

EMERGING INFECTIOUS DISEASES[®]



Viruses

January 2020



Charles Burchfield, *Orion in December, 1959*. Watercolor and pencil on paper. 39 7/8 in x 32 7/8 in/101.2 x 83.4 cm. Smithsonian American Art Museum, Gift of S.C. Johnson & Son, Inc., 1969.47.53.

EMERGING INFECTIOUS DISEASES®

EDITOR-IN-CHIEF

D. Peter Drotman

ASSOCIATE EDITORS

Charles Ben Beard, Fort Collins, Colorado, USA
 Ermias Belay, Atlanta, Georgia, USA
 David M. Bell, Atlanta, Georgia, USA
 Sharon Bloom, Atlanta, Georgia, USA
 Richard Bradbury, Bratislava, Slovakia
 Mary Brandt, Atlanta, Georgia, USA
 Corrie Brown, Athens, Georgia, USA
 Charles H. Calisher, Fort Collins, Colorado, USA
 Benjamin J. Cowling, Hong Kong, China
 Michel Drancourt, Marseille, France
 Paul V. Effler, Perth, Australia
 Anthony Fiore, Atlanta, Georgia, USA
 David O. Freedman, Birmingham, Alabama, USA
 Peter Gerner-Smidt, Atlanta, Georgia, USA
 Stephen Hadler, Atlanta, Georgia, USA
 Matthew J. Kuehnert, Edison, New Jersey, USA
 Nina Marano, Atlanta, Georgia, USA
 Martin I. Meltzer, Atlanta, Georgia, USA
 David Morens, Bethesda, Maryland, USA
 J. Glenn Morris, Jr., Gainesville, Florida, USA
 Patrice Nordmann, Fribourg, Switzerland
 Johann D.D. Pitout, Calgary, Alberta, Canada
 Ann Powers, Fort Collins, Colorado, USA
 Didier Raoult, Marseille, France
 Pierre E. Rollin, Atlanta, Georgia, USA
 Frederic E. Shaw, Atlanta, Georgia, USA
 David H. Walker, Galveston, Texas, USA
 J. Todd Weber, Atlanta, Georgia, USA
 J. Scott Weese, Guelph, Ontario, Canada

Managing Editor

Byron Breedlove, Atlanta, Georgia, USA

Copy Editors Kristina Clark, Dana Dolan, Karen Foster,
 Thomas Gryczan, Amy Guinn, Michelle Moran,
 Shannon O'Connor, Jude Rutledge, P. Lynne Stockton,
 Deborah Wenger

Production Thomas Ehemann, William Hale, Barbara Segal,
 Reginald Tucker

Journal Administrator Susan Richardson

Editorial Assistant Kristine Phillips

Communications/Social Media Sarah Logan Gregory,
 Tony Pearson-Clarke, Deanna Altomara (intern)

Founding Editor

Joseph E. McDade, Rome, Georgia, USA

EDITORIAL BOARD

Barry J. Beaty, Fort Collins, Colorado, USA
 Martin J. Blaser, New York, New York, USA
 Andrea Boggild, Toronto, Ontario, Canada
 Christopher Braden, Atlanta, Georgia, USA
 Arturo Casadevall, New York, New York, USA
 Kenneth G. Castro, Atlanta, Georgia, USA
 Vincent Deubel, Shanghai, China
 Christian Drosten, Charité Berlin, Germany
 Isaac Chun-Hai Fung, Statesboro, Georgia, USA
 Kathleen Gensheimer, College Park, Maryland, USA
 Rachel Gorwitz, Atlanta, Georgia, USA
 Duane J. Gubler, Singapore
 Richard L. Guerrant, Charlottesville, Virginia, USA
 Scott Halstead, Arlington, Virginia, USA
 David L. Heymann, London, UK
 Keith Klugman, Seattle, Washington, USA
 Takeshi Kurata, Tokyo, Japan
 S.K. Lam, Kuala Lumpur, Malaysia
 Stuart Levy, Boston, Massachusetts, USA
 John S. Mackenzie, Perth, Australia
 John E. McGowan, Jr., Atlanta, Georgia, USA
 Jennifer H. McQuiston, Atlanta, Georgia, USA
 Tom Marrie, Halifax, Nova Scotia, Canada
 Nkuchia M. M'ikanatha, Harrisburg, Pennsylvania, USA
 Frederick A. Murphy, Bethesda, Maryland, USA
 Barbara E. Murray, Houston, Texas, USA
 Stephen M. Ostroff, Silver Spring, Maryland, USA
 Mario Raviglione, Milan, Italy, and Geneva, Switzerland
 David Relman, Palo Alto, California, USA
 Guenaël R. Rodier, Saône-et-Loire, France
 Connie Schmaljohn, Frederick, Maryland, USA
 Tom Schwan, Hamilton, Montana, USA
 Rosemary Soave, New York, New York, USA
 P. Frederick Sparling, Chapel Hill, North Carolina, USA
 Robert Swanepoel, Pretoria, South Africa
 David E. Swayne, Athens, Georgia, USA
 Phillip Tarr, St. Louis, Missouri, USA
 Duc Vugia, Richmond, California, USA
 Mary Edythe Wilson, Iowa City, Iowa, USA

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

The conclusions, findings, and opinions expressed by authors contributing to this journal do not necessarily reflect the official position of the U.S. Department of Health and Human Services, the Public Health Service, the Centers for Disease Control and Prevention, or the authors' affiliated institutions. Use of trade names is for identification only and does not imply endorsement by any of the groups named above.

All material published in *Emerging Infectious Diseases* is in the public domain and may be used and reprinted without special permission; proper citation, however, is required.

Use of trade names is for identification only and does not imply endorsement by the Public Health Service or by the U.S. Department of Health and Human Services.

EMERGING INFECTIOUS DISEASES is a registered service mark of the U.S. Department of Health & Human Services (HHS).

EMERGING INFECTIOUS DISEASES®



Viruses

January 2020



On the Cover

Charles Burchfield, *Orion in December, 1959*. Watercolor and pencil on paper. 39 7/8 in x 32 7/8 in/101.2 x 83.4 cm. Smithsonian American Art Museum, Gift of S.C. Johnson & Son, Inc., 1969.47.53.

About the Cover p. 187

Synopses

Spatial Epidemiologic Trends and Hotspots of Leishmaniasis, Sri Lanka, 2001–2018

N.D. Karunaweera et al.

1

Candidatus Mycoplasma haemohominis in Human, Japan

N. Hattori et al.

11

Nutritional Care for Patients with Ebola Virus Disease

M. Ververs, M. Gabra

20

Research

Medscape
EDUCATION
ACTIVITY

Paid Leave and Access to Telework as Work Attendance Determinants during Acute Respiratory Illness, United States, 2017–2018

Practices that actively encourage employees to stay home when ill are crucial to reduce transmission in workplaces.

F. Ahmed et al.

26

Preclinical Detection of Prions in Blood of Nonhuman Primates Infected with Variant Creutzfeldt-Jakob Disease

L. Concha-Marambio et al.

34

Effect of Acute Illness on Contact Patterns, Malawi, 2017

J.R. Glynn et al.

44

Outbreak of Peste des Petits Ruminants Virus among Critically Endangered Mongolian Saiga and Other Wild Ungulates, Mongolia, 2016–2017

M. Pruvot et al.

51

Elephant Endotheliotropic Herpesvirus Hemorrhagic Disease in Asian Elephant Calves in Logging Camps, Myanmar

Z.M. Oo et al.

