay-Vanier, P.N. Newton, D.H. Paris); Faculty of Tropical Medicine, Mahidol University, Bangkok, Thailand (T. Anantatat, S.D. Blacksell, D.H. Paris); Service Fraternel d'Entraide–Laos Medical Projects, Luang Namtha, Laos (G. Slesak); Tropenklinik Paul-Lechler-Krankenhaus, Tübingen, Germany (G. Slesak); Aix Marseille University, Medical University, Marseille, France (A. Dubot-Pérès); and The Geelong Hospital, Geelong, Victoria, Australia (J. Stenos)

DOI: http://dx.doi.org/10.3201/eid2008.131308

References

1. Phongmany S, Rolain JM, Phetsouvanh R, Blacksell SD, Soukkhaseum V, Rasachack B, et al. Rickettsial infections and fever, Vientiane, Laos. Emerg Infect Dis. 2006;12:256–62. http://dx.doi.org/10.3201/eid1202.050900
2. Mayxay M, Castonguay-Vanier J, Chansamouth V, Dubot- Pérés A, Paris DH, Phetsouvanh R, et al. Causes of non-malarial fever in Laos: a prospective study. Lancet Glob Health. 2013;1:e46–54. http://dx.doi.org/10.1016/S2214-109X(13)70008-1
3. Nilsson K, Wallmenius K, Hartwig S, Norlander T, Pahlson C. Bell's palsy and sudden deafness associated with *Rickettsia* spp. infection in Sweden. A retrospective and prospective serological survey including PCR findings. Eur J Neurol. 2014;21:206–14. http://dx.doi.org/10.1111/ene.12218
4. Jiang J, Maina AN, Knobel DL, Cleaveland S, Laudisoit A, Wamburu K, et al. Molecular detection of *Rickettsia felis* and *Candidatus* Rickettsia asemboensis in fleas from human habitats, Asembo, Kenya. Vector Borne Zoonotic Dis. 2013;13:550–8. http://dx.doi.org/10.1089/vbz.2012.1123
5. Maina AN, Knobel DL, Jiang J, Halliday J, Feikin DR, Cleaveland S, et al. *Rickettsia felis* infection in febrile patients, western Kenya, 2007–2010. Emerg Infect Dis. 2012;18:328–31. http://dx.doi.org/10.3201/eid1802.111372
6. Kernif T, Socolovschi C, Wells K, Lakim MB, Inthalad S, Slesak G, et al. Bartonella and Rickettsia in arthropods from the Lao PDR and from Borneo, Malaysia. Comp Immunol Microbiol Infect Dis. 2012;35:51–7. http://dx.doi.org/10.1016/j.cimid.2011.10.003
7. Joshi N, Caputo GM, Weitekamp MR, Karchmer AW. Infections in patients with diabetes mellitus. N Engl J Med. 1999;341:1906–12. http://dx.doi.org/10.1056/NEJM199912163412507
8. Greenwood BM, Bradley-Moore AM, Bryceson AD, Palit A. Immunosuppression in children with malaria. Lancet. 1972;1:169–72. http://dx.doi.org/10.1016/S0140-6736(72)90569-7
9. Socolovschi C, Pages F, Ndiath MO, Ratmanov P, Raoult D. *Rickettsia* species in African *Anopheles* mosquitoes. PLoS ONE. 2012;7:e48254. http://dx.doi.org/10.1371/journal.pone.0048254
10. Choi YJ, Lee EM, Park JM, Lee KM, Han SH, Kim JK, et al. Molecular detection of various *rickettsiae* in mites (acari: *trombiculidae*) in southern Jeolla Province, Korea. Microbiol Immunol. 2007;51:307–12. http://dx.doi.org/10.1111/j.1348-0421.2007.tb03912.x

Address for correspondence: Daniel H. Paris, Mahidol-Oxford Tropical Medicine Research Unit, Faculty of Tropical Medicine, Mahidol University, 3rd Floor, 60th Anniversary Chalermprakiat Building, 420/6 Ratchawithi Rd., Ratchathewi District, 10400-Bangkok, Thailand; email: parigi@tropmedres.ac

Chikungunya Outbreak in Bueng Kan Province, Thailand, 2013

To the Editor: Chikungunya fever is a dengue-like syndrome characterized by acute fever, arthralgia, and maculopapular rash. The causative agent is chikungunya virus (CHIKV), which is transmitted by *Aedes aegypti* and *Aedes albopictus* mosquitoes (*1*). Based on the genome and the viral envelope E1 sequences, CHIKV is classified into 3 genetic lineages: Asian, West African, and East/Central/South African (ECSA) genotypes (*2*).

In Thailand, the first report of CHIKV infection occurred in Bangkok in 1958 (*3*); later, sporadic cases of chikungunya fever occurred in many provinces during 1976–1995 (*4*). All of the CHIKV strains found in Thailand at that time were of the Asian genotype. The virus has since reemerged during 2008–2009 and caused large outbreaks in southern Thailand, affecting >50,000 persons (*5*). These outbreaks were attributed to the ECSA genotype. We report an outbreak of CHIKV infection in the northeastern province of Bueng Kan in 2013.

Bueng Kan Province is located on the Mekong River on the foothills of the mountainous region of Laos to the north. An outbreak of suspected dengue cases was reported during the rainy season during April–September 2013(*6*). Beginning in September, however, hospital physicians noticed that patients were reporting fever with moderate to severe joint pain resulting in limitation of movement that lasted for weeks. Serum samples were collected from 109 persons (hospitalized and outpatient) in October. Clinical data showed that 38 (34.9%) had moderate to severe joint pain; median duration of illness was 4 days (range 1–7). Median timing of sample collection from the onset of illness was 8 days (range 1–21).

Samples were sent to Chulalongkorn University Hospital in Bangkok to screen for mosquitoborne viruses. The study protocol was approved by the Institutional Review Board of Chulalongkorn University and consents were waived because all samples were stored as anonymous. Viral genomic RNA was assayed by using seminested reverse transcription PCR (RT-PCR) for CHIKV nucleic acid (*7*). Serum samples were tested for IgM against CHIKV by using SD BIOLINE Chikungunya IgM Test (Standard Diagnostics Inc., Kyonggi-do,

South Korea) (*8*). In our study, the criteria for diagnosis of CHIKV infection included the detection of CHIKV nucleic acid by RT-PCR or IgM antibodies against CHIKV.

Of the 109 samples tested, 51 (46.8%) had evidence of CHIKV infection, as 25 (22.9%) were positive for CHIKV RNA by RT-PCR, and 32 (29.3%) were positive for IgM antibodies. Both CHIKV nucleic acid and IgM were found in 6 samples. To further characterize the phylogenetic relationships between the CHIKV strains in this outbreak with strains previously found in Thailand and neighboring regions, we performed full-length viral genomic sequencing from srains from 4 samples by primer walking (*4*). We subjected sequences to BLAST analysis (BLAST, http://blast.ncbi.nlm.nih.gov), aligned using the BioEdit program v7.1.9 (http://www.mbio.ncsu.edu/bioedit/bioedit.html), and performed sequence assembly using the DNASTAR v6.0 (DNA Star, Madison, WI, USA). We performed maximum likelihood phylogenetic analysis of a set of 11,710 nt, including the sequences identified in this study (THA/Bueng Kan/BK46/2013, THA/Bueng Kan/BK57/2013, THA/Bueng Kan/BK63/2013, THA/Bueng Kan/BK68/2013; accession nos. KJ579184–7) by using the MEGA program v6.0 (http://www.megasoftware.net). Taking into consideration possible co-circulation of the Asian and ECSA genotypes in Thailand, we also examined intergenotypic recombination using the Recombination Detection Program, v4.22 (http://en.bio-soft.net/tree/RDP.html).

Phylogenetic analysis, comparing the 4 strains of CHIKV identified from Bueng Kan to 52 additional whole genomic sequences from GenBank, revealed that the 4 strains are closely related and share >99.8% pairwise nucleotide identity (Figure). The Bueng Kan isolates grouped to the ECSA genotype within the recent Indian Ocean clade (*9*). This relationship

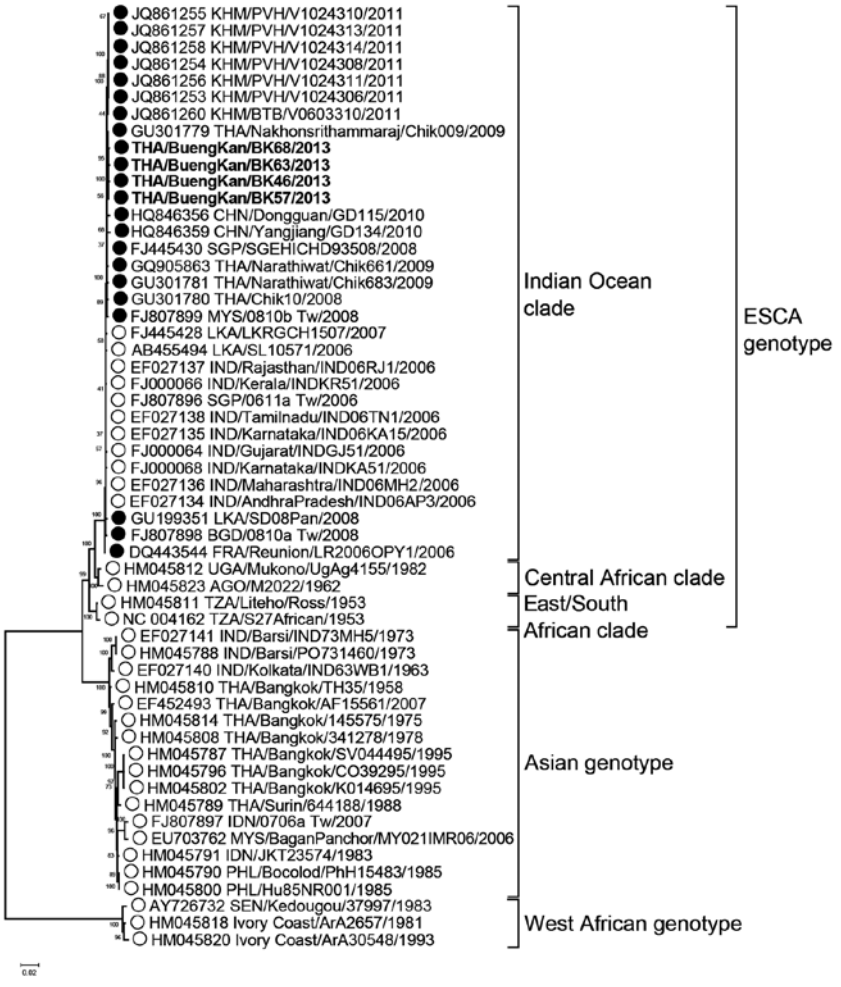


Figure. Phylogenetic analysis of whole genome nucleotide sequences of chikungunya virus (CHIKV) isolated during the 2013 outbreak in Bueng Kan Province, Thailand. The trees were generated by maximum-likelihood method, and the numbers along the branches indicate bootstrap values. Scale bar denotes nucleotide substitutions per site. Branch support and nodal confidence was assessed by using a general time reversible +I-4 nt substitution model with 1,000 bootstrap resampling. All sequences are labeled with GenBank accession number, country (3 letter code) and city of origin, strain name, and year of sampling. Bold text indicates CHIKV isolates identified in this study. Black and white circles on the tree indicate E1-A226V mutant and nonmutant strains, respectively. ESCA, East/Central/South African.

was confirmed by analysis of the nonstructural, structural, and E1 encoding regions (data not shown). The THA/Bueng Kan strains all possessed an alanine to valine change at residue 226 (A226V) in the E1 gene, which was noted as one of the crucial substitutions for increased transmissibility by *Aedes albopictus* mosquitoes reported for the Réunion Island isolates (*9*). This substitution was shared among strains isolated in Thailand in 2008, which were responsible for the previous CHIKV outbreaks in southern Thailand (*7*), and those in the recent outbreak in Cambodia (*10*). This suggests the presence and continued circulation of the E1-A226V virus in Thailand since 2008. However, another mutation specific to the Indian Ocean isolates, D284E in the E1 gene, was not found in THA/Bueng Kan strains. No evidence of recombination has been found in the THA/Bueng Kan strains and other strains identified in Thailand thus far.

Viral infections by mosquitoes continue to challenge public health in Thailand. In evidence of this, we demonstrated that CHIKV has become established in northeastern Thailand. Surveillance and clinical recognition will not prevent future outbreaks, but rather will assist in organizing an early response to outbreaks and thus minimize unnecessary illness and death.

Acknowledgments

We thank Padet Siriyasatien for providing us with laboratory test kits and protocols. We thank the staff of Bueng Kan Provincial Hospital for their contribution in sample collection and the staff at the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, for their hard work in performing laboratory tests.

This research is supported by National Research University Project; Office of Higher Education Commission (WCU-007-HR-57); Integrated Innovation Academic Center, Chulalongkorn University; Centenary Academic Development Project (CU56-HR01); the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (RES560530093); the Outstanding Professor of the Thailand Research Fund (DPG5480002); and King Chulalongkorn Memorial Hospital Thai Red Cross Society; the Ratchadaphiseksomphot Endowment Fund (S. Vongpunsawad and P. Linsuwanon); MK Restaurant Company Limited; and The Siam Cement Pcl.

Nasamon Wanlapakorn, Thanunrat Thongmee, Piyada Linsuwanon, Paiboon Chattakul, Sompong Vongpunsawad, Sunchai Payungporn, and Yong Poovorawan

Author affiliations: Chulalongkorn University, Bangkok, Thailand (N. Wanlapakorn, T. Thongmee, P. Linsuwanon, S. Vongpunsawad, S. Payungporn, Y. Poovorawan); and Bueng Kan Provincial Hospital, Bueng Kan, Thailand (P. Chattakul)

DOI: http://dx.doi.org/10.3201/eid2008.140481

References

1. Weaver SC, Osorio JE, Livengood JA, Chen R, Stinchcomb DT. Chikungunya virus and prospects for a vaccine. Expert Rev Vaccines. 2012;11:1087–101. http://dx.doi.org/10.1586/erv.12.84
2. Volk SM, Chen R, Tsetsarkin KA, Adams AP, Garcia TI, Sall AA, et al. Genome-scale phylogenetic analyses of chikungunya virus reveal independent emergences of recent epidemics and various evolutionary rates. J Virol. 2010;84:6497–504 http://dx.doi.org/10.1128/JVI.01603-09.
3. Hammon WM, Rudnick A, Sather GE. Viruses associated with epidemic hemorrhagic fevers of the Philippines and Thailand. Science. 1960;131:1102–3. http://dx.doi.org/10.1126/science.131.3407.1102
4. Pongsiri P, Auksornkitti V, Theamboonlers A, Luplertlop N, Rianthavorn P, Poovorawan Y. Entire genome characterization of Chikungunya virus from the 2008–2009 outbreaks in Thailand. Trop Biomed. 2010;27:167–76.
5. Suangto P, Uppapong T. Chikungunya fever. Annual epidemiological surveillance report 2009, Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health. [cited 2014 Mar 18]. http://www.boe.moph.go.th/Annual/Annual%202552/Main.html
6. Epidemiological Information Center, Communicable Disease Epidemiological Section, Bureau of Epidemiology. Reported Cases of Diseases under Surveillance 506, 53rd week [cited 2014 Mar 18]. http://203.157.15.4/wesr/file/y56/F56533_1393.pdf
7. Rianthavorn P, Prianantathavorn K, Wuttirattanakowit N, Theamboonlers A, Poovorawan Y. An outbreak of chikungunya in southern Thailand from 2008 to 2009 caused by African strains with A226V mutation. Int J Infect Dis. 2010;3:e161–5. http://dx.doi.org/10.1016/j.ijid.2010.01. 001.
8. Kosasih H, Widjaja S, Surya E, Hadiwijaya SH, Butarbutar DP, Jaya UA, et al. Evaluation of two IgM rapid immunochromatographic tests during circulation of Asian lineage Chikungunya virus. Southeast Asian J Trop Med Public Health. 2012;43:55–61.
9. Schuffenecker I, Iteman I, Michault A, Murri S, Frangeul L, Vaney MC, et al. Genome microevolution of chikungunya viruses causing the Indian Ocean outbreak. PLoS Med. 2006;3:e263. http://dx.doi.org/10.1371/journal.pmed.0030263
10. Duong V, Andries AC, Ngan C, Sok T, Richner B, Asgari-Jirhandeh N, et al. Reemergence of Chikungunya virus in Cambodia. Emerg Infect Dis. 2012;18:2066–9 http://dx.doi.org/10.3201/eid1812.120471.

Address for correspondence: Yong Poovorawan, Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand; email: Yong.P@chula.ac.th

Decline in Japanese Encephalitis, Kushinagar District, Uttar Pradesh, India

To the Editor: Kakkar et al. recently concluded that the low-quality surveillance data on acute encephalitis syndrome (AES)/Japanese encephalitis (JE) in Kushinagar District, India, provide little evidence to support development of prevention and control measures and to estimate the effect of interventions (*1*). Analysis of the surveillance data, however, does provide evidence supporting the effect of an ongoing intervention (i.e., JE vaccination).

In accordance with the surveillance protocol, cerebrospinal fluid and/or serum samples from AES patients admitted to the Baba Raghav Das Medical College (Gorakhpur, India) are tested for IgM against JE at the field laboratory of National Institute of Virology (NIV) at Gorakhpur (*2*). The samples are tested by using the ELISA developed by NIV Pune (Pune, India), which has a specificity of 85% (range 77%–95%) and sensitivity of 71% (range 71%–75%) (*3*). Patients with samples negative for JE are considered to have JE-negative AES.

We obtained the line-list of AES patients from the NIV laboratory at Gorakhpur for 2008–2012. Analysis of the surveillance data indicated that 251 (8.2%, range 4%–14.7%) of the 3,047 AES patients from Kushinagar

were positive for JE IgM and therefore considered to be JE case-patients. JE incidence per 100,000 persons in the district declined from 2.3 cases in 2010 to 0.81 in 2011 to 0.58 in 2012 (Figure). The decline in JE incidence since 2010 could be a consequence of JE vaccination activities in Kushinagar. In 2010, a mass vaccination campaign with 1 dose of JE vaccine (SA 14–14–2 strain) was conducted among children 1–15 years of age. Subsequently, the vaccine was introduced into the childhood vaccination program as a 1-dose strategy in 2011 and a 2-dose strategy in 2013. Unfortunately, information about evaluated coverage of JE vaccine is not available from the district. On the other hand, the average annual incidence of JE-negative AES during the same period was 16 cases per 100,000 persons (95% CI 14.8–17.2), and this incidence has remained relatively stable since 2008.