63





EMERGING INFECTIOUS DISEASES®

January 2020

- Effect of Pediatric Influenza Vaccination on Antibiotic Resistance, England and Wales**
C. Chae et al. 138
- Locally Acquired Human Infection with Swine-Origin Influenza A(H3N2) Variant Virus, Australia, 2018**
Y.-M. Deng et al. 143

Research Letters

- Use of Ambulance Dispatch Calls for Surveillance of Severe Acute Respiratory Infections**
S. Monge et al. 148
- Hantavirus Pulmonary Syndrome in Traveler Returning from Nepal to Spain**
E. Sulleiro et al. 150
- Visceral Leishmaniasis, Northern Somalia, 2013–2019**
M.K. Aalto et al. 153
- Autochthonous Human Fascioliasis, Belgium**
S. Milas et al. 155
- Recombinant Nontypeable Genotype II Human Noroviruses in the Americas**
K. Tohma et al. 157
- Legionella pneumophila* as Cause of Severe Community-Acquired Pneumonia, China**
H. Yi et al. 160
- Training for Foodborne Outbreak Investigations by Using Structured Learning Experience**
F. Burckhardt, E. Kissling 162
- Emergence of *Vibrio cholerae* O1 Sequence Type 75 in Taiwan**
Y.-H. Tu et al. 164

- Risk Factors for and Seroprevalence of Tickborne Zoonotic Diseases among Livestock Owners, Kazakhstan**
J.R. Head et al. 70
- High Azole Resistance in *Aspergillus fumigatus* Isolates from Strawberry Fields, China, 2018**
Y. Chen et al. 81
- Tick-Borne Encephalitis Virus, United Kingdom**
M. Holding et al. 90
- Phenotypic and Genotypic Correlates of Penicillin Susceptibility in Nontoxigenic *Corynebacterium diphtheriae*, British Columbia, Canada, 2015–2018**
J. Zou et al. 97
- High Pathogenicity of Nipah Virus from *Pteropus Iylei* Fruit Bats, Cambodia**
M. Gaudino et al. 104

Dispatches

- Varicella in Adult Foreigners at a Referral Hospital, Central Tokyo, Japan, 2012–2016**
S. Takaya et al. 114
- Geographic Distribution and Incidence of Melioidosis, Panama**
A.B. Araúz et al. 118
- Shigella* Bacteremia, Georgia, USA, 2002–2012**
M. Tobin-D'Angelo et al. 122
- Distribution of Japanese Encephalitis Virus, Japan and Southeast Asia, 2016–2018**
R. Kuwata et al. 125
- Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt**
K.E. Hassan et al. 129
- Infectivity of Norovirus GI and GII from Bottled Mineral Water during a Waterborne Outbreak, Spain**
S. Guix et al. 134





185

EMERGING INFECTIOUS DISEASES®

January 2020

Letter

Vaccine Effectiveness against DS-1–Like Rotavirus Strains

T. Nakaggomi 184

In Memoriam

Jay Stephen Keystone (1943–2019)

D.O. Freedman 185

About the Cover

Hunters Searching among Starry Nights and at the Edges of Life

B. Breedlove 187

Diabetes Mellitus, Hypertension, and Death among 32 Patients with MERS-CoV Infection, Saudi Arabia

K.H. Alanazi et al.

166

Influenza D Virus of New Phylogenetic Lineage, Japan

S. Murakami et al.

168

Diagnosis of Syphilitic Bilateral Papillitis Mimicking Papilloedema

A. Gonzalez-Martinez et al.

171

Influenza A Virus Infections in Dromedary Camels, Nigeria and Ethiopia, 2015–2017

D.K.W. Chu et al.

173

Bovine Kobuvirus in Calves with Diarrhea, United States

L. Wang et al.

176

Imported Schistosomiasis, China, 2010–2018

S.-M. Dai et al.

179

Rare Case of Enteric *Ancylostoma caninum* Hookworm Infection, South Korea

B.-K. Jung et al.

181

Etymologia

Picobirnavirus

Y.S. Malik, S. Ghosh 89

Online Report

Plans for Nationwide Serosurveillance Network in Vietnam

D.D. Anh et al.

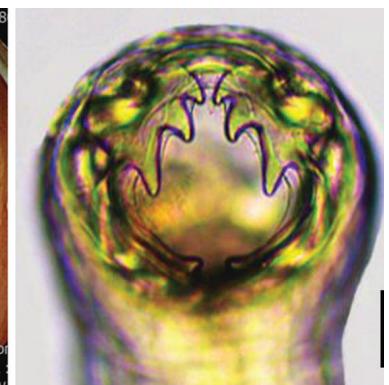
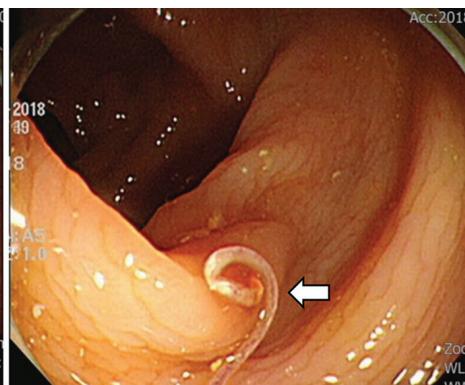
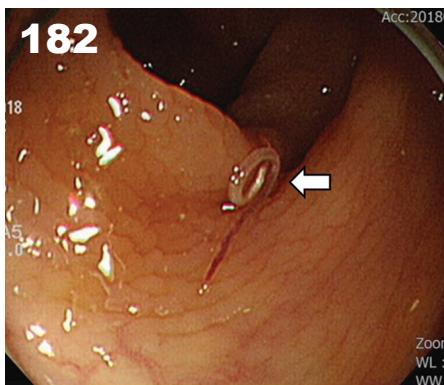
https://wwwnc.cdc.gov/EID/article/26/1/19-0641_article

Conference Summary

Data Behind Vaccine Hesitancy and Latest Updates on Vaccines in the Pipeline—2019 Annual Conference on Vaccinology Research, April 3–5, 2019

W. Schaffner et al.

https://wwwnc.cdc.gov/EID/article/26/1/19-1246_article



DECENNIAL

2020

Global Solutions to Antibiotic Resistance in Healthcare



decennial2020.org • [#Decennial2020](https://twitter.com/Decennial2020)

6th Decennial International
Conference on Healthcare
Associated Infections

March 26–30, 2020
Marriott Marquis
Atlanta, GA

**REGISTER
TODAY!**

Co-Hosted by:



Spatial Epidemiologic Trends and Hotspots of Leishmaniasis, Sri Lanka, 2001–2018

Nadira D. Karunaweera,¹ Samitha Ginige, Sanath Senanayake, Hermali Silva, Nuwani Manamperi, Nilakshi Samaranyake, Yamuna Siriwardana, Deepa Gamage, Upul Senerath, Guofa Zhou¹

Leishmaniasis, a neglected tropical disease, is on the decline in South Asia. However, cases of cutaneous leishmaniasis have risen in Sri Lanka since 2001, and the lack of in-depth research on its epidemiologic characteristics hampers control efforts. We analyzed data collected from patients with cutaneous leishmaniasis in Sri Lanka during 2001–2018 to study temporal and geographic trends and identify and monitor disease hotspots. We noted a progression in case rates, including a sharp rise in 2018, showing temporal expansion of disease-prevalent areas and 2 persistent hotspots. The northern hotspot shifted and shrank over time, but the southern hotspot progressively expanded and remained spatially static. In addition, we noted regional incidence differences for age and sex. We provide evidence of temporally progressive and spatially expanding incidence of leishmaniasis in Sri Lanka with distinct geographic patterns and disease hotspots, signaling an urgent need for effective disease control interventions.

Leishmaniasis are diseases caused by *Leishmania* spp. parasites transmitted through the bites of infected female phlebotomine sand flies. A neglected tropical disease that mainly affects the tropics and subtropics, leishmaniasis has 3 forms: cutaneous, visceral, and mucocutaneous (1). Cutaneous leishmaniasis (CL) is the most common form, causing skin lesions that can leave scars and cause lifelong disability (1). Visceral leishmaniasis (VL) is the most serious form and has a case-fatality rate >95% in untreated cases; globally, 50,000–90,000 new cases and 20,000–40,000 deaths occur annually, making VL one of the

largest killers among neglected tropical diseases (1–3). Approximately 0.7–1 million new CL cases and a few thousand mucocutaneous leishmaniasis cases occur worldwide each year (1–3).

South Asia has the highest incidence of VL; India, Nepal, and Bangladesh are predominantly affected. Leishmaniasis in this region is caused by *Leishmania donovani* transmitted by *Phlebotomus argentipes* sand flies (2–4). Driven by the goal to eliminate VL in South Asia by 2020, the 3 countries once highly endemic for VL have made remarkable progress, bringing down reported cases from 50,898 in 2007 to 6,174 in 2017; Nepal had an 84% case reduction, India an 87% reduction, and Bangladesh a 96% reduction (2,4). Such efforts have contributed greatly to the ≈80% reduction in global VL incidence during 2007–2017 (2,4,5).

Local and international health policy makers do not view leishmaniasis as an urgent health issue in Sri Lanka, possibly because of the perceived nonserious nature of CL and relatively small numbers of reported cases (1,2,4). Locally acquired CL was not reported in Sri Lanka before 1992 (6), and only a few sporadic cases were reported before incidence rates began to escalate in 2001 (7). Since then, locally acquired VL and mucocutaneous forms also have been reported, although most leishmaniasis cases in the country are cutaneous (7–11). Typical symptoms of CL are single, nontender, nonitchy lesions in the form of nodules, papules, or ulcers (Figure 1, panels A–C) that affect exposed body parts (7,10). Occasional atypical symptoms include dermal plaques (Figure 1, panel D), erythematous ulcerative patches (12,13), and mucosal tissue involvement (14). In Sri Lanka, initial treatment for CL is weekly intralesional inoculations of sodium stibogluconate administered through dermatology units of the government health sector at a physician's discretion.

Author affiliations: University of Colombo, Colombo, Sri Lanka (N.D. Karunaweera, S. Senanayake, H. Silva, N. Samaranyake, Y. Siriwardana, U. Senerath); Ministry of Health, Colombo (S. Ginige, D. Gamage); University of Kelaniya, Ragama, Western Sri Lanka (N. Manamperi); University of California Irvine, Irvine, California, USA (G. Zhou)

DOI: <https://doi.org/10.3201/eid2601.190971>

¹These authors were co-principal investigators for this article.

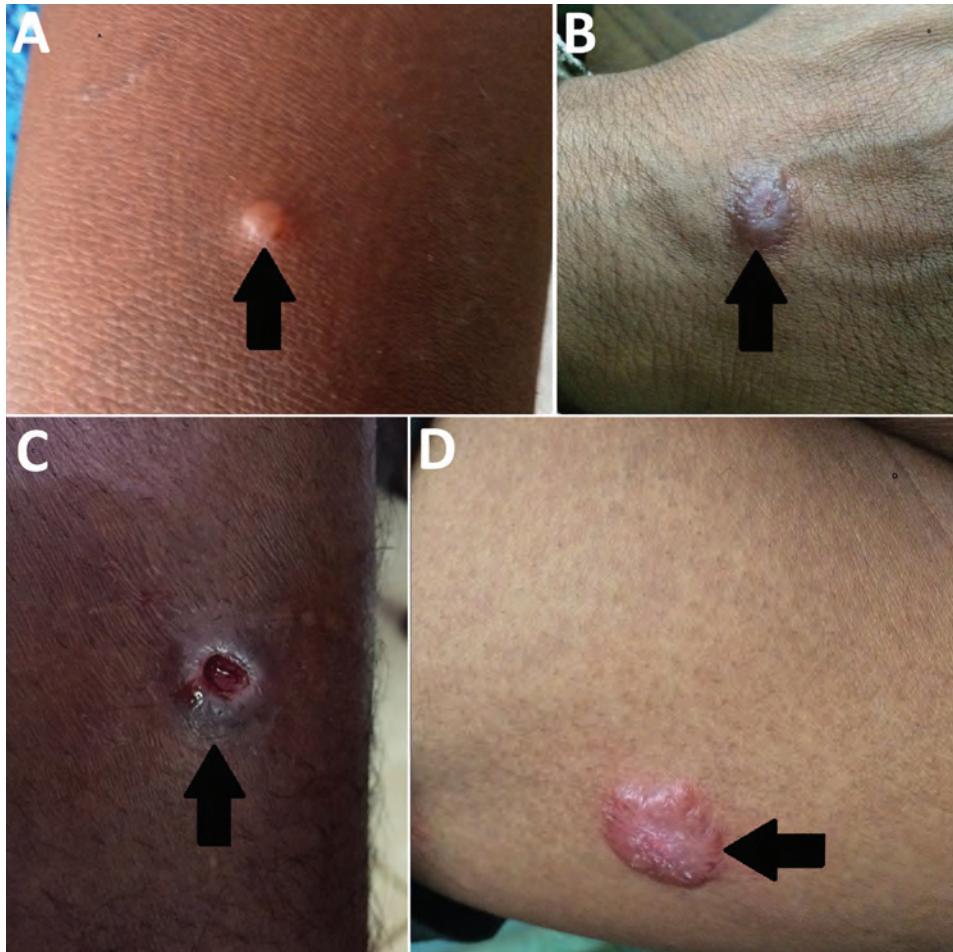


Figure 1. Types of skin lesions observed in cutaneous leishmaniasis patients, Sri Lanka, 2001–2018. Arrows indicate A) papule; B) nodule; C) ulcer; D) plaque.

L. donovani MON 37, a dermatropic variant of the species, is the causative agent of CL in Sri Lanka but is better known to cause VL elsewhere (4,9,15,16). Although the exact basis of dermatropism is unknown, evidence suggests a parasite gene mutation with atypical phenotypic properties manifesting as cutaneous disease devoid of visceralizing features, as noted in long-term patient followup studies (15–17). The probable vector is the *P. argentipes glaucus* sand fly, which demonstrates zoophilic behavior (18,19) and differs morphologically and genetically from the *P. argentipes* sensu lato sand fly species found in South India (20).

Local leishmaniasis transmission occurs either outdoors or peridomestically and spatial clustering is seen in highly disease-endemic areas (10,21,22). Despite the global and regional decline in reported leishmaniasis cases, CL incidence has progressed in Sri Lanka since 2001 (7,11,22,23). Previous studies on the clinical spectrum, sex and age distribution, spatial clustering of cases, and possible links between climatic and environmental variables led to discussions

on challenges to curbing leishmaniasis in the country (9–11,22,24). However, no systematic or in-depth studies have been conducted on the epidemiologic characteristics of leishmaniasis in Sri Lanka and its >2-decade progression in the country. Lack of epidemiologic data endangers the health of the population and threatens disease elimination efforts in South Asia, making it a regional, if not global, priority (3,4,7).