With the isolation of enteroviruses from JE-negative AES patients, waterborne transmission has been hypothesized, and the focus of intervention has shifted toward improving sanitation and water quality. However, enteroviruses were detected only in a small proportion of AES patients. Although the quality of AES surveillance needs to be improved, as Kakkar et al. suggested (*1*), further studies are needed to understand the etiology of JE-negative AES in the district and the risk factors for transmission. These studies might include systematically investigating patients and environmental samples for enteroviral and other etiologic agents.

Prashant Ranjan, Milind Gore, Sriram Selvaraju, K.P. Kushwaha, D.K. Srivastava, and Manoj Murhekar

Author affiliations: Indian Council of Medical Research National Institute of Epidemiology, Chennai, India (P. Ranjan, S. Selvaraju, M. Murhekar); Indian Council of Medical Research National Institute of Virology, Gorakhpur, India (M. Gore); and Baba Raghav Das Medical Collage, Gorakhpur (K.P. Kushwaha, D.K. Srivastava)

DOI: http://dx.doi.org/10.3201/eid2008.131403

References

1. Kakkar M, Rogawski ET, Abbas SS, Chaturvedi S, Dhole TN, Hossain SS, et al. Acute encephalitis syndrome surveillance, Kushinagar District, Uttar Pradesh, India, 2011–2012. Emerg Infect Dis. 2013;19:1361–7 .
2. Directorate of National Vector Borne Diseases Control Programme. Guidelines for surveillance of acute encephalitis syndrome (with special reference to Japanese encephalitis) [cited 2013 Sep 11]. http://www.nvbdcp.gov.in/Doc/AES%20guidelines.pdf
3. World Health Organization Regional Office for Southeast Asia. Fourth biregional meeting on the control of Japanese encephalitis. Report of the meeting Bangkok, Thailand, 7–8 June 2009 [cited 2013 Aug 15]. http://www.wpro.who.int/immunization/documents/docs/JEBiregionalMeetingJune2009final.pdf

Address for correspondence: Manoj Murhekar, National Institute of Epidemiology, Indian Council of Medical Research, Chennai-600 070, India; email: mmurhekar@gmail.com

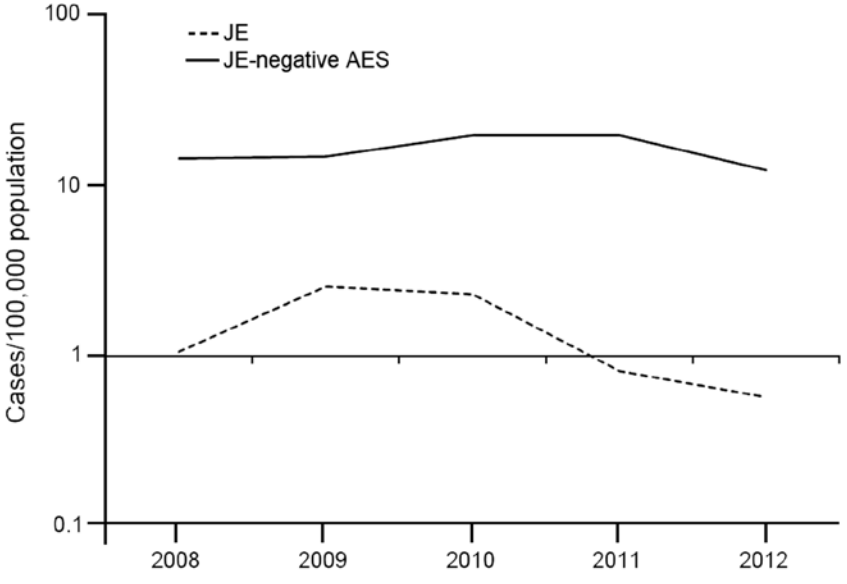


Figure. Annual incidence of Japanese encephalitis (JE) and JE-negative acute encephalitis syndrome (AES), Kushinagar District, Uttar Pradesh, India, 2008–2012.

Babesiosis Surveillance, New Jersey, USA, 2006–2011

To the Editor: Since zoonotic babesiosis was first identified in the United States in 1966 (*1*), its incidence and geographic range have increased (*2*). Previous studies have demonstrated increases in transfusion-associated cases in recent years (*3*). In 2011, babesiosis became nationally notifiable as its emergence and the potential for transfusion-associated cases were recognized (*2*,*4*). We assessed New Jersey, USA, surveillance data for 2006–2011 to characterize case information (incidence, potential transfusion associations, geographic distribution) in a state where babesiosis is endemic.

In New Jersey, babesiosis case reporting began in 1985. A retrospective study identified an upward trend during 1993–2001; eight of 21 counties reported cases (*5*). In 2005, the New Jersey Department of Health

established the Communicable Disease Reporting Surveillance System (CDRSS) to collect detailed information for all reportable communicable diseases from clinicians, hospitals, and laboratories. Babesiosis was classified as confirmed for persons who had clinically compatible illnesses and *Babesia* parasites were detected by blood smear examination and as probable for persons who had clinically compatible illness, including documented anemia or thrombocytopenia, and total antibodies, shown by immunoglobulin or IgG titers of ≥1:256 against *B. microti* by indirect fluorescent test. Cases were considered possibly transfusion associated if patients had documented cellular transfusions with no (or unlikely) other risk factors (e.g., tick bites) reported in CDRSS within 6 months before illness onset. To identify possible transfusion-associated cases, we searched CDRSS text fields for "blood," "transfusion," and "receipt of blood donation." We obtained supportive evidence, when available, for transfusion transmission from medical records or blood center reports. We calculated incidence rates using US Census population data for 2000 (*6*).

During 2006–2011, a total of 568 babesiosis cases were reported (Figure); 521 (92%) were classified as confirmed and 47 (8%) as probable. In 2006 and 2011, 64 and 166 cases were reported, a 260% increase in reported cases; respective incidence rates were 0.76 and 1.97 cases per 100,000 population. Seven of New Jersey's 21 counties accounted for 462 (81%) of all reported cases and for 128 (77%) of the 166 cases occurring during 2011. However, all counties reported at least 1 case within the study period, whereas only 8 counties reported cases during 1993–2001 (*5*) (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/20/8/13-1591-Techapp1.pdf). Incidence for 2006–2011 ranged from 0.4 to 39.4 cases per 100,000 population; counties

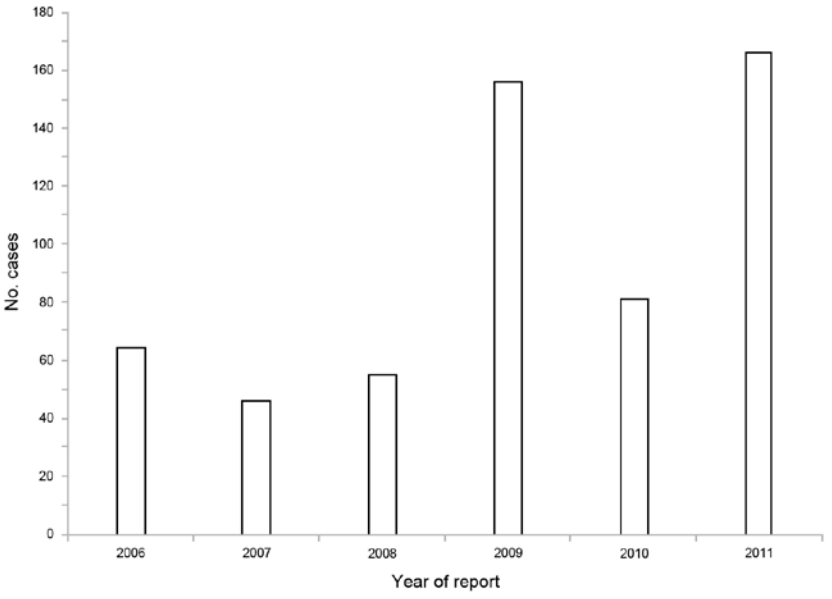


Figure. Reported confirmed and probable babesiosis cases, New Jersey, USA, 2006–2011. N = 568.

in southern New Jersey had the majority of cases and also reported a high incidence of Lyme disease.

Case-patients' median age was 66 years (range 1 month–98 years). Two confirmed cases occurred in infants who were believed to have become infected by congenital transmission (*7*). One infant's mother was asplenic and had confirmed babesiosis. The other mother was asymptomatic and did not meet case criteria but had reported tick bites.

A total of 371 (65%) case-patients were aged ≥60 years of age; 395 (70%) were male. Of the 568 case patients, 401 (71%) had been hospitalized at least once. Of the 303 case-patients for whom information was available 48 (16%) were admitted to an intensive care unit. The all-cause case-fatality rate was 2% (7/357). All 7 persons who died had been hospitalized, 3 of whom had been admitted to intensive care units.

We identified 12 possible transfusion-associated cases (2 in 2006, 1 in 2007, 3 in 2009, 2 in 2010, and 4 in 2011). Two additional transfusion-associated transmissions (1 each in 2006 and 2009) were identified, but these persons were asymptomatic and not included in this study. Risk factors for possible transfusion-associated cases included surgical procedures with complications requiring transfusions. Median age and case-fatality rate were higher for patients with possible transfusion-associated babesiosis, and these patients were significantly more likely to have acquired infection outside the summer months (online Technical Appendix Table).

Our study has some limitations. Increasing awareness, electronic reporting and testing, and environmental or ecologic factors might have contributed to the upward trend and incidence fluctuations. However, neighboring jurisdictions also observed a similar geographic expansion and overall increase in incidence (*8*,*9*). Moreover, New Jersey's Lyme disease surveillance system shows similar incidence fluctuations for Lyme disease during the study period.

Continued surveillance for detecting babesiosis and investigating possible transfusion-associated cases is needed nationwide (*10*). Although most cases in our study were reported during summer months, possible

transfusion-associated cases were reported throughout the year, underscoring the need for constant awareness. The 2 cases of probable congenital infection highlight the need to consider *Babesia* infection for newborns who have compatible clinical manifestations, especially if the mother had risk factors for infection.

Prompt identification of babesiosis is essential to prevent disease transmission from infected blood donors to recipients. Although we modified New Jersey surveillance to include transfusion as a risk factor, collaboration with stakeholders (including blood centers) will further facilitate case detection and confirmation and identification of infected donors. Including babesiosis on the list of nationally notifiable diseases will improve national disease reporting and clarify the geographic distribution and incidence of tickborne and possible transfusion-associated cases. With increasing public awareness and screening, public health professionals and stakeholders might consider dedicating public health resources for babesiosis surveillance.

Acknowledgments

We thank Stella Tsai for her assistance and Kris Bisgard and Barbara Herwaldt for their helpful comments.

Andria Apostolou, Faye Sorhage, and Christina Tan

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A. Apostolou); New Jersey Department of Health, Trenton, New Jersey, USA (A. Apostolou, F. Sorhage, C. Tan)

DOI: http://dx.doi.org/10.3201/eid2008.131591

References

1. Scholtens RG, Braff EH, Healey GA, Gleason N. A case of babesiosis in man in the United States. Am J Trop Med Hyg. 1968;17:810–3.
2. Centers for Disease Control and Prevention. Babesiosis surveillance—18 states, 2011. MMWR Morb Mortal Wkly Rep. 2012;61:505–9.
3. Herwaldt BL, Linden JV, Bosserman E, Young C, Olkowska D, Wilson M. Transfusion-associated babesiosis in the United States: a description of cases. Ann Intern Med. 2011;155:509–19. http://dx.doi.org/10.7326/0003-4819-155-8-201110180-00362
4. Centers for Disease Control and Prevention. National Notifiable Diseases Surveillance System. National notifiable infectious conditions [cited 2013 Sep 15]. http://www.cdc.gov/NNDSS/script/conditionsummary.aspx?CondID=24
5. Herwaldt BL, McGovern PC, Gerwel MP, Easton RM, MacGregor RR. Endemic babesiosis in another eastern state, New Jersey. Emerg Infect Dis. 2003;9:184–8. http://dx.doi.org/10.3201/eid0902.020271
6. US Census Bureau. Census 2000 for the state of New Jersey [cited 2013 Sep 15]. http://www.census.gov/census2000/states/nj.html
7. Sethi S, Alcid D, Kesarwala H, Tolan RW Jr. Probable congenital babesiosis in infant, New Jersey, USA. Emerg Infect Dis. 2009;15:788–91. http://dx.doi.org/10.3201/eid1505.070808
8. Joseph JT, Roy SS, Shams N, Visintainer P, Nadelman RB, Hosur S, et al. Babesiosis in lower Hudson Valley, New York, USA. Emerg Infect Dis. 2011;17:843–7. http://dx.doi.org/10.3201/eid1705.101334
9. New York State Department of Health. Communicable disease annual reports and related information [cited 2014 Feb 15]. http://www.health.ny.gov/statistics/diseases/communicable/
10. Centers for Disease Control and Prevention. Investigation toolkit: transfusion-transmitted infections (TTI) [cited 2013 Sep 15]. http://www.cdc.gov/bloodsafety/tools/investigation-toolkit.html

Address for correspondence: Andria Apostolou, New Jersey Department of Health, Communicable Disease Service, 135 E State St, PO Box 369, Trenton, NJ 08625-0369, USA; email: aapostolou@scimetrika.com

Antibodies against West Nile and Shuni Viruses in Veterinarians, South Africa

To the Editor: Many arboviruses are zoonotic; humans acquire infection from the bites of arthropod vectors or through exposure to the tissues and body fluids of infected animals. West Nile virus (WNV), a widely endemic zoonotic agent in South Africa, occurs wherever the principal vector (*Culex univittatus* mosquitoes) and avian hosts are present (*1*). Serosurveys based on hemagglutination inhibition and neutralization assays conducted during 1950–1970 indicated that 17%–20% of long-term rural residents in the Karoo, 4%–8% in the Highveld, and 1%–3% in the Natal and the Eastern Cape areas had antibodies against WNV (*1*). Most human infections tend to be sporadic and are characterized by mild febrile illness (*2*); however, severe disease has been documented (*3*). WNV has caused severe neurologic disease of horses in South Africa (*4*), and zoonotic transmission was recorded in a veterinary student who performed a necropsy on an infected horse (*5*).

Shuni virus (SHUV) (genus O*rthobunyavirus*, family *Bunyaviridae*) was first isolated in Nigeria in 1966 during surveys of livestock, *Culicoides* midges, and mosquitoes, SHUV also once was isolated from a febrile child (*6*,*7*). SHUV recently was identified as a previously undetected cause of neurologic disease in horses in southern Africa (*8*) and is thus of interest in comparison to WNV.

To determine the potential for human infections, we tested veterinarians as a high-risk group for evidence of infection with these 2 viruses. Veterinarians with regular exposure to horses, livestock, or wildlife—and thus to vectors because of an

outdoor lifestyle—were invited to donate blood samples at specialist veterinary conferences in South Africa in 2011 and 2012.

The Kunjin MRM61C strain of WNV (*9*) and SHUV isolate SAE 18/09 (*8*) were cultured and harvested when the cytopathic effect (CPE) reached 80%. Stock virus was titrated in 100-mL volumes in 6 replicate wells per serial 10-fold dilution (10^{-1} to 10^{-9}) in Leibowitz medium with 5% fetal calf serum (Invitrogen, Carlsbad, CA, USA), with 100 mL of medium added in place of test serum dilution, and 25 mL of Vero cells (8×10^5 cells/mL) added per well. Plates were incubated at 37°C; CPE was monitored; and 50% tissue culture infectious dose per milliliter endpoints were calculated. For neutralization tests, serum were inactivated at 56°C for 30 min; duplicate 100-mL volumes of doubling dilutions were prepared in Leibowitz medium (1:10–1:640) and incubated with equal volumes of medium containing a calculated 100 50% tissue culture infectious dose virus at 37°C for 45 min, and 25 mL Vero cells (8×10^5 cells/mL) were added per well. Medium was added in place of virus to replicate test serum controls at a 1:10 dilution to monitor for toxicity of the serum, and the virus used in the test was back titrated. Tests were monitored for 10 days. Neutralization titers were expressed as the reciprocal of the serum dilution that inhibited ≥75% of CPE in both replicates, and only titers ≥20 were recorded as positive for virus antibodies.

Serum samples were received from 123 veterinarians in South Africa and 4 from neighboring countries. Ten (7.9%) serum samples tested positive for antibody to WNV and 5 (3.9%) for antibody to SHUV; all positive serum samples (titers 20–80) were from South African veterinarians. Prevalence of WNV antibody in men (5/81 [6.2%]) and women (5/46 [10.9%]) did not differ significantly. The veterinarians ranged in age from 23 to 71 years and had practiced an average of ≈23 years; the prevalence of WNV antibody was similar in age groups23–50 years (6/74 [8.1%]) and 51–71 years (4/53 [7.5%)].

Most veterinarians came from periurban practices in Gauteng (51/123) and Western Cape Provinces (18/123); the comparatively small numbers of samples from elsewhere preclude valid comparisons with the historical surveys of rural residents. However, indications that veterinarians might be at increased risk for infection in some areas included a 23.1% (3/13) prevalence of WNV antibody in KwaZulu-Natal veterinarians and a similar prevalence of antibody in much smaller sample groups in the Free State and Northern Cape Provinces. In Gauteng, where most horses reside, 6% of veterinarians tested positive for WNV, which reflects the prevalence described for the Highveld region in the 1970s.

Four of the 5 veterinarians positive for SHUV antibody were men; 2 were in the 23–50-year age group, and 3 were in the 50–71-year age group. Of the veterinarians who tested positive, 3 were identified in Gauteng (3/51 [5.9%]) and 1 each in the Eastern Cape (1/8) and Limpopo (1/8) Provinces. No clear histories of disease compatible with the infections could be elicited from any of the veterinarians whose samples contained antibodies. Nevertheless, the 2 viruses, and related arboviruses, tended to be overlooked as animal and human pathogens in southern Africa until recently, and greater awareness is needed of their potential as zoonotic agents. Investigations of neurologic illness in humans identified several WNV cases that had been overlooked in hospitals in Gauteng (*10*). Similar investigations of febrile and neurologic illness in humans might shed light on the possible clinical significance of SHUV infection in humans.