The aim of this study was to conduct a retrospective review of the epidemiologic characteristics of leishmaniasis through patient data collected during 2001–2018. The information revealed could inform interventional strategies to address the expansion of leishmaniasis in Sri Lanka, which would improve the likelihood of meeting the goal of South Asia VL-elimination plans.

Methods

We obtained nationwide leishmaniasis data from the national diagnostic and research laboratory at the University of Colombo Faculty of Medicine (Colombo, Sri Lanka), which maintains data on laboratory-

confirmed CL cases. Laboratory confirmation of CL was made through visualization of *Leishmania* sp. amastigotes upon microscopic examination of Giemsa-stained lesion aspirate smears or slit-skin scrapings (22). We also accessed data from the repository of notifiable diseases maintained at the Epidemiology Unit of the Ministry of Health and through communication with medical health officers in small health administrative units in each district.

Leishmaniasis was made a notifiable disease in Sri Lanka in 2008, at which time notification of cases to the central epidemiology unit became a mandatory requirement. Dermatology units, led by consultant dermatologists, make CL notifications on the basis of strong clinical suspicion with or without laboratory confirmation and have >90% accuracy in local settings (25).

To avoid overlapping patient data, we cross-checked data accessed through different sources. District-level annual CL case counts covered the entire country during 2001–2018. We estimated district- and division-level populations using census data from 2001 and 2012 and projected population levels by assuming a linear annual growth rate. We calculated annual incidence rates for each district as cases per 100,000 population. We mapped case distribution by using ArcGIS 10.1 (Esri, <https://www.arcgis.com>) and used monthly district-level data from each year to analyze leishmaniasis seasonality.

To analyze disease hotspots, we collected patient data from each division for 2015–2017. We determined hotspots and coldspots by using the Optimized HotSpot Analysis tool of ArcGIS, which calculates Getis-Ord G_i^* spatial statistics (26,27). We determined hotspots from positive z-scores and coldspots from negative z-scores and CI values of 90%, 95%, and 99% for both. We used Ripley's K function and the Multi-Distance Spatial Cluster Analysis tool of ArcGIS to determine the average cluster size (28). To calculate K function, we used division-level population data from the 2012 Sri Lanka census weighted against leishmaniasis incidence as the clustering variable for each division.

We estimated the population in each district by using census data from 2001 and 2012 with projections for each year as given by the government of Sri Lanka (<http://www.statistics.gov.lk>). We considered persons from districts with ≥ 10 cases/100,000 persons/year as at-risk populations.

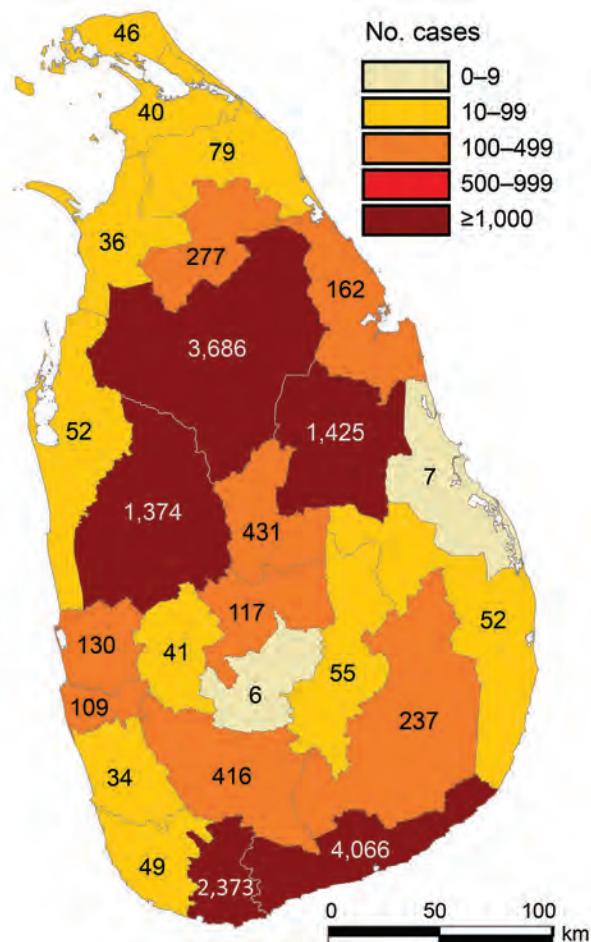
To analyze age and sex distribution over time and by region, we selected 3 health divisions with the highest incidence rates in the Southern Province, Dickwella, Tangalle, and Beliatta, and 2 health divisions with the highest incidence rates in the North-

Central Province, Thalawa and Thamankaduwa. We collected data from 2 periods, 2001–2003 and 2015–2018. We classified age into 3 categories, 0–14, 15–49, and ≥ 50 years (7,10,29). We used 2001 and 2012 census data from these divisions for data analysis. We compared differences in sex and age distribution in each region, between regions, and between years by using χ^2 test. We also compared age distribution against census data by using z-score test with standard residual. We did not analyze sex and age distribution for 2001–2003 because of the low disease incidence.

Results

Increasing Trends in Incidence and Spatial Expansion

During 2001–2018, island-wide spatial distribution of reported CL cases was 15,300 (Figure 2). Five districts reported >1,000 cases, which accounted for 84.5% (12,924/15,300) of all cases (Figure 2). We noted a slow but steady increase in case numbers from 2001 to 2010, which expanded from 37 to 426 cases/100,000



persons with the majority (73.8%; 2,306/3,125) reported from 3 districts, Hambantota, Anuradhapura, and Matara (Figure 3, panel A). We also noted an increase in incidence during 2010–2011; case counts reached >1,000 during 2012 and remained stable until 2017 (Figure 3, panel B). Most cases (88.3%; 7,865/8,904) were reported from 5 districts, Hambantota, Anuradhapura, Matara, Polonnaruwa, and Kurunegala (Figure 2; Figure 3, panel A). In 2018, we saw an alarming uptick in cases, doubling to 3,271 cases from 1,508 cases in 2017 (Figure 3), and 2 additional districts, Matale and Ratnapura, reached case counts >200 within 1 year. Of the total cases reported during 2018, a total of 86.5% (2,830/3,271) occurred in those 7 districts, and the annual incidence rate was >100 cases/100,000 population in Hambantota District for the first time (Figure 3, panel A). We analyzed monthly data for each year but did not see a uniform seasonal pattern of case distribution at the district, regional, or national level, but we noted that case counts in the north peaked during July–September each year (data not shown).

We saw a marked expansion in spatial distribution of leishmaniasis cases over time (Figure 4). In 2001, the case incidence rate per district was

<10 cases/100,000 population, but in 2009, the population living at risk for leishmaniasis increased to >2 million in 3 districts with incidence rates between 10 and 50 cases/100,000 population (Table 1). By 2018, 8 districts had incidence rates of >10 cases/100,000 population, including Hambantota, where the incidence rate reached 117.2 cases/100,000 population (Table 1; Figure 3, panel A). Using 2018 case counts, we estimate 6,622,843 persons, nearly one third of the total population of Sri Lanka, live at considerable risk for leishmaniasis.

Shifts in Spatial Distribution and Hotspots

The increased incidence of leishmaniasis that started in 2001 in 2 districts, Anuradhapura in the North-Central Province and Matara in the Southern Province, extended to other provinces in subsequent years (Figure 4). The disease-endemic area in the North-Central Province expanded during 2001–2018 and its epicenter shifted during 2007–2018 (Figure 4). In the Southern Province, a similar expansion occurred, with a marked increase in incidence rates from 1.2 cases/100,000 persons in 2001 to 117.2 cases/100,000 persons in 2018, but the epicenter remained spatially static (Figure 4).