Charmaine van Eeden, Robert Swanepoel, and Marietjie Venter[1]

Author affiliation: University of Pretoria Department of Medical Virology, Pretoria, South Africa.

DOI: http://dx.doi.org/10.3201/eid2008.131724

References

1. Jupp PG. The ecology of West Nile virus in South Africa and the occurrence of outbreaks in humans. Ann N Y Acad Sci. 2001;951:143–52. http://dx.doi.org/10.1111/j.1749-6632.2001.tb02692.x
2. Jupp PG, Blackburn NK, Thompson DL, Meenehan GM. Sindbis and West Nile virus infections in the Witwatersrand-Pretoria region. S Afr Med J. 1986;70:218–20.
3. Burt FJ, Grobbelaar AA, Leman PA, Anthony FS, Gibson GV, Swanepoel R. Phylogenetic relationships of southern African West Nile virus isolates. Emerg Infect Dis. 2002;8:820–6. http://dx.doi.org/10.3201/eid0808.020027
4. Venter M, Swanepoel R. West Nile virus lineage 2 as a cause of zoonotic neurological disease in humans and horses in southern Africa. Vector-Borne Zoonotic Dis. 2010;10:659–64. http://dx.doi.org/10.1089/vbz.2009.0230
5. Venter M, Steyl J, Human S, Weyer J, Zaayman D, Blumberg L, et al. Transmission of West Nile virus during horse autopsy. Emerg Infect Dis. 2010;16:573–5. http://dx.doi.org/10.3201/eid1603.091042
6. Causey OR, Kemp GE, Causey CE, Lee VH. Isolations of Simbu-group viruses in Ibadan, Nigeria 1964–69, including the new types Sango, Shamonda, Sabo and Shuni. Ann Trop Med Parasitol. 1972;66:357–62.
7. Moore DL, Causey OR, Carey DE, Reddy S, Cooke AR, Akinkugbe FM, et al. Arthropod-borne viral infections in man in Nigeria: 1964–1970. Ann Trop Med Parasitol. 1975;69: 49–64.
8. van Eeden C, Williams JH, Gerdes TGH, van Wilpe E, Vijoen A, Swanepoel R, et al. Shuni virus as cause of neurologic disease in horses. Emerg Infect Dis. 2012;18:318–21. http://dx.doi.org/10.3201/eid1802.111403
9. Zaayman D, Human S, Venter M. A highly sensitive method for the detection and genotyping of West Nile virus by real-time

[1]Current affiliation: Global Disease Detection, US Centers for Disease Control and Prevention, Pretoria, South Africa.

PCR. J Virol Methods. 2009;157:155–60. http://dx.doi.org/10.1016/j.jviromet.2008.12.014
10. Zaayman D, Venter M. West Nile virus neurologic disease in humans, South Africa, September 2008–May 2009. Emerg Infect Dis. 2012;18:2051–4. http://dx.doi.org/10.3201/eid1812.111208

Address for correspondence: Marietjie Venter, Global Disease Detection, US-CDC, PO Box, 9536, Pretoria, 0001, South Africa; email: yds8@cdc.gov

Isolation of *Rickettsia typhi* from Human, Mexico

To the Editor: Murine typhus is a febrile illness caused by *Rickettsia typhi*. The clinical manifestations are nonspecific, and the signs and symptoms resemble those of several other febrile illnesses. Murine typhus can be a self-limiting infection; however, it should be diagnosed and treated because complications and even death can result (*1*). In Mexico, particularly in Yucatan State, cases of murine typhus in humans and high prevalence of antibodies in healthy blood donors have been reported (*2*,*3*). In 2012, we isolated *R. typhi* from a human patient in southeastern Mexico by using a simple and effective method, an adaptation of the centrifugation shell vial method to cell culture plates.

The patient, a 23-year-old man from Dzibzantun (21°15′00″N, 89°03′00″W), in the northeastern part of Yucatan State, was referred for possible diagnosis of rickettsial infection. He had a low-grade fever (37.6°C) and a maculopapular rash on the thorax and upper and lower extremities. The patient reported having cats in the house, but no fleas or ticks were observed. Clinical laboratory findings were within reference ranges. Test results were negative for dengue virus, but the Weil-Felix (Proteus OX19) test result was positive (titer 1:164). Single-step PCR amplification was performed by using genus-specific primers for the 17-kDa lipoprotein and the citrate synthase gene (*gltA*), as described previously, to obtain amplicons of 434 bp and 380–385 bp (*4*). PCR was positive for *R. typhi*, and 100 mg of oral doxycycline 2 times per day for 7 days was prescribed; the rash cleared.

We subjected 5 mL of blood to centrifugation for 1 hour at 1,000 rpm and then stored the plasma at –80°C. Blood samples from other patients were used as controls. A total of 50,000 Vero cells were grown in 8 central wells of a 24-well cell culture cluster (Corning Incorporated, Corning, NY, USA) with minimal essential medium (MEM; Biowest, Nuaillé, France) supplemented with 10% fetal bovine serum (Biowest) and incubated at 37°C with 5% CO_2 for 48 hours to obtain 95% confluence. We then thawed 700 mL of the plasma in a 37°C water bath. The MEM was discarded, and the wells were refilled with 250 mL each of a mixture of the plasma and fresh medium at a 1:3 ratio. The plaque was covered with parafilm and centrifuged at 700 *g* for 60 minutes at 22°C. The supernatant was discarded and replaced with 1 mL of MEM supplemented with 5% fetal bovine serum, 100 U penicillin, 100 μg streptomycin, and 250 ng amphotericin B (Sigma Aldrich, St. Louis, MO, USA) and incubated at 33°C with 5% CO_2.

On day 3 after sample inoculation, the antimicrobial drug–containing medium was removed and replaced with MEM without antimicrobial drug and supplemented with 5% fetal calf serum (HyClone Laboratories, Inc., South Logan, UT, USA). Medium was changed every 3 days until day 15. A cell sample from each well was tested for infection at days 9 and 15 by using Gimenez stain and PCR with *17 kDa* and *gltA* primers.

Gimenez staining on day 15 yielded numerous red-stained bacteria in the cytoplasm of Vero cells in the 8 wells used. A single scraping of the cells from the positive wells was inoculated onto confluent layers of Vero cells, which enabled establishment of the isolate.

Three PCR amplicons of the *17kDa*- and *gltA*-specific primers (*4*–*6*) from positive wells were fully sequenced. After removing primer sequences, we compared amplicon sequences by conducting a gapped BLAST 2.0 (http://blast.st-va.ncbi.nlm.nih.gov/Blast.cgi) search of the GenBank database; the *17-kDa* (accession no. JX198507) and *gltA* (accession no. KC469611) gene fragment sequences showed 100% identity with *R. typhi* strain Wilmington (accession no. AE017197.1).

Murine typhus has been reemerging in southeastern Mexico for the past 6 years (*3*,*7*). Active epidemiologic surveillance led to early detection of human cases and opportune treatment, thereby decreasing the rate of severe illness. However, the prevalent social and cultural conditions in small villages, with close contact with domestic, peridomestic, and wild animals, facilitate the transmission of this fleaborne rickettsiosis; human infections, such as the case presented here, still occur.

We replaced shell vials with cell culture plates and isolated rickettsiae from a biological sample from a patient with acute murine typhus. The method is as simple as the shell vial centrifugation technique and is highly sensitive and easy to perform, making it an excellent choice for rickettsiae isolation when shell vials are not available.

In the United States, isolation of *R. typhi* from a human was last reported >50 years ago (*8*). The case reported here reinforces the need to extend surveillance to small towns and villages

in Yucatan State. It also shows that a shell vial alternative method for *R. typhi* isolation is simple and effective.

Acknowledgments

We thank the members of the Red Iberoamericana para la Investigación y Control de las Enfermedades Rickettsiales for providing useful information for the preparation of this manuscript.

We dedicate this article to the memory of Jorge Zavala Velázquez, who pioneered *Rickettsia* research in Mexico and Latin America.

This research was supported by grants from the Consejo Nacional de Ciencia y Tecnologia (CB-2008-1-99890) to J.E.Z.-C. and facilitated by the Ciencia y Tecnologia para el Desarrollo.

Jorge E. Zavala-Castro, Karla R. Dzul-Rosado, Gaspar Peniche-Lara, Raúl Tello-Martín, and Jorge E. Zavala-Velázquez

Author affiliation: Universidad Autónoma de Yucatán, Mérida, México

DOI: http://dx.doi.org/10.3201/eid2008.130095

References

1. Gillespie JJ, Ammerman NC, Beier-Sexton M, Sobral BS, Azad AF. Louse- and flea-borne rickettsioses: biological and genomic analyses. Vet Res. 2009;40:12. http://dx.doi.org/10.1051/vetres:2008050
2. Acuna-Soto R, Calderón-Romero L, Romero-López D, Bravo-Lindoro A. Murine typhus in Mexico City. Trans R Soc Trop Med Hyg. 2000;94:45. http://dx.doi.org/10.1016/S0035-9203(00)90432-2
3. Zavala-Castro JE, Zavala-Velázquez J, Sulú-Uicab J. Murine typhus in child, Yucatan, Mexico. Emerg Infect Dis. 2009;15:972–4. http://dx.doi.org/10.3201/eid1506.081367
4. Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol. 1991;173:1576–89.
5. Williams SG, Sacci JB Jr, Schriefer ME, Anderson EM, Fujioka KK, Sorvillo FJ, et al. Typhus and typhus-like rickettsiae associated with opossums and their fleas in Los Angeles County, California. J Clin Microbiol. 1992;30:1758–62.
6. Webb L, Carl M, Mallow DC, Dasch GA, Azad AF. Detection of murine typhus in fleas by using the polymerase chain reaction. J Clin Microbiol. 1990;28:530–4.
7. Dzul-Rosado K, González-Martínez P, Peniche-Lara G, Zavala-Velázquez J, Zavala-Castro J. Murine typhus in humans, Yucatan, Mexico. Emerg Infect Dis. 2013;19:1021–2. http://dx.doi.org/10.3201/eid1906.121400
8. Kordová N. Plaque assay of rickettsiae. Acta Virol. 1966;10:278.

Address for correspondence: Jorge E. Zavala-Castro, Centro de Investigaciones Regionales Dr. Hideyo Noguchi, Universidad Autónoma de Yucatán, Av Itzaés no. 490 x Calle 59 Colonia Centro, Mérida, Yucatán 97000, México; email: zcastro@uady.mx

Zika Virus Infection after Travel to Tahiti, December 2013

To the Editor: Zika virus (ZIKV), a member of the family *Flaviviridae*, is a mosquito-borne virus that is endemic to Africa and Southeast Asia. ZIKV causes illness that is similar to dengue fever, characterized by joint pain, myalgia, headache, and rash (*1*). ZIKV has caused several recent outbreaks, including one in Micronesia in 2007 (*2*) and one in French Polynesia (≈30,000 cases) ongoing since October 2013 (*3*) and spreading to New Caledonia and Easter Island (*4*). We report the clinical and laboratory findings for a patient with ZIKV infection imported from Tahiti, French Polynesia.

The previously healthy 31-year-old woman from Norway was admitted to the Oslo University Hospital, Norway, on December 13, 2013. Six days earlier, she had returned from a 14-day vacation to Tahiti, where she mainly stayed in the capital, Pape'ete, and took a short trip to the island of Mo'orea. One day after her return to Norway, she experienced fever, intense joint pain, and myalgia. Subsequently, a maculopapular rash developed. At the time of admission, her temperature was 37.7°C, and she had enlarged nuchal lymph nodes; injected conjunctivae; and a maculopapular rash on her trunk, extremities, and face (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/14-0302-Techapp1.pdf). Clinical examination findings were otherwise unremarkable. Laboratory tests showed leukopenia of 2.7×10^9 cells/L (reference range 3.5–10 $\times$ 10^9/L), with mild lymphopenia of 1.0×10^9 cells/L (reference range 1.5.–4.0 $\times$ 10^9/L) and neutropenia of 1.4×10^9 cells/L (reference range 1.5–7.3 $\times$ 10^9/L). No thrombocytopenia or elevated liver enzyme levels were detected. C-reactive protein levels (1.4 mg/L) were within reference range.

Because of the patient's clinical picture and travel history, an acute ZIKV infection was suspected and several diagnostic tests were ordered. In a serum sample taken 5 days after symptom onset, no IgM or IgG against ZIKV, dengue virus (DENV), Japanese encephalitis virus, yellow fever virus, or chikungunya virus was detected by in-house indirect immunofluorescence (*5*,*6*). Only a weak IgG titer of 1:20 (and no IgM) against tickborne encephalitis virus was found (cutoff <1:20). Test results for DENV nonstructural protein 1 antigen (Platelia; Bio-Rad, Hercules, CA, USA) and generic flavivirus reverse transcription PCR (RT-PCR) (*6*) were negative. Thus, for increased sensitivity, quantitative ZIKV-specific real-time RT-PCR (*6*) with the AgPath-ID One-Step RT-PCR Kit (Life Technologies, Carlsbad, CA, USA) was performed according to the manufacturer's instructions, and results were positive. ZIKV RNA load was 1.6×10^5 copies/mL; in vitro–transcribed RNA from a

reference plasmid was used as a quantification standard.

Attempts to isolate ZIKV in cell culture failed. Therefore, the serum sample was used to obtain the partial ZIKV genome sequence with primers designed from multiple alignments of partial ZIKV genomes retrieved from databases. Primer sequences used for partial genome amplification of ZIKV are available on request (to J. S.-C.). The partial ZIKV genome (strain Tahiti, GenBank accession no. KJ461621) was successfully amplified from the serum sample, and phylogenetic analysis of an ≈200-bp long genomic fragment of the nonstructural protein 3 gene demonstrated that strain Tahiti clusters within the Asian ZIKV lineages and is closely related to a strain from Malaysia (Figure).

In a follow-up serum sample collected 36 days after symptom onset, IgG and IgM seroconversion against ZIKV was demonstrated; IgM titer was 1:1,280 and IgG titer was 1:2,560 (cutoff <1:20). In the same sample, low IgG titers against tick-borne encephalitis virus and DENV (1:40 and 1:80, respectively) were noted (cutoffs <1:20). Real-time RT-PCR for ZIKV in this serum sample was negative.

Travel-related imported ZIKV infections have been reported after travel from Thailand to Germany (*6*) and Canada (*7*), from Indonesia to Australia (*8*), and from Senegal to the United States (*9*). Linked to the current outbreak in French Polynesia, infections in 2 travelers who had returned from Bora Bora to Japan have recently been described (*10*). The clinical findings for the patient reported here (fever, rash, arthralgia, myalgia) were similar to those previously reported for patients with imported cases (*6*,*10*). Available laboratory data are meager, but mild thrombocytopenia has been reported for some patients with Zika fever (*10*), but not for others (*6*,*8*). Outbreaks of dengue fever also occur in French Polynesia (*10*), making dengue fever clinically and epidemiologically the most important differential diagnosis. Elevated liver enzymes, which are found in patients with acute dengue fever, are found in some, but not all, patients with Zika fever (*6*,*8*).

The measured viral load for the patient reported here (5 days after symptom onset) would not be high enough for efficient transmission of ZIKV to susceptible vectors such as *Aedes aegypti* or *Ae. albopictus* mosquitoes (S. Becker, pers. comm.). This finding is consistent with previously reported findings of ZIKV RNA loads of 930–728,800 copies/mL (*2*). However, *Ae. aegypti* and *Ae. albopictus* mosquitoes are not present in Norway; thus, transmission in Norway seems unlikely. ZIKV infection should be considered as a differential diagnosis for febrile dengue fever–like syndromes in travelers who have returned from Southeast Asia and the Pacific region.

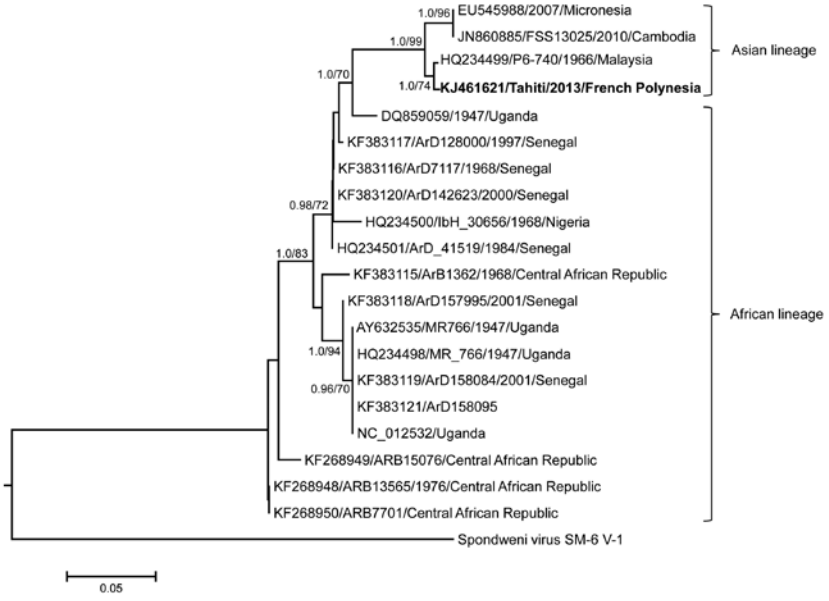


Figure. Phylogenetic analysis of partial (≈200 bp) nonstructural protein 3 gene sequences of Zika virus strains performed by using maximum-likelihood and Bayesian methods. A substitution model was based on a general time-reversible model with gamma-distributed rate variation and a proportion of invariant sites. Numbers at the nodes represent posterior probability values (clade credibilities ≥90%) and percentage bootstrap support values (≥70%) based on 1,000 replicates. GenBank accession numbers, strain name, year of isolation, and country of origin for sequences used to construct the tree are indicated on the branches. The tree was rooted with Spondweni virus (GenBank accession no. DQ859064). Strain Tahiti (from patient who had traveled to Tahiti, this study) is indicated in boldface. The scale bar represents genetic distance in nucleotide substitutions per site. The lineage of each virus is indicated to the right of the tree.

Acknowledgments

We thank Claudia Poggensee, Mathis Petersen, Alexandra Bialonski, Insa Bonow, Corinna Thomé, and Birgit Hüsing for technical assistance.

This letter is dedicated to the late Ursula Herrmann (1927–2014), who made this study possible.