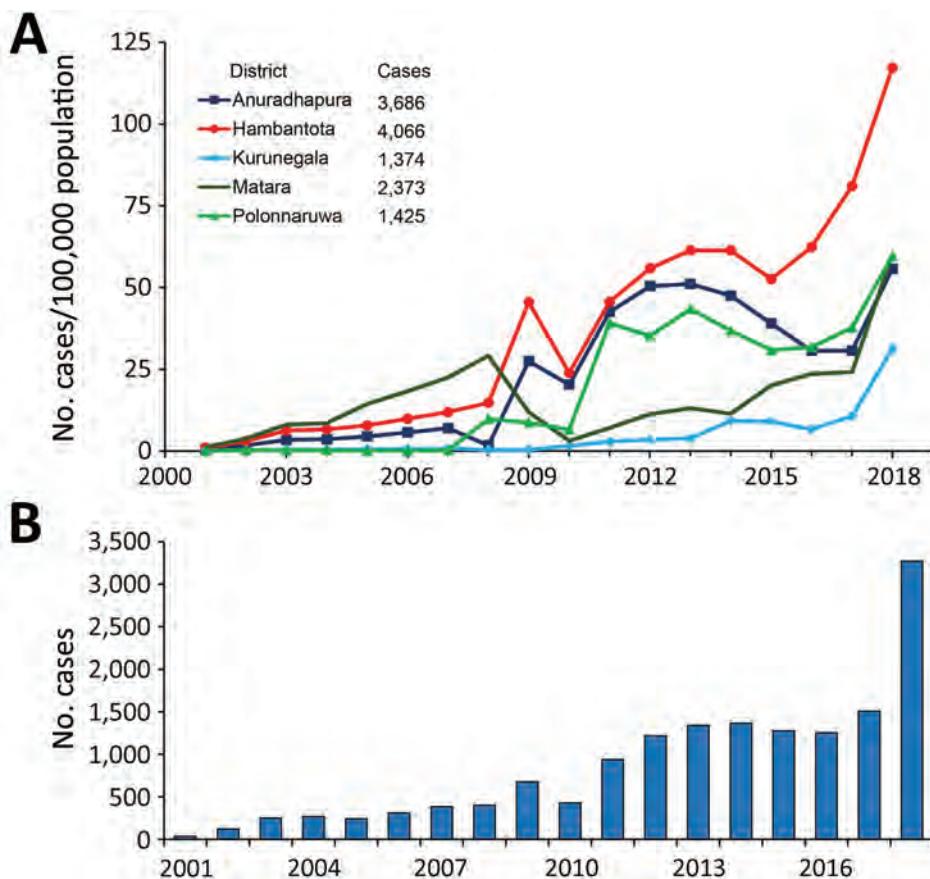


Figure 3. Changes in leishmaniasis incidence and case counts, Sri Lanka, 2001–2018. A) Leishmaniasis incidence rates for 5 districts with the highest numbers of reported cases. B) Nationwide reported leishmaniasis cases by year.

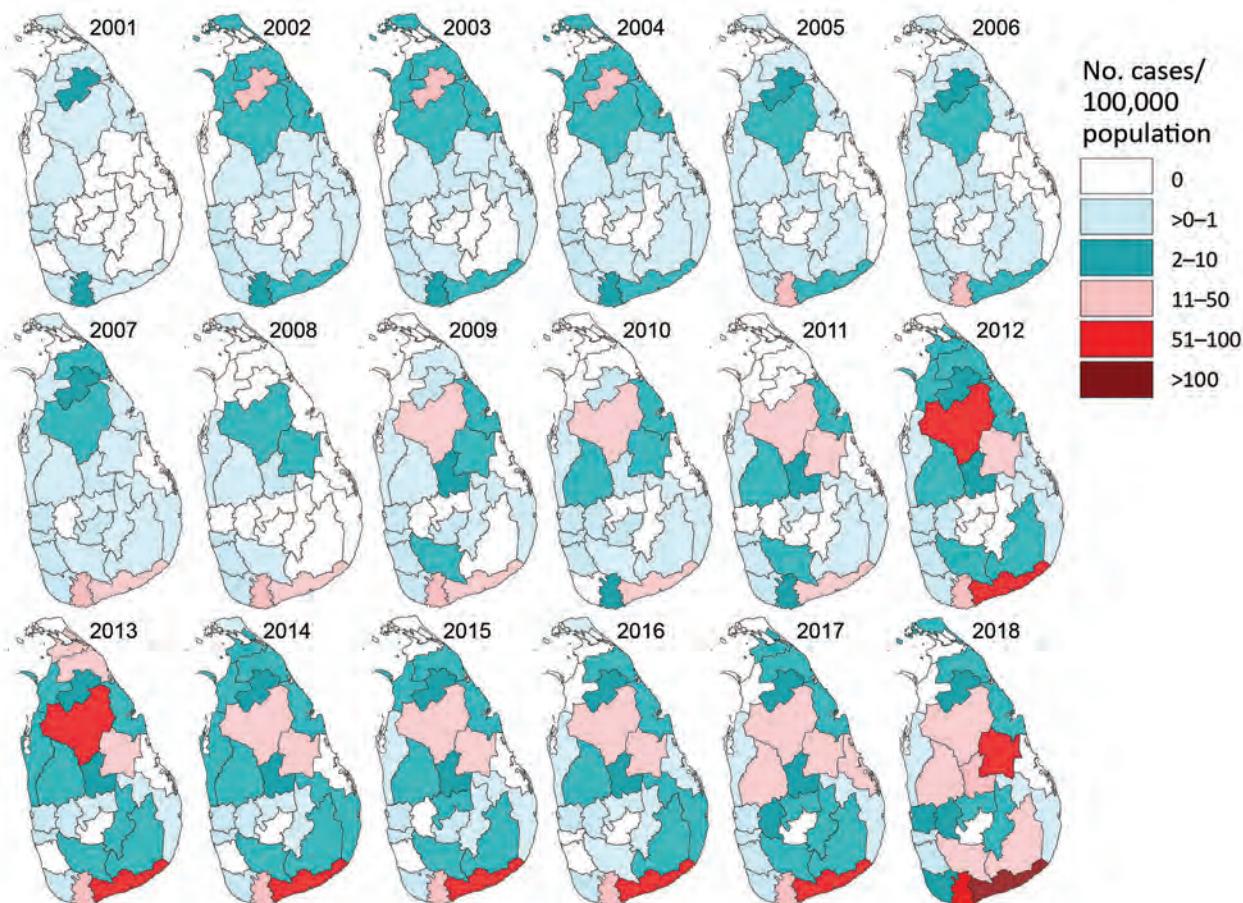


Figure 4. Leishmaniasis incidence rates by district and year, Sri Lanka, 2001–2018.

Fine-scale cluster analysis revealed 2 major hotspots in the North-Central and Southern provinces and a coldspot in the central region that spreads across the island from west to east (Figure 5). The size of the hotspot in the North-Central Province gradually shrank, but the one in the Southern Province expanded during 2015–2017 (Figure 5). The average size of the southern hotspot was ≈ 40 km in radius in 2015 and ≈ 70 km in 2017 (Figure 6).