Torgun Wæhre, Anne Maagard, Dennis Tappe,[1] Daniel Cadar,[1] and Jonas Schmidt-Chanasit[1]

Author affiliations: Oslo University Hospital, Oslo, Norway (T. Wæhre, A. Maagard); WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Hamburg, Germany (D. Tappe, D. Cadar, J. Schmidt-Chanasit); and German Centre for Infection Research, Hamburg (J. Schmidt-Chanasit)

DOI: http://dx.doi.org/10.3201/eid2008.140302

[1]These authors contributed equally to this article.

References

1. Hayes EB. Zika virus outside Africa. Emerg Infect Dis. 2009;15:1347–50. http://dx.doi.org/10.3201/eid1509.090442
2. Lanciotti RS, Kosoy OL, Laven JJ, Velez JO, Lambert AJ, Johnson AJ, et al. Genetic and serologic properties of Zika virus associated with an epidemic, Yap State, Micronesia, 2007. Emerg Infect Dis. 2008;14:1232–9. http://dx.doi.org/10.3201/eid1408.080287
3. European Centre for Disease Prevention and Control. Zika virus—Pacific (08). ProMed. 2014 Mar 16. http://www.promedmail.org, archive no. 20140316.2335754.
4. Schwan K. Zika virus—Pacific (07). ProMed. 2014 Mar 9. http://www.promedmail.org, archive no. 20140309.2322907.
5. Tappe D, Schmidt-Chanasit J, Ries A, Ziegler U, Müller A, Stich A. Ross River virus infection in a traveller returning from northern Australia. Med Microbiol Immunol (Berl). 2009;198:271–3. http://dx.doi.org/10.1007/s00430-009-0122-9
6. Tappe D, Rissland J, Gabriel M, Emmerich P, Gunther S, Held G, et al. First case of laboratory-confirmed Zika virus infection imported to Europe, November 2013. Euro Surveill. 2014;19:20685.
7. Fonseca K. Zika virus—Canada ex Thailand. ProMed. 2013 May 29. http://www.promedmail.org, archive no. 20130529.1744108.
8. Kwong JC, Druce JD, Leder K. Zika virus infection acquired during brief travel to Indonesia. Am J Trop Med Hyg. 2013;89:516–7. http://dx.doi.org/10.4269/ajtmh.13-0029
9. Foy BD, Kobylinski KC, Chilson Foy JL, Blitvich BJ, Travassos da Rosa A, Haddow AD, et al. Probable non-vector-borne transmission of Zika virus, Colorado, USA. Emerg Infect Dis. 2011;17:880–2. http://dx.doi.org/10.3201/eid1705.101939
10. Kutsuna S, Kato Y, Takasaki T, Moi ML, Kotaki A, Uemura H. Two cases of Zika virus imported from French Polynesia to Japan, December 2013 to January 2014. Euro Surveill. 2014;19:pi=20683.

Address for correspondence: Jonas Schmidt-Chanasit, Bernhard Nocht Institute for Tropical Medicine, WHO Collaborating Centre for Arbovirus and Haemorrhagic Fever Reference and Research, Bernhard-Nocht-Strasse 74, 20359 Hamburg, Germany; email: jonassi@gmx.de

Yersinia pestis in *Pulex irritans* Fleas during Plague Outbreak, Madagascar

To the Editor: *Yersinia pestis* (family *Enterobacteriaceae*) is a bacterium that can cause high rates of death in susceptible mammals and can provoke septicemic, pneumonic, and bubonic plague in humans (*1*). This zoonotic pathogen can be transmitted directly by infectious droplets or by contact with contaminated fluid or tissue or indirectly through flea bites (*1*).

Plague was introduced into Madagascar in 1898 from rat-infested steamships that had sailed from affected areas (*2*). Now, Madagascar is 1 of 2 countries in Africa that have reported cases of human plague every year since 1991 (*3*). During January 2008–January 2013, the number of human plague cases reported in Madagascar ranged from 312 to 648 per year. Of these, 61.8%–75.5% were laboratory confirmed (National Plague Laboratory of the Ministry of Health, pers. comm.). Most (>83%) confirmed cases were bubonic plague, which most commonly results from flea bites, suggesting that these bites were the most common mode of *Y. pestis* transmission. In Madagascar, *Xenopsylla cheopis* fleas have been known as the primary plague vector in urban areas, whereas *Synopsyllus fonquerniei* fleas have been usually involved in plague transmission in rural areas (*2*).

In January 2013, a total of 9 suspected bubonic plague cases, 3 confirmed, were reported in Soavina, a rural area in the district of Ambatofinandrana, Madagascar. Domestic fleas were collected with candle traps inside 5 houses during 3 nights (Table). Fleas were also caught on small mammals trapped inside houses and outside in the sisal fences and rice fields (Table). A total of 319 fleas belonging to 5 species in 5 genera were collected inside and outside the houses, an average of 44 per house (maximum 71): *Pulex irritans, Echidnophaga gallinacea*, and *Ctenocephalides canis* fleas were collected inside the houses (244, 76.5%), and *S. fonquerniei* and *X. cheopis* fleas were collected outside (75, 23.5%). The human flea, *P. irritans*, was the most collected flea species (233, 73.3%), followed by *S. fonquerniei* (62, 19.4%), *X. cheopis* (13, 4.1%), *E. gallinacea* (10, 3.1%), and *C. canis* (1, 0.3%).

Bacterial DNA was extracted from 277 fleas of 5 species: 233 *P. irritans*, 24 *S. fonquerniei,* 9 *X. cheopis*, 10 *E. gallinacea*, and 1 *C. canis*. PCR to detect *Y. pestis* was performed by using primers YP1 (5′-ATC TTA CTT TCC GTG AGA AG-3′) and YP2 (5′-CTT GGA TGT TGA GCT TCC TA-3′) to amplify a 478-bp fragment (*4*). *Y. pestis* DNA was then amplified and genotyped by Beckman Coulter Genomics Inc. (Takeley, United Kingdom). The positive control was *Y. pestis* reference strain (strain 6/69, 3 × 10^8 bacteria/mL; Institut Pasteur de Madagascar).

Detection of *Y. pestis* was carried out on 274 fleas belonging to 5 flea species: 230 *P. irritans* (181 unfed and 49 engorged), 24 *S. fonquerniei* (15 unfed and 9 engorged), 9 *X. cheopis* (8 unfed and 1 engorged), 10 *E. gallinacea* (blood-feeding status not identified), and 1 unfed *C. canis*. *Y. pestis*

Table. Fleas collected inside and outside houses in Soavina, Madagascar, January 2013

Species	Total no. (%)	No. (%) inside	No. (%) outside
Pulex irritans	233 (73.0)	233 (95.5)	0
Ctenocephalides canis	1 (0.3)	1 (0.4)	0
Echidnophaga gallinacea	10 (3.1)	10 (4.1)	0
Synopsyllus fonquerniei	62 (19.4)	0	62 (82.7)
Xenopsylla cheopis	13 (4.1)	0	13 (17.3)
Total	319 (100)	244 (100)	75 (100)

was detected in 9 *P. irritans* fleas (7 male [6 unfed and 1 engorged] and 2 [engorged] female) from 3 houses, including the house where a confirmed human case of plague had occurred (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/13-0629-Techapp1.pdf). Eight sequences (GenBank accession nos. KJ361938–KJ361945) were obtained and share 99% nucleotide homology with plasminogen activator genes of *Y. pestis* published in GenBank (accession nos. AF528537, AY305870). No *Y. pestis* was detected in the 24 *S. fonquerniei,* 9 *X. cheopis,* 10 *E. gallinacea*, or 1 *C. canis* fleas collected.

Although only *X. cheopis* and *S. fonqueniei* fleas had previously been described as plague vectors in Madagascar, *P. irritans* fleas were most commonly collected during this field study; engorged and unfed male and female *P. irritans* fleas carried *Y. pestis*. Other studies have found *P. irritans* fleas in the plague risk area in other countries in Africa (*5,6*); one study found that *P. irritans* fleas may play a role in plague epidemiology in Tanzania (*5*). Data on *P. irritans* fleas in rats make it unlikely that these fleas are involved in rat-to-human transmission of *Y. pestis* in Madagascar. During 1922–1995, a total of 118,608 rats were caught and examined in Madagascar, but only 148 *P. irritans* fleas were identified, and none have been found on rats since 1996 (http://www.pasteur.mg/spip.php?rubrique124). The high density of *P. irritans* fleas we observed in villages where plague outbreaks occurred in late 2012 and early 2013 (http://www.pasteur.mg/spip.php?rubrique124) supports the possibility that *P. irritans* fleas played a role in domestic human-to-human transmission of *Y. pestis* during these outbreaks.

Jocelyn Ratovonjato, Minoarisoa Rajerison, Soanandrasana Rahelinirina, and Sébastien Boyer

Author affiliations: Institut Pasteur de Madagascar, Antananarivo, Madagascar (J. Ratovonjato, S. Boyer); and World Health Organization Collaborating Centre, Institut Pasteur de Madagascar, Antananarivo (M. Rajerison, S. Rahelinirina)

DOI: http://dx.doi.org/10.3201/eid2008.130629

References

1. Bulter T. Plague and other *Yersinia* infections. In: Greenough WB III, Merigan TC, editors. Current topics in infectious disease. New York: Plenum: 1983. p. 71–92.
2. Brygoo ER. Epidemiology of the plague at Madagascar [in French]. Arch Inst Pasteur Madagascar. 1966;35:7–147.
3. World Health Organization. Report on global surveillance of epidemic-prone infectious diseases—plague [cited 2013 Apr 15]. http://www.who.int/csr/resources/publications/plague/CSR_ISR_2000_1/en/index5.html
4. Hinnebusch J, Schwan TG. New method for plague surveillance using polymerase chain reaction to detect *Yersinia pestis* in fleas. J Clin Microbiol. 1993;31:1511–4.
5. Laudisoit A, Leirs H, Makundi RH, Van Dongen S, Davis S, Neerinckx S, et al. Plague and the human flea, Tanzania. Emerg Infect Dis. 2007;13:687–93. http://dx.doi.org/10.3201/eid1305.061084
6. Eisen RJ, Gage KL. Transmission of flea-borne zoonotic agents. Annu Rev Entomol. 2012;57:61–82. http://dx.doi.org/10.1146/annurev-ento-120710-100717

Address for correspondence: Jocelyn Ratovonjato, Medical Entomology Unit, Institut Pasteur de Madagascar, BP 1274, Antananarivo 101, Madagascar; email: ratov@pasteur.mg

Serologic Surveillance for West Nile Virus in Dogs, Africa

To the Editor: West Nile fever is caused by the West Nile virus (WNV), a mosquito-borne member of the genus *Flavivirus*. Birds are the natural reservoir of the virus, which is maintained in nature in a mosquito–bird–mosquito transmission cycle. WNV has been detected in many regions worldwide, including North America, Europe, Africa, the Near East, and Asia (*1*). WNV has been shown to cause meningoencephalitis in humans and horses. In the United States, seroconversion in dogs was detected 6 weeks before a human case was reported (*2*). Thus, dogs could be considered as sentinels for WNV infection, but their role as reservoir is unlikely because of short-term and low levels of viremia (*3*). In this study, we determined the seroprevalence of WNV in dogs living close to humans in different environments to assess their role as sentinels of this potentially severe zoonosis.

During 2003–2012, blood samples were collected from 753 adult dogs from France and 6 countries in Africa (Table). Samples were centrifuged within 24 h after collection, separated, frozen at –20°C, and sent to the virology laboratory of the Institut de Recherche Biomédicale des Armées (Marseille, France). Each sample was systematically tested for IgG against WNV by using an in-house ELISA with inactivated WNV as antigen. Serum samples were considered positive if the optical density at 450 nm was >3-fold the mean of that for negative antigen. Because of the antigenic cross-reactivity among flaviviruses, all positive samples were further tested by Western blot for WNV-specific antibodies (*4*); seroprevalence was calculated on the basis of Western blot–confirmed cases only.

Table. Prevalence of West Nile virus antibodies in dog populations, France and Africa, 2003–2012

Country and area	No. dogs, N = 753	No. positive for IgG by ELISA	No. results confirmed by Western blot	Prevalence, % (95% CI)
France				
Corsica	35*	3	0	0 (0–10)
Var	25*	3	3	12.0 (2.5–31.2)
Gard	11*	1	1	9.1 (0.2–41.3)
Imported from				
Germany/the Netherlands	9*	0	0	0 (0–33.6)
Hungary	24*	6	3	12.5 (2.7–32.4)
Djibouti	47*	8	6	12.8 (4.8–25.7)
N'Djamena, Chad	50*	13	12	24.0 (13.1–38.2)
	5	5	5	100.0 (47.8–100.0)
Senegal				
Dakar	11*	0	0	0 (0–28.5)
	16†	3	3	18.7 (4.1–45.6)
Siné-Saloum	33	6	1	3.0 (0.1–15.8)
Casamance	81	3	3	3.7 (0.8–10.4)
Abidjan, Côte d'Ivoire	137	7	3	2.2 (0.5–6.3)
Kinshasa, Democratic Republic of the Congo	24	4	3	12.5 (2.7–32.4)
Haut-Ogooué, Gabon	245	0	0	0 (0–1.5)

*French military working dogs.
†Senegalese gendarmerie working dogs.

For the statistical analysis, we used the exact binomial method to calculate 95% CIs of the proportions and the Fisher exact test to calculate p values and compare the seroprevalence rates between countries; significance was set at $p<0.05$.

Seropositive dogs were found in all portions of Africa and France surveyed except northeastern Gabon and Corsica (Table). Seroprevalence of WNV in native dogs was significantly higher in Chad than in the Democratic Republic of the Congo (DRC) ($p<0.001$), Senegal ($p<0.00001$), Côte d'Ivoire ($p<0.000001$), and Gabon ($p<0.000001$). Seroprevalence was low in Kinshasa, DRC (12.5%), and Dakar, Senegal (11.1%), but in N'Djamena, Chad, all 5 native dogs tested had specific antibodies against WNV.

As part of the study, we tested 50 military dogs from France twice, before and after a 4-month mission in Chad; 12 (24.0%) became seropositive after the stay. In addition, 12.5% of military working dogs in France imported from Hungary were seropositive on initial testing. We also found that, in France, dogs are the sentinels of WNV circulation in the Var (12.0%) and Gard (9.1%) departments. All dogs we tested that were positive for IgG were negative for IgM, a finding that indicates infection by the virus did not occur recently.

The results and the statistical analysis reveal notable differences in the seroprevalence rates, according to the geographic area. N'Djamena, Chad, where all native dogs tested positive for WNV, is located at the confluence of the Chari and Logone Rivers and is an area with high densities of residential and migratory birds. In contrast, the northeastern region (Haut-Ogooué) of Gabon, where no native dogs tested positive for WNV, is an ecosystem of wet forests without migratory birds, unfavorable to virus circulation. In Dakar, 18.7% of native dogs were seropositive. In these central parts of Senegal, characterized by a semi-arid climate and vegetation composed of steppe plants and bluegrass, several WNV strains have been isolated from birds and mosquitoes. The seroprevalence was lower (0%–12.5%) in the sub-Saharan area, including Côte d'Ivoire, Gabon, DRC, and Senegal (Siné-Saloum and Casamance), where the humid or semihumid climate is linked with tropical rain forests or woodland savannah known to favor sedentary birds (*5*).

In a large proportion of the human and animal population of Africa, immunity to WNV has developed (*1*). A serologic survey of dogs from the Highveld region of South Africa showed that 37% (138/377) had neutralizing antibodies against WNV (*6*). Similarly, seroprevalence of antibodies against WNV is high among dogs in the United States, for example, 55.9% (218/390) in the Gulf Coast region (*7*). In Turkey, an area where many birds stop over during migration, seroprevalence among dogs was high (37.7%, 43/114) (*8*).

Our study highlights the role of dogs as sentinels for WNV circulation, particularly in southeastern France (Gard and Var departments), where WNV epidemics and epizootics occurred in 2000 and 2003. In addition, we observed that military working dogs purchased from Hungary, where WNV infection is common (*9*), may be seropositive. Seroconversion in dogs returning from short missions in WNV-endemic countries such as Chad was also observed. Therefore, our data emphasize the usefulness and convenience of WNV seroprevalence surveys in dogs for studying WNV epidemiology and circulation. It is possible that dogs living close to humans could attract infected mosquitoes, thereby reducing human infection.

Acknowledgments

We thank Olivier Bourry, José Gomez-Peñate, and Hubert Bassene for their help during the conduct of field work, Jean-Paul Durand, and all the persons who contributed to the study, especially the French military veterinarians. We thank also the team of the virology laboratory of the Institut de recherche biomédicale des armées (William Daries, Patrick Gravier, and Olivier Merle) for processing the samples.

Financial support was provided in part by the French Defense Medical Service.

Bernard Davoust, Isabelle Leparc-Goffart, Jean-Paul Demoncheaux, Raphaël Tine, Mamadou Diarra, Grégory Trombini, Oleg Mediannikov, and Jean-Lou Marié

Author affiliations: Groupe de Travail en Épidémiologie Animale du Service de Santé des Armées, Toulon, France (B. Davoust, J.-P. Demoncheaux, G. Trombini, J.L. Marié); Unité de Recherche sur les Maladies Infectieuses et Tropicales Émergentes (IRD 198), Dakar, Senegal (B. Davoust, O. Mediannikov); Centre National de Référence des Arbovirus–Institut de Recherche Biomédicale des Armées, Marseille, France (I. Leparc-Goffart); and Services Vétérinaires de la Gendarmerie Nationale, Dakar (R. Tine, M. Diarra)

DOI: http://dx.doi.org/10.3201/eid2008.130691

References

1. Dauphin G, Zientara S, Zeller H, Murgue B. West Nile: worldwide current situation in animals and humans. Comp Immunol Microbiol Infect Dis. 2004;27:343–55. http://dx.doi.org/10.1016/j.cimid.2004.03.009
2. Resnick MP, Grunenwald P, Blackmar D, Hailey C, Bueno R, Murray KO. Juvenile dogs as potential sentinels for West Nile virus surveillance. Zoonoses Public Health. 2008;55:443–7.
3. Kile JC, Panella NA, Komar N, Chow CC, MacNeil A, Robbins B, et al. Serologic survey of cats and dogs during an epidemic of West Nile virus infection in humans. J Am Vet Med Assoc. 2005;226:1349–53. http://dx.doi.org/10.2460/javma.2005.226.1349
4. Cabre O, Grandadam M, Marié JL, Gravier P, Prangé A, Santinelli Y, et al. West Nile virus in horses, sub-Saharan Africa. Emerg Infect Dis. 2006;12:1958–60. http://dx.doi.org/10.3201/eid1212.060042
5. Traore-Lamizana M, Zeller HG, Mondo M. Isolations of West Nile and Bagaza viruses from mosquitoes (Diptera: *Culicidae*) in Center Senegal (Ferlo). J Med Entomol. 1994;31:934–8.
6. Blackburn NK, Reyers F, Berry WL, Shepherd AJ. Susceptibility of dogs to West Nile virus: a survey and pathogenicity trial. J Comp Pathol. 1989;100:59–66. http://dx.doi.org/10.1016/0021-9975(89)90090-X
7. Levy JK, Lappin MR, Glaser AL, Birkenheuer AJ, Anderson TC, Edinboro CH. Prevalence of infectious diseases in cats and dogs rescued following Hurricane Katrina. J Am Vet Med Assoc. 2011;238:311–7. http://dx.doi.org/10.2460/javma.238.3.311
8. Ozkul A, Yildirim Y, Pinar D, Akcali A, Yilmaz V, Colak D. Serological evidence of West Nile Virus (WNV) in mammalian species in Turkey. Epidemiol Infect. 2006;134:826–9. http://dx.doi.org/10.1017/S0950268805005492
9. Paz S, Semenza JC. Environmental drivers of West Nile epidemiology in Europe and Western Asia—a review. Int J Environ Res Public Health. 2013;10:3543–62. http://dx.doi.org/10.3390/ijerph10083543

Address for correspondence: Bernard Davoust, Unité de Recherche en Maladies Infectieuses et Tropicales Emergentes (URMITE) CNRS UMR 7278 IRD 198 INSERM U1095 Aix-Marseille Université, Faculté de Médecine, 27 bd Jean Moulin, 13385 Marseille CEDEX 5, France; email: bernard.davoust@gmail.com

Another Dimension

EID publishes 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.

Severe Encephalitis Caused by Toscana Virus, Greece

To the Editor: In late June 2012, a previously healthy, 49-year-old woman was admitted to the emergency department of Trikala General Hospital in Trikala, Greece, with confusion and delirium. A few hours before admission, she had had a grand mal seizure; she had experienced gastroenteritis with fever (38°C) 5 days earlier. On admission, she was intubated and transferred to the intensive care unit, where she underwent mechanical ventilation and sedation.

The patient was a resident of Genesi village (350 m altitude), 22 km west of Trikala in the Thessaly region. She had not traveled abroad or to other area of Greece. Results of blood and cerebrospinal fluid (CSF) laboratory testing were unremarkable except slight leukocytosis (leukocytes 11,330 cells/mm^3, 92% neutrophils) and slightly elevated serum lactate dehydrogenase level (240 U/L). Brain imaging showed edema (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/20/8/14-0248-Techapp1.pdf), which resolved 48 hours after admission. The patient was awakened on day 3 of hospitalization and extubated on day 4. Treatment included anticonvulsants, mannitol, antimicrobial drugs (vancomycin and ceftriaxone), acyclovir, and corticosteroids. The patient fully recovered and was discharged from the hospital on day 12 with short-term antiepileptic medication.

Because West Nile virus (WNV) infections emerged in 2010 in Greece and outbreaks have recurred (*1*), serum and CSF samples from the patient were sent for testing to the National Reference Centre for Arboviruses. Antibodies against WNV were not detected. Reverse transcription nested PCR was conducted by using generic primers for flaviviruses,

enteroviruses, and phleboviruses. PCR for phleboviruses (*2*) resulted in a PCR product of the expected size, and the sequence was most closely related to those of isolates belonging to the *Sandfly fever Naples virus* (SFNV) species (Figure). The sequence also had the highest homology (85%) with a Toscana virus (TOSV) strain belonging to lineage C that had been obtained from a patient with central nervous system infection in Croatia in 2008 (*3*). The TOSV sequence derived from the patient in this report was submitted to GenBank (accession no. KJ418710). On the basis of a partial sequence comparison (202 nt in the polymerase gene), we found that TOSV lineage C differs from lineages A and B by 29% and 30%, respectively.

Forty-one days after symptom onset, a second serum sample was taken from the patient and tested in parallel with the first serum sample by indirect immunofluorescence to detect IgM and IgG antibodies against 4 phleboviruses: TOSV, SFNV, sandfly fever Sicilian virus (SFSV), and Cyprus virus (Sandfly Fever Virus Mosaic 1; Euroimmun, Lübeck, Germany). The first sample yielded negative results, but the follow-up sample showed IgM and IgG against TOSV and SFNV (both belonging to SFNV serocomplex). Neutralization testing to differentiate TOSV and SFNV was not performed because PCR and sequencing confirmed the TOSV infection.

Sandfly-borne phleboviruses (family *Bunyaviridae*) are endemic in Mediterranean countries, and at least 3 serotypes are associated with disease in humans: TOSV, SFNV, and SFSV. Among these, TOSV is associated with neurotropism, a major cause of meningitis and encephalitis in the Mediterranean region (*4*). Recent studies in Greece showed that the seroprevalence of TOSV (and antigenically related viruses) ranges from 0% to 60%; the higher levels are found in the islands and the coastal regions (*5*–*7*).

A study conducted in 2 Greek islands (Lefkas and Corfu, where Corfu virus was isolated) showed that the sandfly species with the widest distribution was *Phlebotomus neglectus* (31.2%), followed by *P. similis* (25.1%) and *P. tobbi* (15.3%) (*8*). In Thessaly region, where the case we report occurred, a faunistic study of sandflies showed that *P. perfiliewi* and *P. papatasi* (known vectors of TOSV) accounted for 83.4% and 3.93%, respectively, of the sandflies collected (*9*). Another phlebovirus, Adria virus (belonging to the Salehabad serocomplex), which was initially detected in sandflies collected in Albania, was detected in a febrile child with seizure in Thessaloniki in northern Greece (*10*). Concerning TOSV, however, although seroconversion has been previously observed in patients in Greece, RNA has not been detected.

For this patient, TOSV was detected by using phlebovirus generic primers. The TOSV sequence found in Greece differs greatly from other TOSV sequences, even from the genetically closer Croatian TOSV sequence (15%). To avoid false-negative results, the high genetic diversity among TOSV strains must be taken into consideration when using TOSV-specific primers.

In conclusion, a novel variant of TOSV has been detected in Greece. Further studies are needed to obtain a whole-genome sequence of the Greek TOSV strain and to identify the vector(s) of the virus. TOSV is a highly variable neurotropic phlebovirus, a characteristic that must be taken into account by laboratory scientists. Clinicians should be aware of the possibility of phlebovirus infections in Mediterranean countries and should include these viruses in the differential diagnosis of febrile illnesses observed during the warm seasons, especially in patients who exhibit neurologic symptoms.

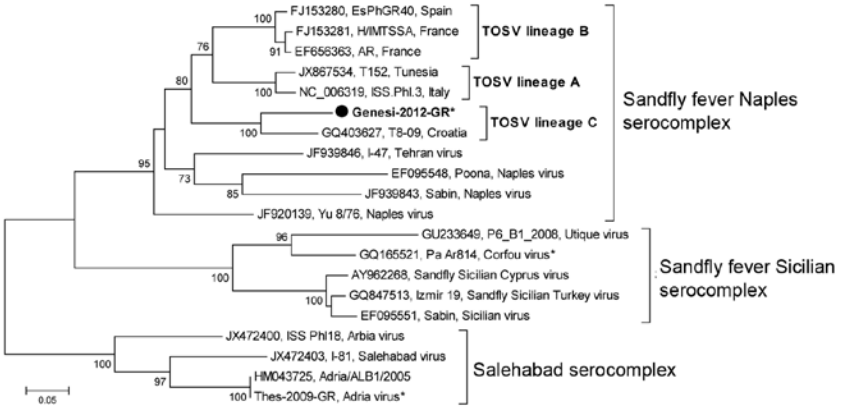


Figure. Neighboring-joining tree constructed on the basis of a 202-bp fragment of the large RNA segment of sandfly-borne phleboviruses. Black circle indicates Toscana virus strain detected in this study in a patient in Greece; asterisks (*) indicate phleboviruses detected in Greece. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches. Evolutionary analyses were conducted in MEGA5 (http://www.megasoftware.net). Scale bar indicates substitutions per nucleotide position.

The National Reference Centre for Arboviruses and Hemorrhagic Fever viruses in Thessaloniki, Greece, is financially supported by the Hellenic Center for Disease Control and Prevention.

Anna Papa, Theoniki Paraforou, Ioannis Papakonstantinou, Kiriaki Pagdatoglou, Anastasia Kontana, and Triantafilia Koukoubani

Author affiliations: National Reference Centre for Arboviruses and Hemorrhagic Fever Viruses, Aristotle University of Thessaloniki, Thessaloniki, Greece (A. Papa, A. Kontana); and Trikala General Hospital, Trikala, Greece (T. Paraforou, I. Papakonstantinou, K. Pagdatoglou, T. Koukoubani)

DOI: http://dx.doi.org/10.3201/eid2008.140248

References

1. Papa A. West Nile virus infections in Greece: an update. Expert Rev Anti Infect Ther. 2012;10:743–50. http://dx.doi.org/10.1586/eri.12.59
2. Sánchez-Seco MP, Echevarria JM, Hernandez L, Estevez D, Navarro-Mari JM, Tenorio A. Detection and identification of Toscana and other phleboviruses by RT-nested-PCR assays with degenerated primers. J Med Virol. 2003;71:140–9. http://dx.doi.org/10.1002/jmv.10465
3. Punda-Polić V, Mohar B, Duh D, Bradaric N, Korva M, Fajs L, et al. Evidence of an autochthonous Toscana virus strain in Croatia. J Clin Virol. 2012;55:4–7. http://dx.doi.org/10.1016/j.jcv.2012.06.006
4. Charrel RN, Gallian P, Navarro-Mari JM, Nicoletti L, Papa A, Sanchez-Seco MP, et al. Emergence of Toscana virus in Europe. Emerg Infect Dis. 2005;11:1657–63. http://dx.doi.org/10.3201/eid1111.050869
5. Anagnostou V, Papa A. Prevalence of antibodies to phleboviruses within the sand fly fever Naples virus species in humans, Northern Greece. Clin Microbiol Infect. 2013;19:566–70.
6. Anagnostou V, Papa A. Seroprevalence of Toscana virus among residents of Aegean Sea islands, Greece. Travel Med Infect Dis. 2013;11:98–102. http://dx.doi.org/10.1016/j.tmaid.2012.11.006
7. Papa A, Andriotis V, Tzilianos M. Prevalence of Toscana virus antibodies in residents of two Ionian islands, Greece. Travel Med Infect Dis. 2010;8:302–4. http://dx.doi.org/10.1016/j.tmaid.2010.09.002
8. Xanthopoulou K, Anagnostou V, Ivovic V, Djurkovic-Djakovic O, Rogozi E, Sotiraki S, et al. Distribution of sandflies (Diptera, Psychodidae) in two Ionian Islands and northern Greece. Vector Borne Zoonotic Dis. 2011;11:1591–4. http://dx.doi.org/10.1089/vbz.2011.0750
9. Ivović V, Patakakis M, Tselentis Y, Chaniotis B. Faunistic study of sandflies in Greece. Med Vet Entomol. 2007;21:121–4. http://dx.doi.org/10.1111/j.1365-2915.2006.00649.x
10. Anagnostou V, Pardalos G, Athanasiou-Metaxa M, Papa A. Novel phlebovirus in febrile child, Greece. Emerg Infect Dis. 2011;17:940–1. http://dx.doi.org/10.3201/eid1705.101958

Address for correspondence: Anna Papa, Department of Microbiology, Medical School, Aristotle University of Thessaloniki, 54124, Thessaloniki, Greece; email: email: annap@med.auth.gr

Phylogenetic Analysis of West Nile Virus Genome, Iran

To the Editor: West Nile virus (WNV) is a single-stranded, positive-sense RNA virus (≈11 kb) that is taxonomically classified within the family *Flaviviridae*, genus *Flavivirus*. WNV is found in Africa, Eurasia, Australia, and North America (*1*).

Comprehensive studies on phylogenetic relatedness of WNV strains have showed that WNV can be grouped into 5 lineages. Lineage 1 contains WNV strains from different regions, including northern, western, and central Africa; southern and eastern Europe; India; and the Middle East. Lineage 1 is subdivided into 3 clades. Clade 1A contains strains from Europe, northern Africa, the United States, and Israel, clade 1B contains Kunjin virus from Australia. Lineage 2 contains isolates from west, central, and eastern Africa and Madagascar. There is evidence that lineage 2 circulates in some regions of Europe (e.g., Italy, Austria, and Greece) (*2*,*3*). Lineage 3 contains Rabensburg virus 97–103, which was isolated in 1997 from *Culex pipiens* mosquitoes in South Moravia in the Czech Republic. Lineage 4 contains a new variant of WNV (strain LEIVKrnd88–190), which was isolated in 1998 from *Dermacentor marginatus* ticks in a valley in the northwestern Caucasus Mountains of Russia. Lineage 5 contains an WNV isolate from India (strain 804994) (*4*,*5*). In this study, we compared the phylogenetic relationships of WNV circulating in Iran to other WNV strains by using a partial WNV sequence isolated from an Iranian patient.

WNV was obtained from a blood sample from an Iranian patient who had encephalitis and was hospitalized in 2009 in Isfahan in the central highlands of Iran. The patient reported no history of animal contact, insect bites, blood transfusions, transplantations, and travel. He exhibited fever, headache, hypertension, and vomiting. On initial examination, he had a body temperature of 40°C. Laboratory investigations on the day of admission showed a leukocyte count of 240 cells/mL, a protein level of 52 mg/dL, and a glucose level of 50 mg/dL in a cerebrospinal fluid sample.

Further examinations were undertaken, and samples were sent to the Arboviruses and Viral Hemorrhagic Fevers Laboratory at Pasteur Institute of Iran in Teheran. For an IgG ELISA, wells in test plates were coated overnight with mouse hyperimmune ascitic fluid. Native antigen was added, and wells were incubated and washed. Test samples and peroxidase-labeled anti-human or anti-animal immunoglobulin were added. After incubation for 10 min, optical densities were read (*6*).

Viral RNA was extracted by using the QIAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany) from serum of the patient. A reverse transcription PCR was conducted by using a One-Step RT-PCR Kit (QIAGEN). Samples were subjected to 1 cycle at 50°C for 30 min to synthesize cDNA; 95°C for 15 min; and 95°C for 30 s, 54°C for 30 s, and 72°C for 60 s; and a final extension at 72°C for 5 min (*6*). The serum sample was positive for IgG against WNV. Molecular tests showed positive results for WNV.

The PCR product was sequenced by using the Big Dye Terminator V3.1 Cycle Sequencing Kit (Applied

Biosystems, Foster City, CA, USA), the modified Sanger sequencing method, and an ABI Genetic Analyzer 3130 (Applied Biosystems) (*7*). Multiple alignments of nucleotide sequences were made by using ClustalW (http://www.clustal.org/). A phylogenetic tree was constructed by using 27 representative sequences of WNV and Japanese encephalitis virus (available in GenBank) and a 358-nt sequence of WNV (GenBank accession no. KJ486150), from the patient, which corresponds to nt 259–616 in the late region of the capsid gene and the early region of the membrane gene. Phylogenetic status of the sequence for the WNV strain from the patient was assessed by using the neighbor-joining algorithm in MEGA5 (*8*). Reliability of phylogenetic groupings was evaluated by using the bootstrap test (1,000 replications). Japanese encephalitis virus SA14 sequence was used as an outgroup in phylogenetic analysis of partial genome sequences (*8*).

The phylogenetic tree identified clustering of isolates in 5 lineages. Lineage 1 had 2 sublineage (clade 1A and clade 1B). All sequences in lineage 1 were geographically distinguishable. Clade 1A contained strains from Europe, Africa, the Middle East, and the United States; clade 1B included the Australian strain NSW 2011 (JN887352). The WNV sequence from Iran (KJ486150) was grouped into lineage 2 and had 99% identity with the 358-bp region of WNV strain ArB3573/82 from the Central African Republic. Although it was believed that lineage 2 strains circulate only in Africa, reports of their emergence primarily in Balkan countries (*9*) and in our study support the presence of a lineage WNV 2 strain in the Middle East, particularly in western Asia (Figure).

Although the patient did not report any mosquito bites, this infection route cannot be excluded because he was a farmer and spent most of his time outdoors. Previous studies have demonstrated that nearly all human WNV infections were a consequence of mosquito bites (*10*). Our study should increase awareness of WNV infections as a public health threat. In future studies, priority should be given to investigations of the ecology, occurrence, and epidemiology of the different WNV strains circulating in Iran.

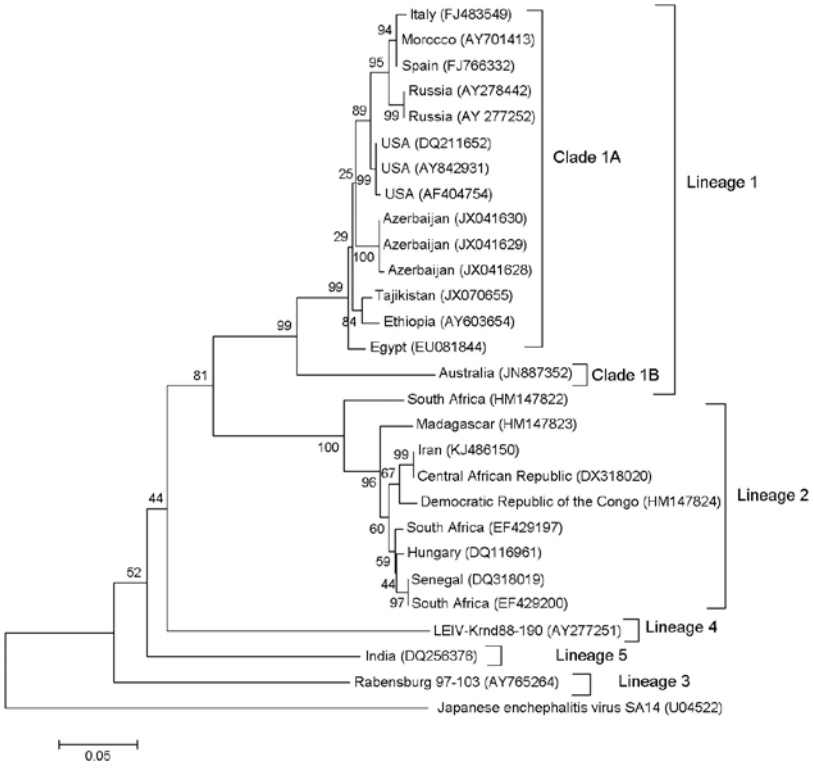


Figure. Phylogenetic tree based on a 358-nt sequence (nt 259–616) of 27 strains of West Nile virus (WNV) generated by using the neighbor-joining algorithm in MEGA5 (*8*). Japanese encephalitis virus was used as an outgroup. Location of virus isolation and GenBank accession numbers (in parentheses) are provided. The WNV sequence from Iran (KJ486150) was grouped into lineage 2 and had 99% identity with the 358-bp region of WNV strain ArB3573/82 from the Central African Republic. Scale bar indicates nucleotide substitutions per site.