Changes in Age and Sex Distribution over Time

We analyzed anthropometric data of 2,379 cases to study the age and sex distributions and make comparisons between the early disease period, 2001–2003, and the late period, 2015–2018, and between the North-Central and Southern provinces (Table 2). We noted 200 cases during 2001–2003 and 2,179 cases during 2015–2018 and a statistically significant change in the age and sex distribution in the North-Central Province between the early and late periods (Table 2). During 2001–2003, most cases (94.7%) in the North-Central Province were in male patients, but the

proportion of male cases dropped to 68.8% during 2015–2018 ($\chi^2 = 38.75$, $df = 1$, $p < 0.0001$). Similarly, in this region most cases (86.3%) were in persons 15–49 years of age during the early period but dropped to 53.7% in this group during the late period ($\chi^2 = 47.73$, $df = 1$, $p < 0.0001$) (Table 2). In contrast, in the Southern Province the sex distribution remained the same in the early and late periods ($\chi^2 = 0.0001$, $df = 1$, $p = 0.9784$) (Table 2), but the age range of the highest incidence group shifted from persons ≥ 50 years of age (40.6%) during the early period to those 15–49 years of age (54.7%) during the late period ($\chi^2 = 10.62$, $df = 2$, $p = 0.0049$) (Table 2).

Further analysis of data from the late period revealed statistically significant differences in distribution of the disease by sex within and between the North-Central and Southern provinces (Table 3). Further examination revealed fewer cases in persons < 14 years of age, but more male patients in this age group, a finding common to both regions (Table 3). Regardless of sex, we noted a higher incidence rate in those ≥ 50 years of age in the North-Central Province

Table 1. Estimates of population living at risk for leishmaniasis during 2001, 2009, and 2018, Sri Lanka*

Cases/100,000 population	2001		2009		2018	
	No. districts	Population	No. districts	Population	No. districts	Population
0	12	7,728,455	7	3,899,690	2	1,306,933
<1	11	10,157,597	11	11,453,975	6	6,988,158
1–10	2	911,205	4	2,290,870	9	6,622,843
11–50	0	0	3	2,194,176	4	3,862,871
51–100	0	0	0	0	3	2,211,753
>100	0	0	0	0	1	639,340
Total	25	18,797,257	25	19,838,711	25	21,631,898

*Determined by using cutaneous leishmaniasis incidence rates per district and census data from the government of Sri Lanka (<http://www.statistics.gov.lk>) from 2001 to project potential incidence rates for 2009 and from 2012 to project incidence rates for 2018.

compared with census data, but we did not see this pattern in the south (Table 3). In addition, we did not see a difference in age distribution of female patients between the 2 regions ($\chi^2 = 0.69$, $df = 2$, $p = 0.7065$), but we saw a statistically significant difference in male patients ($\chi^2 = 27.74$, $df = 2$, $p < 0.0001$) (Table 3) relative to the age distribution reported in census data. Our findings demonstrate a statistically high incidence rate in persons ≥ 50 in the North-Central Province compared with the Southern Province.

Discussion

Leishmaniasis was seldom reported in Sri Lanka before the 1990s (30), and neither local nor international health authorities have considered it a serious public health threat in the country (1,2,4). However, as the case incidence and spread intensifies, leishmaniasis is increasingly becoming a concern, especially among residents of Sri Lanka (7,11,24,31). Furthermore, 2 major disease hotspots emerged during 2001–2003 and disease-affected areas expanded during 2011–2018,

reaching >3,000 cases nationwide in 2018, a drastic increase from preceding years. The actual picture might be worse because reports from health facilities reflect only a fraction of the true incidence (7,23,31,32). Many questions regarding leishmaniasis in Sri Lanka remain unanswered and no organized efforts are in place for its control at a national level, or even in disease hotspots.

The alarming case expansion in 2018 could reflect a buildup of asymptomatic or early-stage symptomatic infections in the preceding years, but no field data are available to support this hypothesis. The infection-reservoir pool also might have grown because of poor treatment response, a growing problem in local healthcare settings (13). The 2001 increase in cases began in an army camp adjoining a jungle in the North-Central Province during a civil war (7). The initial cases could be attributed to known risk factors, such as nonimmune hosts entering the vector’s habitat (33,34). Although it was suspected at the outset, no zoonotic reservoir has been proven to be the cause

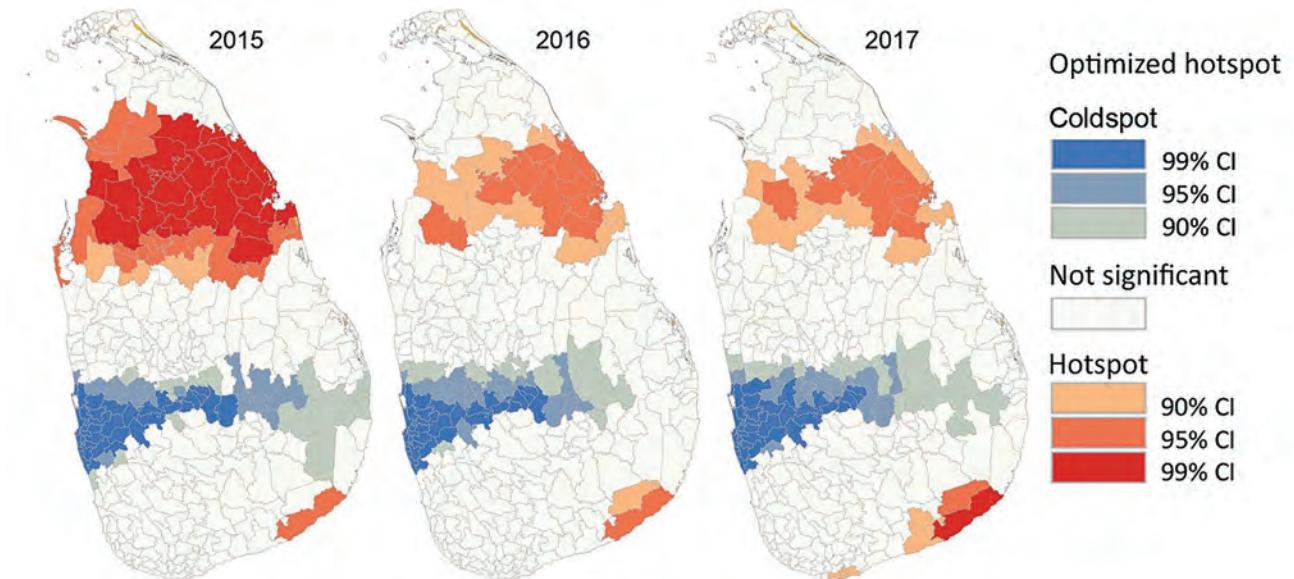


Figure 5. Optimized hotspots and coldspots of leishmaniasis in districts of Sri Lanka during 2015–2017. Hotspots and coldspots were calculated by using the Optimized Hot Spot Analysis tool of ArcGIS (Esri, <https://www.arcgis.com>); hotspots had large positive z-scores and coldspots had negative z-scores.

of the upsurge in cases, but leishmaniasis later was detected in dogs in Sri Lanka (35,36). Increased population mobility in the country after the civil war ended in 2009, along with enhanced infrastructure developments and easy road access, could have facilitated the spread of leishmaniasis. Activities raising CL awareness among the population also might have increased self-referrals and improved case diagnoses, thus contributing to progressive increases in case numbers. However, none of these factors, taken singly or in combination, can explain the case upsurge observed in 2018, highlighting the need for in-depth longitudinal studies.