Acknowledgments

We thank the staff of the Arboviruses and Viral Hemorrhagic Fevers Laboratory, Pasteur Institute of Iran, for assistance during this study.

Nariman Shah-Hosseini, Sadegh Chinikar, Behroz Ataei, Anthony R. Fooks, and Martin H. Groschup

Author affiliations: Pasteur Institute of Iran, Tehran, Iran (N. Shah-Hosseini, S. Chinikar); Isfahan University of Medical Sciences, Isfahan, Iran (B. Ataei); Veterinary Laboratories Agency, Addlestone, UK (A.R. Fooks); and Friedrich-Loeffler-Institut, Greifswald-Insel Riems, Germany (M.H. Groschup)

DOI: http://dx.doi.org/10.3201/eid2008.131321

References

1. Bakonyi T, Hubalek Z, Rudolf I, Nowotny N. Novel flavivirus or new lineage of West Nile virus, central Europe. Emerg Infect Dis. 2005;11:225–31. http://dx.doi.org/10.3201/eid1102.041028
2. Papa A, Bakonyi T, Xanthopoulou K, Vázquez A, Tenorio A, Nowotny N. Genetic characterization of West Nile virus lineage 2, Greece, 2010. Emerg Infect Dis. 2011;17:920–2. http://dx.doi.org/10.3201/eid1705.101759

3. Papa A, Xanthopoulou K, Gewehr S, Mourelatos S. Detection of West Nile virus lineage 2 in mosquitoes during a human outbreak in Greece. Clin Microbiol Infect. 2011;17:1176–80. http://dx.doi.org/10.1111/j.1469-0691.2010.03438.x
4. Bondre VP, Jadi R, Mishra A, Yergolkar P, Arankalle V. West Nile virus isolates from India: evidence for a distinct genetic lineage. J Gen Virol. 2007;88:875–84. http://dx.doi.org/10.1099/vir.0.82403-0
5. Botha EM, Markotter W, Wolfaardt M, Paweska JT, Swanepoel R, Palacios G, et al. Genetic determinants of virulence in pathogenic lineage 2 West Nile virus strains. Emerg Infect Dis. 2008;14:222–30. http://dx.doi.org/10.3201/eid1402.070457
6. Chinikar S, Javadi A, Ataei B, Shakeri H, Moradi M, Mostafavi E, et al. Detection of West Nile virus genome and specific antibodies in Iranian encephalitis patients. Epidemiol Infect. 2012;140:1525–9.
7. Chinikar S, Shah-Hosseini N, Bouzari S, Jalali T, Shokrgozar MA, Mostafavi E. New circulating genomic variant of Crimean-Congo hemorrhagic fever virus in Iran. Arch Virol. 2013; 158:1085–8.
8. Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics. 2001;17:1244–5. http://dx.doi.org/10.1093/bioinformatics/17.12.1244
9. Bakonyi T, Ivanics E, Erdelyi K, Ursu K, Ferenczi E, Weissenbock H, et al. Lineage 1 and 2 strains of encephalitic West Nile virus, central Europe. Emerg Infect Dis. 2006;12:618–23. http://dx.doi.org/10.3201/eid1204.051379
10. Chinikar S, Shah-Hosseini N, Mostafavi E, Moradi M, Khakifirouz S, Jalali T, et al. Seroprevalence of West Nile virus in Iran. Vector Borne Zoonotic Dis. 2013;13:586–9. http://dx.doi.org/10.1089/vbz.2012.1207

Address for correspondence: Sadegh Chinikar, Laboratory of Arboviruses and Viral Haemorrhagic Fevers, Pasteur Institute of Iran, 60 Pasteur Ave, Teheran 1316943551, Iran; email: sadeghchinikar@yahoo.com

Human Infection with West Nile Virus, Xinjiang, China, 2011

To the Editor: West Nile virus (WNV) is a mosquito-borne flavivirus in the Japanese encephalitis serocomplex of the family *Flaviviridae* (*1*). It has been reported in Africa, Asia, Europe, Australia, and North America, and is recognized as the most globally widespread mosquito-borne flavivirus (*2*). Isolation of WNV has previously been attempted in China, Japan and South Korea; however, no virus has been isolated (*3*–*5*). We report isolation of WNVs from mosquitoes in Xinjiang Uyghur Autonomous Region in western China. We also provide evidence of WNV human infections confirmed by IgM ELISA and seroconversion by 90% plaque reduction neutralization tests of paired serum samples obtained from persons with febrile illness and viral encephalitis in 2011.

Arbovirus surveillance was performed in the Kashi Region, Xinjiang, China, in August 2011. Mosquitoes were captured by using light traps and gravid traps for 15 days (days 1–5, 11–15, and 21–25) at 9 collection sites in 9 villages in 2 townships. A total of 7,122 mosquitoes, representing 3 genera and 7 species in 118 pools, were tested. Mosquitoes collected were *Culex pipiens pipiens* (65.0%, 4,629/7,122), *Aedes flavidorsalis* (24.1%, 1,717/7,122), *Ae. caspius* (10.1%, 716/7,122), and other *Aedes* and *Culex* species (0.8%, 40/7,122) mosquitoes.

Mosquitoes were homogenized, and viral RNA was extracted directly from mosquito pools and amplified by using PCR and primers specific for WNV envelope (E) and nonstructural protein 5 genes as described (*6*). A total of 12 pools of *Cx. p. pipiens* mosquitoes were positive for WNV, which was confirmed by nucleotide sequencing. The minimum infection rate for *Cx. p. pipiens* mosquitoes was 2.56 infections/1,000 specimens tested.

In addition, supernatants of the 12 WNV-positive mosquito pools were inoculated onto Vero cells. Five pools yielded 5 virus isolates designated XJ11129–3, XJ11138–6, XJ11141–4, XJ11146–4, and XJ11148–2. The Vero cells aggregated and began shedding virus by 72 h postinfection.

Phylogenetic comparisons of complete nucleotide sequences of E gene from the 5 Xinjiang isolates (Figure, panel A) showed a high degree of genetic identity of lineage 1 with other highly pathogenic WNV strains, such as WNV NY99 and isolates from Russia. Nucleotide and amino acid sequences showed ≥99% identity with isolates from Russia (1999–2004) (*7*).

The complete nucleotide sequence of XJ11129–3 contained 11,029 nt, and the phylogenetic tree of the nucleotide coding region showed similar topology with the E gene tree (Figure, panel B). Nucleotide sequences of E genes from XJ11138–6, XJ11141–4, XJ11146–4, and XJ11148–2 and the complete genome sequence of XJ11129–3 were submitted to GenBank under accession nos. JX442280, JX442281, JX442282, JX442278, and JX442279, respectively.

To determine whether humans were infected with WNV, we obtained acute-phase serum samples within 1–7 days of onset of illness from persons visiting an outpatient clinic in Kashi during June 11–August 25, 2011. All patients had fever (37°C–39°C) or viral encephalitis with or without symptoms of encephalitis. Serum samples were obtained from 254 patients with fever of unknown origin and 9 patients with encephalitis.

All acute-phase serum samples were initially screened for IgM against WNV (WNV IgM Capture DxSelect; Focus Diagnostics Inc., Cypress, CA, USA) and against Japanese encephalitis virus (JEV) (JEV IgM Capture ELISA Kit; Panbio, Sinnamon Park, Queensland, Australia). A total of 38

patients (2 with viral encephalitis and 36 with fever of unknown etiology) had IgM against WNV. All samples were negative for WNV and JEV RNA.

A total of 23/38 patients positive for IgM against WNV provided convalescent-phase serum samples (obtained 18–83 days after acute-phase serum samples were obtained). All 23 paired serum samples were tested by using a 90% plaque reduction neutralization test and the XJ11029–3 strain of WNV and the P3 strain of JEV. Of these 23 serum samples, 11 had a 4-fold increase in titer of WNV-neutralizing antibody; neutralizing antibody against JEV was not detected.

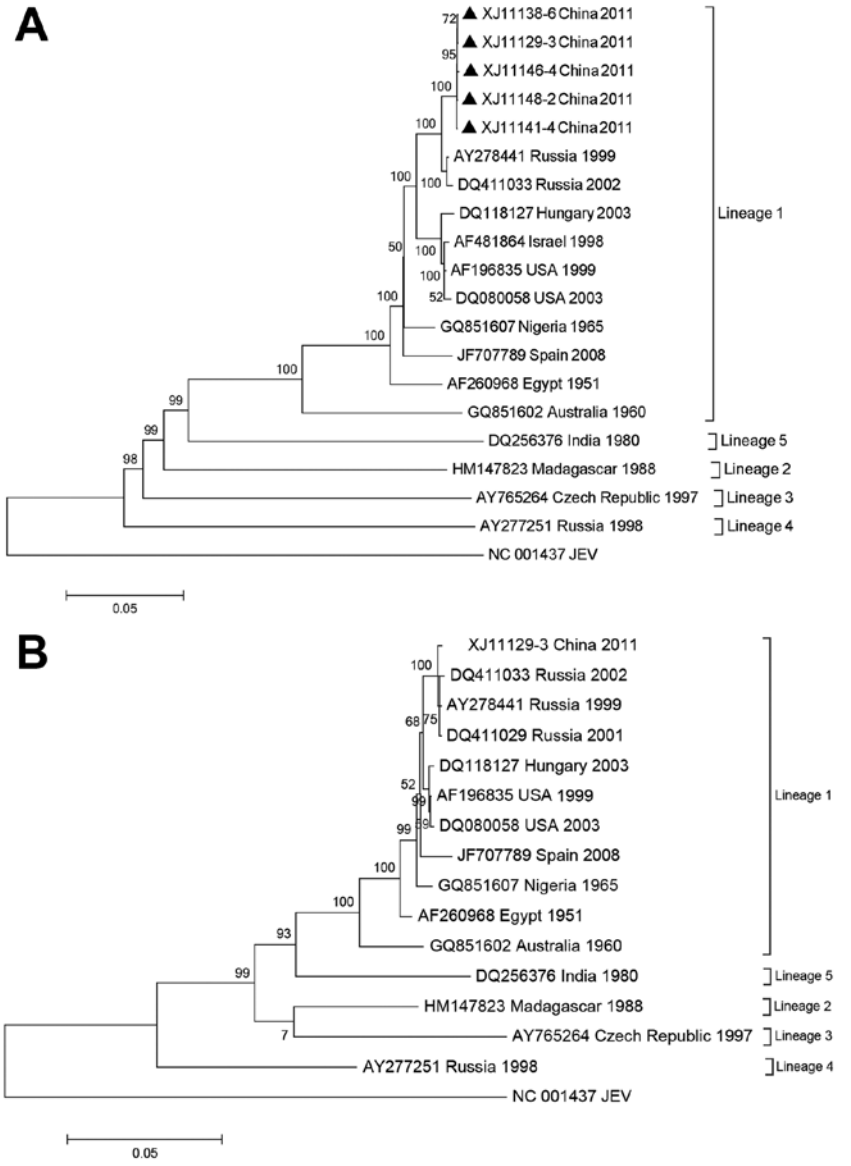


Figure. Phylogenetic analyses of A) envelope gene nucleotide sequence from 5 West Nile virus isolates (black triangles) from Xianjiang, Uyghur Autonomous Region, China, 2011, and B) nucleotide sequence of complete coding region of 1 isolate from Xinjiang (XJ11129–3). Trees were constructed by using MEGA 5.05 (http://www.megasoftware.net/) and maximum-likelihood with Kimura 2-parameter model parameter distances. Bootstrap values along branches are for 1,000 replicates. Trees were rooted by using Japanese encephalitis virus (JEV) as the outgroup virus. USA, United States. Scale bars indicate nucleotide substitutions per site.

Among the 11 patients who showed seroconversion, 9 had neutralization antibodies against WNV (titers 1:10 for acute-phase samples and 1:40 for convalescent-phase samples). One patient with encephalitis had a WNV antibody titer of 1:10 for an acute-phase sample and 1:160 for a convalescent-phase sample. Another patient with encephalitis had a WNV antibody titer of 1:640 for an acute-phase sample and of 1:5,120 for a convalescent-phase sample. All 11 case-patients were reported during July 28–August 23, 2011.

This study and other reports of fever and human encephalitis caused by WNV in Xinjiang, China, in 2004 (*8*,*9*) suggest that infections with WNV might be greatly underestimated. In addition, although JEV is present in this region and WNV has not been isolated in China, some patients might have been given misdiagnoses of infection with JEV because of cross-reactivity between these 2 viruses (*10*). Therefore, nationwide surveillance programs for WNV in China are needed.

Acknowledgments

We thank Roger S. Nasci for providing suggestions for the study and comments regarding the manuscript, and Wei-Hong Yang and Yu-Zhen Zhang for collecting mosquitoes.

This study was supported by grants from the National Science Foundation of China (81290342,81171635), the Ministry of Science and Technology of China (no. 2011CB504702), and the State Key Laboratory for Infectious Disease Prevention and Control (Development grants 2014SKLID103 and 2014SKLID205), and the Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases.

Zhi Lu, Shi-Hong Fu, Lei Cao, Cheng-Jun Tang, Song Zhang, Zhao-Xia Li, Mamutijiang Tusong,

Xin-Hua Yao, Hai-Lin Zhang, Pi-Yu Wang, Maimaitijiang Wumaier, Xue-Yan Yuan, Ming-Hua Li, Chang-Zhong Zhu, Li-Ping Fu, and Guo-Dong Liang

Author affiliations: State Key Laboratory for Infectious Disease Prevention and Control, Beijing, China (Z. Lu, S.-H. Fu, C.-J. Tang, M.-H. Li, G.-D. Liang); National Institute for Viral Disease Control and Prevention, Beijing (Z. Lu, S.-H. Fu, C.-J. Tang, M.-H. Li, G.-D. Liang); Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou, China (Z. Lu, S.-H. Fu, C.-J. Tang, M.-H. Li, G.-D. Liang); Chinese Academy of Sciences, Beijing (L. Cao); Xinjiang Center for Disease Control and Prevention, Urumqi, China (S. Zhang, M. Wumaier, L.-P. Fu); Kashi Center for Disease Control and Prevention, Kashi, China (Z.-X. Li, C.-Z. Zhu); Jiashi County Center for Disease Control and Prevention, Jiashi, China (M. Tusong, X-H Yao); Yunnan Institute of Endemic Disease Control and Prevention, Dali City, China (H.-L. Zhang, P.-Y. Wang); and Jiashi County Hospital, Jiashi (X.-Y. Yuan)

DOI: http://dx.doi.org/10.3201/eid2008.131433

References

1. Heinz FX, Collett MS, Purcell RH, Gould EA, Howard CR, Houghton M. Family *Flaviviridae*. In: van Regenmorteel MH, Fauquet CM, Bishop DH, Carstens EB, Estes MK, Lemon SM, et al., editors. Virus taxonomy: classification and nomenclature of viruses. San Diego: Academic Press; 2000. p. 859–78.
2. Hayes EB, Komar N, Nasci RS, Montgomery SP, O'Leary DR, Campbell GL. Epidemiology and transmission dynamics of West Nile virus disease. Emerg Infect Dis. 2005;11:1167–73. http://dx.doi.org/10.3201/eid1108.050289a
3. Gao X, Nasci R, Liang G. The neglected arboviral infections in mainland China. PLoS Negl Trop Dis. 2010;4:e624. http://dx.doi.org/10.1371/journal.pntd.0000624
4. Yeh JY, Kim HJ, Nah JJ, Lee H, Kim YJ, Moon JS. Surveillance for West Nile virus in dead wild birds, South Korea, 2005–2008. Emerg Infect Dis. 2011;17:299–301. http://dx.doi.org/10.3201/eid1702.100551
5. Hoshino K, Takahashi-Nakaguchi A, Isawa H, Sasaki T, Higa Y, Kasai S. Entomological surveillance for flaviviruses at migratory bird stopover sites in Hokkaido, Japan, and a new insect flavivirus detected in *Aedes galloisi* (*Diptera: Culicidae*). J Med Entomol. 2012;49:175–82. http://dx.doi.org/10.1603/ME11123
6. Lanciotti RS, Kerst AJ, Nasci RS, Godsey MS, Mitchell CJ, Savage HM, et al. Rapid detection of West Nile virus from human clinical specimens, field-collected mosquitoes, and avian samples by a TaqMan reverse transcriptase-PCR assay. J Clin Microbiol. 2000;38:4066–71.
7. Fyodorova MV, Savage HM, Lopatina JV, Bulgakova TA, Ivanitsky AV, Platonova OV, et al. Evaluation of potential West Nile virus vectors in Volgograd region, Russia, 2003 (*Diptera: Culicidae*): species composition, bloodmeal host utilization, and virus infection rates of mosquitoes. J Med Entomol. 2006;43:552–63. http://dx.doi.org/10.1603/0022-2585(2006)43[552:EOPWNV]2.0.CO;2
8. Li Z, Zhu C, Yu S, Ka D, Tian L. Epidemiologic analysis of an unexplained outbreak of acute viral meningoencephalitis in Jiashi County, Xinjiang [in Chinese]. Chinese Journal of Public Health. 2007;23:S72–4.
9. Li XL, Fu SH, Liu WB, Wang HY, Lu Z, Tong SX, et al. West Nile virus infection in Xinjiang, China. Vector Borne Zoonotic Dis. 2013;13:131–3. http://dx.doi.org/10.1089/vbz.2012.0995
10. Zheng Y, Li M, Wang H, Liang G. Japanese encephalitis and Japanese encephalitis virus in mainland China. Rev Med Virol. 2012;22:301–22. http://dx.doi.org/10.1002/rmv.1710

Address for correspondence: Guo-Dong Liang, National Institute for Viral Disease Control and Prevention, 155 Changbai Rd, Changping District, Beijing 102206, China; email: gdliang@hotmail.com

Chikungunya in the Caribbean as Threat to Europe

To the Editor: The first evidence of chikungunya virus in the Western Hemisphere was its detection in December 2013 in the French West Indies (*1*). One month later, the virus spread to other Caribbean islands.

Two cases of chikungunya in siblings (an 8-year old girl and a 10-year old boy) were recently identified at Toulouse University Hospital in southwestern France. Two days after these children had returned to France from the island of Martinique (French West Indies), acute fever associated with an arthromyalgic syndrome developed in these children. The children had maculopapular, nonpruriginous rashes on their arms and legs and endobuccal petechiae. The boy had bilateral knee effusions, and the girl had a measles-like rash that became more extended. Both children also had many mosquito bites that they scratched. They were discharged on the day of their admission. These 2 cases reported in metropolitan France after the patients visited Martinique indicate rapid spread of chikungunya virus.

We identified the virus by sequencing a 205-nt fragment within the envelope protein E1 gene of chikungunya virus (*2*) and performing phylogenetic analyses on the basis of reference sequences. This virus was a strain from Asia (Figure), whereas virus detected in 2 children in southeastern France in September 2010 had been imported from Rajasthan, India, and was an East/Central/South Africa strain (*3*). All of these strains did not show the single amino acid substitution in the envelope protein gene (E1-A226V) that favors adaptation for dissemination by *Aedes albopictus* mosquitoes (*4*) and would affect the potential magnitude of this outbreak.

Ae. aegypti mosquitoes are common in the Western Hemisphere,

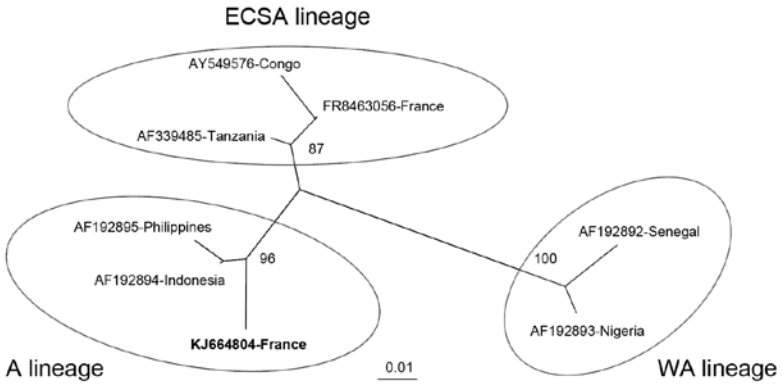


Figure. Phylogenetic tree constructed by using the neighbor-joining method and based on a partial (205 nt) sequence of the envelope protein 1 gene of chikungunya virus that was imported to metropolitan France from Martinique. Phylogenetic analysis includes reference sequences of chikungunya viruses from East/Central/South African (ECSA), West African (WA), and Asian (A) lineages. Sequences are indicated as GenBank accession number and country. The imported chikungunya strain isolated in this study is indicated in boldface. Bootstrap support values (100 replicates) are indicated at major nodes. Scale bar indicates nucleotide substitutions per site.

where they are the major vector of urban dengue and yellow fever, and will facilitate spread of chikungunya in this region. *Ae. albopictus* (Asian tiger mosquito) is also an efficient vector of chikungunya virus and is found in many areas, including southern Europe. This mosquito species was responsible for the extensive chikungunya outbreak on La Réunion Island in the Indian Ocean (*5*) and was involved in the first chikungunya outbreak in Italy in 2007 (*6*). In these 2 outbreaks, human and mosquito virus strains contained mutation A226V in the envelope protein gene.

Ae. albopictus mosquitoes became established in a large area (91,150 km^2) of southern France in 2013, where ≈13 million persons live. This mosquito, which is highly efficient in transmitting chikungunya virus (*7*), has been present in the study area for 2 years. For these reasons, a chikungunya/dengue national control program for continental France was established in 2006. The program involves rapid virologic diagnosis of imported or suspected autochthonous cases and vector control measures. This program operates during May–November, the period when *Ae. albopictus* mosquitoes circulate, and is based on entomologic surveillance data. The area covered by the program in 2013 was >10 times larger than that covered in 2006.

The presence of an effective vector, its progressive spread, and the outbreak of chikungunya in the Western Hemisphere increase concerns of a chikungunya outbreak in Europe (*8*). The greatest challenge is to find a way of interrupting the transmission chain of the virus as soon as possible. This challenge requires an effective policy of informing travelers at risk, early screening based on rapid virologic diagnosis, and effective vector control. Such control measures need an educated population to ensure emptying standing water from flowerpots, gutters, buckets, pool covers, pet water dishes, and discarded tires. They also need global antivector measures (eradication of eggs, larvae, and adults of *Aedes s*pp. mosquitoes).

These measures must be extremely efficient because an outbreak of chikungunya in the Western Hemisphere could spread rapidly. All countries in southern Europe are concerned by this public health challenge, and the battle against chikungunya requires rapid establishment of a supranational organization that should be able in real time to collect and return epidemiologic, virologic, and entomologic data. Although the usual movements of tourists around southern Europe during the summer will increase the number of persons at risk in this area, an even greater threat is the international movement of >600,000 persons expected to attend the next Soccer World Cup in Brazil in 2014 (*9*).

Jean-Michel Mansuy, Erick Grouteau, Catherine Mengelle, Isabelle Claudet, and Jacques Izopet

Author affiliations: Toulouse University Hospital, Toulouse, France (J.-M. Mansuy, E. Grouteau, C. Mengelle, I. Claudet, J. Izopet); Institut National de la Santé et de la Recherche Médicale, Toulouse (J. Izopet); and Université Toulouse III Paul-Sabatier, Toulouse, (J. Izopet)

DOI: http://dx.doi.org/10.3201/eid2008.140650

References

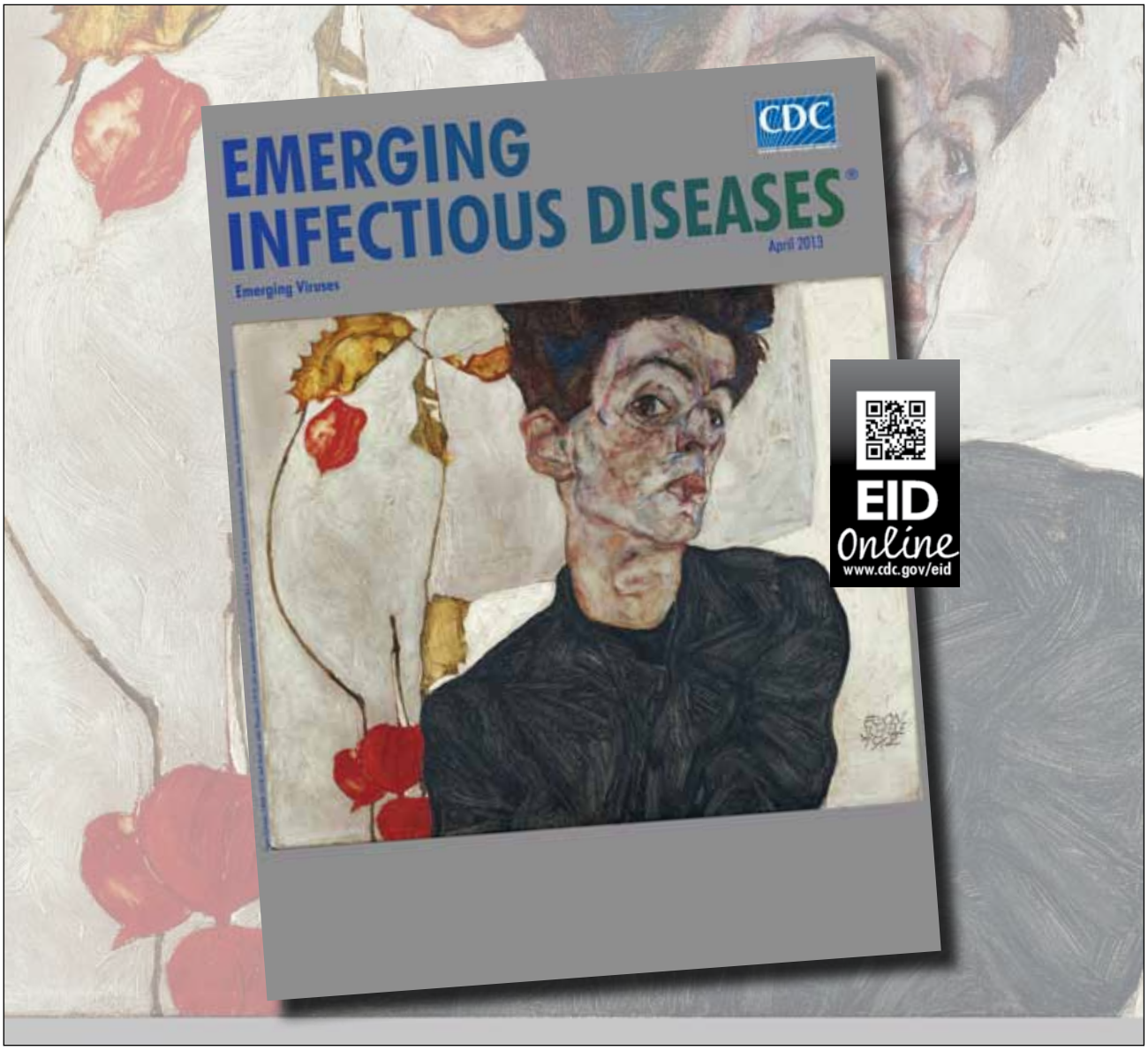
1. Leparc-Goffart I, Nougairede A, Cassadou S, Prat C, de Lamballerie X. Chikungunya in the Americas. Lancet. 2014;383:514. http://dx.doi.org/10.1016/S0140-6736(14)60185-9
2. Pastorino B, Bessaud M, Grandadam M, Murri S, Tolou HJ, Peyrefitte CN. Development of a TaqMan RT-PCR assay without RNA extraction step for the detection and quantification of African chikungunya viruses. J Virol Methods. 2005;124:65–71. http://dx.doi.org/10.1016/j.jviromet.2004.11.002
3. Grandadam M, Caro V, Plumet S, Thiberge JM, Souares Y, Failloux AB, et al. Chikungunya virus, southeastern France. Emerg Infect Dis. 2011;17:910–3. http://dx.doi.org/10.3201/eid1705.101873
4. Tsetsarkin KA, Vanlandingham DL, McGee CE, Higgs S. A single mutation in chikungunya virus affects vector specificity and epidemic potential. PLoS Pathog. 2007;3:e201. http://dx.doi.org/10.1371/journal.ppat.0030201
5. Vazeille M, Moutailler S, Coudrier D, Rousseaux C, Khun H, Huerre M, et al. Two chikungunya isolates from the outbreak

of La Reunion (Indian Ocean) exhibit different patterns of infection in the mosquito, *Aedes albopictus*. PLoS ONE. 2007;2:e1168. http://dx.doi.org/10.1371/journal.pone.0001168
6. Rezza G, Nicoletti L, Angelini R, Romi R, Finarelli AC, Panning M, et al. Infection with chikungunya virus in Italy: an outbreak in a temperate region. Lancet. 2007;370:1840–6. http://dx.doi.org/10.1016/S0140-6736(07)61779-6
7. Vega-Rua A, Zouache K, Caro V, Diancourt L, Delaunay P, Grandadam M, et al. High efficiency of temperate *Aedes albopictus* to transmit chikungunya and dengue viruses in the Southeast of France. PLoS ONE. 2013;8:e59716. http://dx.doi.org/10.1371/journal.pone.0059716
8. Fischer D, Thomas SM, Suk JE, Sudre B, Hess A, Tjaden NB, et al. Climate change effects on chikungunya transmission in Europe: geospatial analysis of vector's climatic suitability and virus' temperature requirements. Int J Health Geogr. 2013;12:51. http://dx.doi.org/10.1186/1476-072X-12-51
9. Brazilian Government Website. World Cup Portal [cited 2014 May 6]. http://www.copa2014govbr/en

Address for correspondence: Jean-Michel Mansuy, Virology Laboratory, University Hospital Purpan, 330 Ave de Grande Bretagne, TSA 40031, Toulouse 31059, France; email: mansuy.jm@chu-toulouse.fr

Correction: Vol. 20, No. 6

In the first paragraph of the article Novel Phlebovirus with Zoonotic Potential Isolated from Ticks, Australia (J. Wang et al.), *Anaplasma phagocytophilum* should be replaced by *Haemaphysalis longicornis*. The article has been corrected online (http://wwwnc.cdc.gov/eid/article/20/6/14-0003_intro.htm).



In Memoriam: Robert Emmons Kissling (1923–2013)

Charles H. Calisher, Frederick A. Murphy, and Thomas P. Monath

Robert Kissling, a pillar of US Center for Disease Control (CDC) infectious diseases programs through an era of great programmatic and institutional growth (1947–1973), died November 13, 2013 at his home in North Carolina. Through his years at CDC, Bob led investigations of great diversity and impact, always with the same sense of openness, integrity, composure, and respect for everyone involved.

The fondest of memories return; they remind the authors, his colleagues, and friends that it was with this attitude that the viral disease programs of CDC were established and have been maintained through the years. Bob, as an eminent virologist and a leader of CDC's viral disease programs, left a permanent legacy.

Born in Toledo, Ohio, Bob attended Otterbein College, was awarded a DVM and an MSc from the College of Veterinary Medicine, Ohio State University, and then spent a year at the graduate school of the University of California at Davis. In 1947, the year after CDC was founded, he joined the US Public Health Service and began a long and distinguished career with CDC, then known as the Communicable Disease Center.

The 1950s was a period of great progress in field and laboratory research in virology and at CDC's Virus and Rickettsia Section in Montgomery, Alabama. Bob began collaborative studies of the natural history, epidemiology, and pathogenesis of vectorborne viral encephalitides and began a career-long interest in rabies research; CDC developed an outstanding reputation in these areas, not the least because of Bob's contributions. While in Montgomery, Bob married Martha Eidson, his laboratory technician, and they had 3 children.

In 1958, Bob and guest researcher Robert Goldwasser succeeded in adapting the then-new immunofluorescence methodology to detect rabies viral antigen in brain specimens of animals suspected of being rabid (*1*). Field trials provided proof of its 100% sensitivity, specificity,

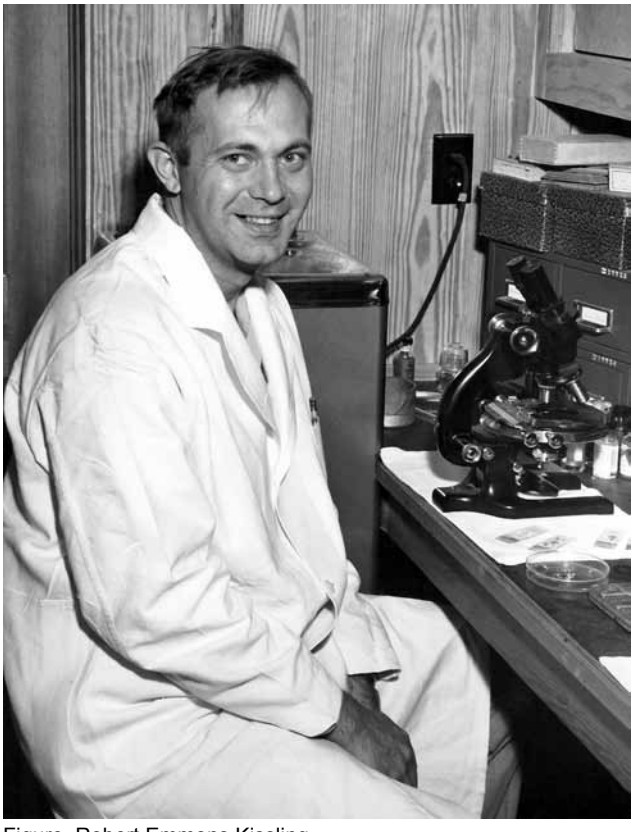


Figure. Robert Emmons Kissling

Author affiliations: Colorado State University, Fort Collins, Colorado, USA (C.H. Calisher); University of Texas Medical Branch, Galveston, Texas, USA (F.A. Murphy); Hookipa BioTech AG, Vienna, Austria (T.P. Monath); and PaxVax Inc., Menlo Park, California, USA (T.P. Monath)

DOI: http://dx.doi.org/10.3201/eid2008.IM2008

and reliability. The assay became the first "rapid" viral diagnostic test, with results reported quickly enough to guide postexposure treatment. Remarkably, this is now the oldest rapid viral diagnostic test in use, and it remains the national and World Health Organization international standard assay. Bob's interest in rabies continued after the Virus and Rickettsia Section was moved into CDC's new facility in Atlanta, Georgia, in 1960. He was the first person to amplify rabies virus in modern cell culture systems; one of his cell culture–adapted virus strains was later used to produce the most popular human rabies vaccine in the United States.

In the new laboratory facilities in Atlanta, CDC's viral and rickettsial disease programs flourished, as did Bob's role as scientist and leader. As was the way in the 1960s and 1970s, virologists such as Bob were quite broad in their outlook and expertise. He and his colleagues worked on rabies, borreliosis, Chagas disease, psittacosis, and more, with a major emphasis on the natural history of arbovirus diseases. With his colleagues, Bob did the original field and laboratory research on eastern equine encephalitis virus, unraveling its complex transmission cycle in birds and mosquitoes.

As the years went by, Bob took on ever-increasing administrative responsibilities at CDC, serving as chief of various CDC organizational units: Veterinary Research, Viropathology, and Hepatitis Activities. From 1968 until 1973, he was chief of the Virus and Rickettsial Disease Section, Bureau of Laboratories. This period saw the emergence of several important new viral diseases (or of diseases at first thought to have a viral etiology) and the emergence of increasingly sophisticated approaches to virus research, virus detection and identification, and the means to guide CDC's mission of disease prevention and control. Two new diseases are exemplary of the importance of his work, Marburg hemorrhagic fever and Lassa fever.