Genetic analysis through microsatellite typing and whole-genome sequencing suggests prolonged existence of *Leishmania* spp. in the country and refutes theories of recent parasite introduction (15,37). The endemic *Leishmania* parasitic population could have been expanding gradually and spreading within foci, forming focal clusters (22). We hypothesize that during this phase of parasitic population growth, asymptomatic disease reservoirs were created and later expanded, contributing to the sudden increase of case numbers in 2018. However, carefully designed cross-sectional field studies are required to confirm this hypothesis.

Malaria cases have declined in Sri Lanka since 2000, and the last indigenous case was reported in 2012 (38). Subsequent restrictions on insecticide use for vector control could have played a role in the increased incidence of leishmaniasis in the intervening years. Leishmaniasis control is a widely recognized by-product of concerted malaria control in the region (39). Reduced vector control for malaria could explain the contrasting pictures, almost mirror images, of declining incidence rates for malaria and increasing rates of leishmaniasis in Sri Lanka during the past 2 decades.

Reporting on patients with leishmaniasis has improved over the years, as has the level of disease awareness among clinicians and healthcare personnel. These factors could have contributed to the increased case documentation, but underdiagnosis remains a concern (11,23,32). Local travel and vector dispersal also are factors that cannot be ruled out and might have contributed to the 2018 case surge, since these expansions were adjacent to the previous areas of disease (23,40,41).

Although leishmaniasis affects both sexes in all age groups, previous studies consistently indicated a male predominance among cases in groups 20–40 years of age (7,12,23,31,32). Our study showed differences in the sex and age distribution between the northern and southern disease foci. In southern

regions, the incidence data deviated from the census data with reduced numbers of disease in patients <14 years of age, and more so in male patients. Other age groups were equally affected by the disease, even in the early period, except this age group, raising concerns of underreporting and undiagnosed cases among children, especially boys, and creating a potential for them to become disease reservoirs. The predominance of young men (20–40 years) infected during the early period easily could be explained by the disease foci located in a military camp in the North-Central Province (7,10). However, for 2015–2018, persons >50 years of age in both sexes had much higher incidence rates compared with other age groups, out of proportion with trends seen in census data. One or more factors, such as changes in the level of infection awareness, behavioral differences, environmental

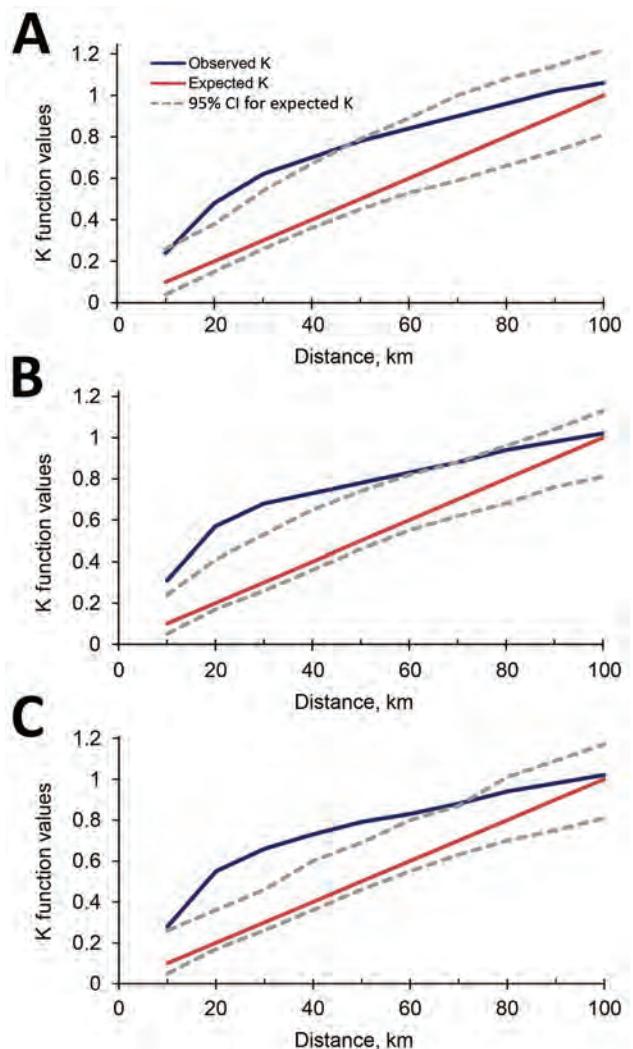


Figure 6. Observed and expected cluster sizes (K values) for incidence of leishmaniasis in Sri Lanka, 2015 (A), 2016 (B), and 2017 (C).

SYNOPSIS

Table 2. Characteristics of leishmaniasis cases in selected highly disease-endemic areas of 2 provinces, Sri Lanka*

Characteristics	North-Central Province		Southern Province	
	2001–2003	2015–2018	2001–2003	2015–2018
Age, y				
0–14	6 (4.6)	70 (11.3)	17 (24.6)	256 (16.4)
15–49	113 (86.3)	332 (53.7)	24 (34.8)	854 (54.7)
≥50	12 (9.2)	216 (35.0)	28 (40.6)	451 (28.9)
Sex				
F	7 (5.3)	198 (32.0)	28 (40.6)	636 (40.7)
M	124 (94.7)	420 (68.0)	41 (59.4)	925 (59.3)
Total	131	618	69	1,561

*Values represent no. (%) in each age and sex category during early and late periods of disease expansion. Data provided by medical health officers in each region.

factors, vector-related factors, and peridomestic transmission patterns, might have contributed to such findings and warrant further investigation (10,22,42). Enhanced surveillance is needed to ensure coverage of all age groups, including children.

We noted a clear expansion of spatial distribution in reported CL cases. Because *L. donovani* also is the causative agent for VL, the expansion of CL in Sri Lanka could be a potential threat to the regional VL elimination efforts (2). The VL elimination target is <10 cases/100,000 population/year, an incidence rate at which the disease is no longer considered a public health concern (2). If similar standards had been applied to *L. donovani*-induced CL in Sri Lanka, leishmaniasis would not have been public health concern in 2001 and might have been considered a minor concern until 2009, when only 1 district had >10 cases/100,000 population. However, under this target, leishmaniasis in Sri Lanka should be considered a major public health threat, especially considering our calculations show that more than one third of the country’s population is at risk for this infection.

We did not see the clear-cut pattern of seasonality described in previous studies, which demonstrated

cases increased in a district in 2 biannual peaks (43). However, peak case numbers in the north during July–September might be related to seasonal vector abundance, which needs confirmation. The hotspots detected in the north and the south are likely caused by the expansion of local disease transmission. Although the epicenters of disease shifted over time, they remained in the same broader areas where they started in 2001. The central highlands appear to act as a barrier for disease spread, probably because environmental factors do not favor the survival of the vector sand flies.