In 1967, cases of hemorrhagic fever occurred among laboratory workers in Germany and Yugoslavia who were processing kidneys for cell culture; the kidneys were from African green monkeys that had been imported from Uganda. A new virus, Marburg virus, was isolated from patients and monkeys, first in Germany but shortly thereafter at CDC. From the beginning, it was realized CDC must have the capacity to deal with this virus and that Marburg virus was dangerous, calling for the best biocontainment of the day. CDC borrowed a mobile containment laboratory from the National Institutes of Health; the containment level provided was about what would be called Biosafety Level 2+ today. The work was led by Bob (*2*); the main safety feature was that only 2 other people would be at risk, Roslyn Robinson and Fred Murphy, each experienced in safe practices for working with dangerous pathogens. The virus was obtained and used to develop diagnostic reagents and for morphologic, pathologic, and ultrastructural pathologic studies. A serologic test was developed and used to try to discern the source of the virus in Uganda. Bob uniquely foresaw the role that CDC had to play in this new field of exotic, dangerous pathogens, a field that would continue to grow in following years.

As understood clearly by Bob, the Marburg virus episode had to be the impetus for the development of permanent biocontainment facilities at CDC. A glove port cabinet line laboratory was constructed, which opened in 1968. Completion of the laboratory coincided with the emergence of Lassa fever in West Africa. Early work on Lassa virus elsewhere was terminated after 1 person died and another nearly died; all virus stocks were sent to CDC. Under Bob's leadership, Tom Monath and others set to work to develop diagnostic systems and uncover the natural history and reservoir host of Lassa virus (*3*). Work expanded from an emphasis on diagnostics to the pathogenesis of several viral hemorrhagic fevers. All this activity put a great strain on the glove port laboratory, and soon Bob led long-gestating planning for a large higher containment laboratory. CDC's present leadership role, globally, in dealing with dangerous pathogens stems from Bob's initial vision.

Retiring to a farm north of Atlanta in 1973, Bob and Martha were at last able to bird-watch, farm a bit, and enjoy the wildflowers. Martha died in 1999 and Bob, whose health was declining, moved to North Carolina to enjoy his children, grandchildren, and great-grandchildren. A gentleman of common sense, practical sense, he was always polite, always helpful, always willing to teach, and greatly respected by his peers. Bob Kissling was one of the giants of CDC.

Respectfully submitted on behalf of all CDC staff members from the era represented by the career of Bob Kissling.

References

1. Goldwasser RA, Kissling RE. Fluorescent antibody staining of street and fixed rabies virus antigens. Proc Soc Exp Biol Med. 1958;98:219–23.
2. Kissling RE, Murphy FA, Henderson BE. Marburg virus. Ann N Y Acad Sci. 1970;174:932–45. http://dx.doi.org/10.1111/j.1749-6632.1970.tb45614.x
3. Monath TP, Mertens PE, Patton R, Moser CR, Baum JJ, Pinneo L, et al. A hospital epidemic of Lassa fever in Zorzor, Liberia, March-April 1972. Am J Trop Med Hyg. 1973;22:773–9.

Address for correspondence: Charles H. Calisher, Department of Microbiology, Immunology, and Pathology, Colorado State University, Delivery Code 1690, Foothills Campus, 3195 Rampart Rd, Fort Collins, CO 80523-1690, USA; email: calisher@cybersafe.net

Medical Entomology for Students, 5th Edition

Mike Service

Cambridge University Press, New York, New York, USA; 2012

ISBN: 978-1107668188

Pages: 317; Price: US $58.00 (paperback)

In the ever-changing field of medical entomology, Mike Service's textbook, Medical Entomology for Students, is now in its fifth edition. The usefulness, adaptability, popularity—and, therefore, longevity—of this textbook is remarkable among the large variety of books on the subject published during the past 20 years.

As in earlier editions, the book is concisely written to provide basic information about recognition, biology, and control methods of arthropods that affect human health—parasites, nuisance pests, and vectors of human diseases—without delving into too much detail and nuance. Almost two thirds of the volume is dedicated to medically important members of the order Diptera (true flies, mosquitoes, and sandflies), and separate sections are devoted to the biology, morphology, and identification of individual mosquito genera. The remaining pages are divided among dscussions of fleas, lice, bedbugs, triatomine bugs, cockroaches, mites, and argasid and ixodid ticks. Although such inequality in coverage seems prejudicial, it is partially justified by the relative epidemiologic importance of dipterans as vectors of arthropodborne diseases in humans.

This edition updates strategies for controlling insects, ticks, and mites. Extensive illustrations and color photographs may help readers recognize arthropods, such as mosquitoes, flies, and myiasis-producing larvae, and distinguish between soft and hard ticks. The book includes a glossary of entomologic and epidemiologic terms and a list of commonly used insecticides and their trade names.

Although the book is aimed primarily at students, it is also suitable as a general introduction into medical entomology for practicing physicians, nurses, public health officials, epidemiologists, and pest control professionals. The book also is valuable to anyone interested in learning basic information about the diversity and biology of medically important arthropods and ways of controlling them.

Michael L. Levin

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA.

Address for correspondence: Michael L. Levin, Centers for Disease Control and Prevention 1600 Clifton Rd, Mailstop G13, Atlanta, GA 30333, USA; email: MLevin@cdc.gov

DOI: http://dx.doi.org/10.3201/eid2008.131738

James Abbott McNeill Whistler (1834–1903) Man at Table beneath Mosquito Net (from Sketchbook) (detail), 1854–55. Black ink on manila-colored wove paper, (4 5/16 × 3 7/16 in/11 × 8.7 cm) Gift of Margaret C. Buell, Helen L. King, and Sybil A. Walk, 1970 (1970.121.40). Image copyright © The Metropolitan Museum of Art. Image source: Art Resource, NY.

Musings on Sketches, Artists, and Mosquito Nets

Byron Breedlove

James Abbott McNeill Whistler was born in Lowell, Massachusetts, on July 11, 1834. When he was 9 years of age, his family moved to St. Petersburg, Russia, and there he studied drawing at the Imperial Academy of Science.

He later attended the United States Military Academy at West Point for 3 years, where he excelled in drawing classes. Because Whistler proved more adept at accumulating 218 demerits than at completing course work or complying with the disciplinary code, West Point Superintendent Colonel Robert E. Lee was obliged to dismiss the young cadet from the academy in 1854.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: http://dx.dopi.org/10.3201/eid2008.AC2008

After a short unhappy stint as a draftsman, Whistler briefly lived with the Winan family in Baltimore, Maryland, where, he "would also ruin all our best pencils, sketching not only on the paper, but also on the smoothly finished wooden backs of the drawing-boards. . . ." During the early 1850s, he began experimenting with techniques, themes, and subjects. Art researcher Nancy Dorfman Pressly wrote, "Drawing less from his imagination, he began to look more carefully around him, catching a moment of unguarded behavior or simply the amusing attitudes of people in everyday situations."

In 1855, Whistler moved back to Europe where he began to establish himself as a painter in Paris and London. He completed more than 500 paintings not only in oils, but also in pastels and watercolors. Among the best known of

his paintings are *Nocturne: Blue and Gold – Old Battersea Bridge* and *Arrangement in Grey and Black No.1*, commonly and mistakenly called *Whistler's Mother*.

In addition to capturing everyday situations, Whistler became recognized for his portrait paintings, including one of Thomas Carlyle. He was celebrated for his etchings of family members, mistresses, and street scenes; some art historians suggest that the quality of his work matched that from the deft hand of Rembrandt.

Whistler was a leader in the Aesthetic Movement, writing and lecturing on the philosophy of "art for art's sake." His work later provided the inspiration for Oscar Wilde's novel *The Picture of Dorian Gray* (1891). Whistler died on July 17, 1903.

During the last decades of the artist's life, a number of great advances in understanding disease transmission occurred. In 1880, Charles Louis Alphonse Laveran, a French physician, identified the protozoan parasite that causes malaria. Seven years later, Ronald Ross and his team discovered the malaria protozoa in anopheles mosquitoes.

Also around this time, in 1900, Walter Reed and a team of colleagues demonstrated that yellow fever was transmitted by mosquitoes. On New Year's Eve, in a letter to his wife, Reed wrote "[I]t has been permitted to me and my assistants to lift the impenetrable veil that has surrounded the causation of this most dreadful pest of humanity and to put it on a rational and scientific basis."

In *Man at Table beneath Mosquito Net*, Whistler himself might be the subject of this black ink drawing, part of a collection of such drawings from 1854–55. Whistler captures the continued struggle of humans versus biting and stinging insects, including those that transmit vector-borne pathogens, from an intimate perspective.

Despite the mosquitoes teeming around him, the man is able to sketch intently and without worry, sheltered by the confines of his personal impenetrable veil. The flurry of cross-hatched, finely scrawled lines in these ephemera could be seen to mimic a mosquito's flight path but this was simply a common technique that Whistler used in his sketches.

Mosquito nets, particularly bed nets or sleeping nets, have, in some shape and form, been used for thousands of years. Herodotus described how people living in marshes in ancient Egypt fished with nets during the day then slept under the same nets to repel insects. Today, pyrethroid-treated mosquito nets are used extensively in malaria-endemic countries in Africa, yielding life-saving returns for little cost.

Personal measures such as using insect repellent, covering exposed skin with clothing, and using mosquito nets also provide simple, cost-effective—albeit not foolproof—protection. Various antimalarial drugs help combat this problem, but none is 100% protective, and they may cause adverse effects. Neither method is perfect but both are essential tools for preventing malaria.

The World Health Organization reported that in 2012, 207 million cases of malaria occurred, causing an estimated 627,000 deaths, mostly in children under 5 years of age. Today, another aspiring young artist working under his or her mosquito net may be sketching formative works that will someday inspire conversation and comment, and be a prelude of greater things to come, as did Whistler's *Man at Table beneath Mosquito Net.*

Bibliography

1. The Metropolitan Museum of Art. James Abbott McNeill Whistler (1834–1903) [cited 2014 Jun 12]. http://www.metmuseum.org/toah/hd/whis/hd_whis.htm
2. Pennell ER, Pennell J. The life of James McNeill Whistler. Philadelphia: J. B. Lippincott Company; 1911.
3. Pressly ND. Whistler in America: an album of early drawings. Metropolitan Museum Journal. 1972;5:125–54. http://dx.doi.org/10.2307/1512629
4. Centers for Disease Control and Prevention. Laveran and the discovery of the malaria parasite [cited 2014 Jul 10]. http://www.cdc.gov/malaria/about/history/laveran.html.
5. Calisher CH. Lifting the impenetrable veil: from yellow fever to Ebola hemorrhagic fever and SARS. Red Feather Lakes (CO): Rockpile Press; 2013.
6. Charlwood D. Did Herodotus describe the first airborne use of mosquito repellents? Trends Parasitol. 2003;19:555–6. http://dx.doi.org/10.1016/j.pt.2003.10.005
7. Brogdon WG, McAllister JC. Insecticide resistance and vector control. Emerg Infect Dis. 1998;4:605–13. http://dx.doi.org/10.3201/eid0404.980410
8. Understanding the environmental, human health, and ecological connections. Workshop summary. Institute of Medicine (US) Forum on Microbial Threats. Washington (DC): National Academies Press; 2008.
9. World Malaria Report - World Health Organization. Geneva: The Organization; 2013.

Address for correspondence: Byron Breedlove, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E41, Atlanta, GA 30329-4027, USA; email: wbb1@cdc.gov

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Molecular Epidemiology of Reemergent Rabies, Yunnan Province, Southwestern China

Passive Surveillance for Azole-Resistant *Aspergillus fumigatus*, United States, 2011–2013

Swine-to-Human Transmission of Influenza A(H3N2) Virus at Agricultural Fairs, Ohio, 2012

Feeding Period Required by *Amblyomma aureolatum* Ticks for Transmission of *Rickettsia rickettsii* to Vertebrate Hosts

Asymptomatic, Mild, and Severe Influenza A(H7N9) Virus Infection in Humans, Guangzhou, China

Methicillin-Sensitive *Staphylococcus aureus* CC398 in Intensive Care Unit

Factors Contributing to Decline in Foodborne Disease Outbreak Reports, United States, 2009

Genetic Variation among African Swine Fever Genotype II Viruses, Eastern and Central Europe.

Family Cluster of Middle East Respiratory Syndrome Coronavirus Infections, Tunisia, 2013

Mutations of Novel Influenza A(H10N8) Virus in Chicken Eggs and MDCK Cells

Novel Circovirus from Mink, China

Risk Factors for Severe Influenza A–Related Pneumonia in Adult Cohort, Mexico, 2013–14

Incidence of *Cronobacter* spp. Infections, USA, 2003–2009

Reemergence of *Brucella melitensis* in Wildlife, France

Antibody against Arenaviruses in Humans, Southwestern United States

Porcine Coronavirus HKU15 Detected in 9 US States, 2014

Genetic Changes of Reemerged Influenza A(H7N9) Viruses, China

Characterization of Highly Pathogenic Avian Influenza Virus A(H5N8) from Waterfowl, South Korea, 2014

Schistosomiasis Haematobium, Corsica, France

Complete list of articles in the September issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

2014

September 5–9, 2014
ICAAC 2014
Interscience Conference on Antimicrobial Agents and Chemotherapy
Washington, DC, USA
http://www.icaac.org

October 8–12, 2014
ID Week 2014
Philadelphia, PA, USA
http://www.idweek.org/

October 31–November 3, 2014
IMED 2014
Vienna, Austria
http://imed.isid.org

November 2–6, 2014
ASTMH
American Society of Tropical Medicine and Hygiene
63rd Annual Meeting
New Orleans, LA, USA
http://www.astmh.org/Home.htm

November 15–19, 2014
APHA 142nd Annual Meeting & Expo
New Orleans, LA, USA
http://www.apha.org/meetings/AnnualMeeting

November 30–December 4, 2014
ASLM 2014 International Conference
Cape Town International Convention Centre, South Africa
http://www.aslm2014.org/

2015

March 8–11, 2015
ICEID
International Conference on Emerging Infectious Diseases
Atlanta, GA, USA

Announcements

To submit an announcement, send an email message to EIDEditor (eideditor@cdc.gov). Include the date of the event, the location, the sponsoring organization(s), and a website that readers may visit or a telephone number or email address that readers may contact for more information.

Announcements may be posted on the journal Web page only, depending on the event date.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to www.medscape.org/journal/eid. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers. You must be a registered user on Medscape.org. If you are not registered on Medscape.org, please click on the "Register" link on the right hand side of the website to register. Only one answer is correct for each question. Once you successfully answer all post-test questions you will be able to view and/or print your certificate. For questions regarding the content of this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@webmd.net. American Medical Association's Physician's Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please refer to http://www.ama-assn.org/ama/pub/about-ama/awards/ama-physicians-recognition-award.page. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the certificate and present it to your national medical association for review.

Article Title

Leptospirosis-Associated Hospitalizations, United States, 1998–2009

CME Questions

1. Which of the following statements is most accurate regarding the clinical presentation of leptospirosis?

A. Infection invariably results in clinical disease
B. Symptom onset can be biphasic in nature
C. Most patients present with genitourinary tract symptoms
D. Nearly three-quarters of infected patients have severe disease

2. Which of the following statements regarding the epidemiology of leptospirosis-associated hospitalizations in the current study is most accurate?

A. The average annual prevalence of leptospirosis-associated hospitalizations was 0.6 cases per 1 million persons for 1998–2009
B. There was a steady increase in the prevalence of leptospirosis-associated hospitalizations between 1998 and 2009
C. Leptospirosis affected women and men approximately equally
D. Children were disproportionately affected with leptospirosis compared with adults

3. Which season accounted for the greatest percentage of leptospirosis-associated hospitalizations in the current study?

A. Spring
B. Summer
C. Fall
D. Winter

4. What does the current study conclude in comparing leptospirosis-associated hospitalizations vs non-leptospirosis infectious disease hospitalizations during the study period?

A. There was no difference in hospital stay data in comparing leptospirosis-associated vs. non-leptospirosis infectious disease hospitalizations
B. Leptospirosis-associated hospitalizations was associated with longer and more costly hospitalizations vs. non-leptospirosis infectious disease hospitalizations
C. Non-leptospirosis infectious disease hospitalizations were associated with longer and more costly hospitalizations vs. leptospirosis-associated hospitalizations
D. Although non-leptospirosis infectious disease hospitalizations were associated with longer lengths of stay compared with leptospirosis-associated hospitalizations, leptospirosis-associated hospitalizations were associated with higher hospital charges

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 is a peer-reviewed journal established expressly to promote the recognition of new and reemerging infectious diseases around the world and improve the understanding of factors involved in disease emergence, prevention, and elimination.

The journal is intended for professionals in infectious diseases and related sciences. We welcome contributions from infectious disease specialists in academia, industry, clinical practice, and public health, as well as from specialists in economics, social sciences, and other disciplines. Manuscripts in all categories should explain the contents in public health terms. For information on manuscript categories and suitability of proposed articles, see below and visit http://wwwnc.cdc.gov/eid/pages/author-resource-center.htm.

Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

Instructions to Authors

Manuscript Submission. To submit a manuscript, access Manuscript Central from the Emerging Infectious Diseases web page (www.cdc.gov/eid). Include a cover letter indicating the proposed category of the article (e.g., Research, Dispatch), verifying the word and reference counts, and confirming that the final manuscript has been seen and approved by all authors. Complete provided Authors Checklist.

Manuscript Preparation. For word processing, use MS Word. Set the document to show continuous line numbers. List the following information in this order: title page, article summary line, keywords, abstract, text, acknowledgments, biographical sketch, references, tables, and figure legends. Appendix materials and figures should be in separate files.

Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

Keywords. Use terms as listed in the National Library of Medicine Medical Subject Headings index (www.ncbi.nlm.nih.gov/mesh).

Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

Tables. Provide tables within the manuscript file, not as separate files. Use the MS Word table tool, no columns, tabs, spaces, or other programs. Footnote any use of boldface. Tables should be no wider than 17 cm. Condense or divide larger tables. Extensive tables may be made available online only.

Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

Videos. Submit as AVI, MOV, MPG, MPEG, or WMV. Videos should not exceed 5 minutes and should include an audio description and complete captioning. If audio is not available, provide a description of the action in the video as a separate Word file. Published or copyrighted material (e.g., music) is discouraged and must be accompanied by written release. If video is part of a manuscript, files must be uploaded with manuscript submission. When uploading, choose "Video" file. Include a brief video legend in the manuscript file.

Types of Articles

Perspectives. Articles should not exceed 3,500 words and 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.

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.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.