Transmission of leishmaniasis in Sri Lanka is likely to progress, unless active interventions for disease containment are put in place. In the absence of proof for the presence of nonhuman reservoirs, infection control measures should focus on early diagnosis and effective treatment for patients; vector control involving chemical and environmental methods; and reducing human–vector contact by educating the population on steps they can take to reduce their risk for infection, such as applying insect repellents and using insecticide-impregnated bed nets. However, specific vector control measures essentially require

Table 3. Differences in sex and age distribution in cases of leishmaniasis against census data for regions of Sri Lanka, 2015–2018*

Characteristics by province	F, no. (%)	M, no. (%)	Total	χ^2	df	p value
North-Central	198 (32.0)	420 (68.0)	618	159.50	1	<0.001
Southern	636 (40.7)	925 (59.3)	1,561	107.01	1	<0.001
North-Central versus Southern				14.20	1	0.002
				Standard residuals†		
				F	M	
Age ranges by province, y						
North-Central						
0–14	39 (19.7)	31 (7.4)		–1.29	–3.85	20.38
15–49	95 (48.0)	237 (56.4)		–0.75	0.56	
≥50	64 (32.3)	152 (36.2)		2.70	3.62	
Southern						
0–14	49 (25.5)	37 (13.9)		–0.96	–2.27	37.76
15–49	76 (39.6)	162 (60.7)		–0.49	1.60	
≥50	67 (34.9)	68 (25.5)		1.55	0.07	
North-Central versus Southern						
F by age group				0.69	2	0.7065
M by age group				27.74	2	<0.001

*Leishmaniasis case count data provided by regional medical health officers. Where not specified, comparisons were made between the number of female and male cases in different age groups within the same region. df, degrees of freedom.

†Age distributions by sex standardized against census data from the same regions. Numbers in bold indicate statistically significant difference from census (p<0.05).

more studies on vector behavior and insecticide susceptibility to inform evidence-based policy decisions.

Carefully designed longitudinal studies are needed in the community to clarify the epidemiology and transmission dynamics of the disease. Intensive awareness programs should be implemented for clinicians and healthcare workers to ensure effective patient management, and for the general public to improve treatment-seeking behavior, backed up by qualitative studies to enhance early case detection. Better accessibility and the use of more cost-effective treatment options, such as radiofrequency heat therapy (44), could improve patient compliance and reduce infection reservoirs. Furthermore, use of modern technological tools, such as satellite remote sensing, could aid in epidemiologic surveillance, identification of probable sandfly-infested areas, and prediction of disease hotspots. In addition, planning and implementation of effective interventions would improve containment efforts for leishmaniasis in Sri Lanka.

Acknowledgments

We are grateful to the head and staff of the Department of Parasitology, Faculty of Medicine, University of Colombo, and director general and staff of the Ministry of Health, especially the medical health officers, other health officials, and epidemiologists for assistance in this study. We thank Raushan Siraj, Ruksala Ranatunga, Dasun M. Dissanayake, Sachee B. Piyasiri, and Nishanthan Chandrasekera for assistance with data entry and Richard Carter for critical comments and suggestions to our manuscript.

Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award no. U01AI136033 to N.D.K. and G.Z. The authors are solely responsible for the content and it does not necessarily represent the official views of the National Institutes of Health.

This study was performed in the Faculty of Medicine department of the University of Colombo, Sri Lanka.

About the Author

Dr. Karunaweera is a senior professor and the chair of parasitology at the University of Colombo, Sri Lanka. Her research interests include biology, pathogenesis, and epidemiology of human parasites, especially *Plasmodium* and *Leishmania* spp.

References

1. World Health Organization. Leishmaniasis. 2019 [cited 2019 Feb 20]. <https://www.who.int/en/news-room/fact-sheets/detail/leishmaniasis>
2. Burza S, Croft SL, Boelaert M. Leishmaniasis. *Lancet*. 2018; 392:951–70. [https://doi.org/10.1016/S0140-6736\(18\)31204-2](https://doi.org/10.1016/S0140-6736(18)31204-2)
3. Karunaweera ND. Leishmaniasis: path toward elimination from the Indian subcontinent. *Trop Parasitol*. 2016;6:2–4. <https://doi.org/10.4103/2229-5070.175023>
4. Rijal S, Sundar S, Mondal D, Das P, Alvar J, Boelaert M. Eliminating visceral leishmaniasis in South Asia: the road ahead. *BMJ*. 2019;364:k5224. <https://doi.org/10.1136/bmj.k5224>
5. Bi K, Chen Y, Zhao S, Kuang Y, John Wu CH. Current visceral leishmaniasis research: a research review to inspire future study. *BioMed Res Int*. 2018:e9872095. <https://doi.org/10.1155/2018/9872095>
6. Athukorale DN, Seneviratne JK, Ihalamulla RL, Premaratne UN. Locally acquired cutaneous leishmaniasis in Sri Lanka. *J Trop Med Hyg*. 1992;95:432–3.
7. Karunaweera ND. *Leishmania donovani* causing cutaneous leishmaniasis in Sri Lanka: a wolf in sheep's clothing? *Trends Parasitol*. 2009;25:458–63. <https://doi.org/10.1016/j.pt.2009.07.002>
8. Abeygunasekara PH, Costa YJ, Seneviratne N, Ratnatunga N, Wijesundera MS. Locally acquired visceral leishmaniasis in Sri Lanka. *Ceylon Med J*. 2007;52:30–1. <https://doi.org/10.4038/cmj.v52i1.1047>
9. Karunaweera ND, Pratloug F, Siriwardane HV, Ihalamulla RL, Dedet JP. Sri Lankan cutaneous leishmaniasis is caused by *Leishmania donovani* zymodeme MON-37. *Trans R Soc Trop Med Hyg*. 2003;97:380–1. [https://doi.org/10.1016/S0035-9203\(03\)90061-7](https://doi.org/10.1016/S0035-9203(03)90061-7)
10. Siriwardana HVYD, Thalagala N, Karunaweera ND. Clinical and epidemiological studies on the cutaneous leishmaniasis caused by *Leishmania (Leishmania) donovani* in Sri Lanka. *Ann Trop Med Parasitol*. 2010;104:213–23. <https://doi.org/10.1179/136485910X12647085215615>
11. Wijerathna T, Gunathilaka N, Gunawardana K, Rodrigo W. Potential challenges of controlling leishmaniasis in Sri Lanka at a disease outbreak. *BioMed Res Int*. 2017;2017:e6931497. <https://doi.org/10.1155/2017/6931497>
12. Siriwardana Y, Zhou G, Deepachandi B, Akarawita J, Wickremarathne C, Warnasuriya W, et al. Trends in recently emerged *Leishmania donovani* induced cutaneous leishmaniasis, Sri Lanka, for the first 13 years. *BioMed Res Int*. 2019;2019:e4093603. <https://doi.org/10.1155/2019/4093603>
13. Refai FW, Madarasingha NP, Fernandopulle R, Karunaweera N. Nonresponsiveness to standard treatment in cutaneous leishmaniasis: a case series from Sri Lanka. *Trop Parasitol*. 2016;6:155–8. <https://doi.org/10.4103/2229-5070.190835>
14. Rajapaksa US, Ihalamulla RL, Karunaweera ND. First report of mucosal tissue localisation of leishmaniasis in Sri Lanka. *Ceylon Med J*. 2005;50:90–1.
15. Samarasinghe SR, Samaranyake N, Kariyawasam UL, Siriwardana YD, Imamura H, Karunaweera ND. Genomic insights into virulence mechanisms of *Leishmania donovani*: evidence from an atypical strain. *BMC Genomics*. 2018;19:843. <https://doi.org/10.1186/s12864-018-5271-z>
16. Kariyawasam UL, Selvapandiyan A, Rai K, Wani TH, Ahuja K, Beg MA, et al. Genetic diversity of *Leishmania donovani* that causes cutaneous leishmaniasis in Sri Lanka: a cross sectional study with regional comparisons. *BMC Infect Dis*. 2017;17:791. <https://doi.org/10.1186/s12879-017-2883-x>
17. Kariyawasam KKGDUL, Selvapandiyan A, Siriwardana HVYD, Dube A, Karunanayake P, Senanayake SASC, et al. Dermotropic *Leishmania donovani* in Sri Lanka: visceralizing potential in clinical and preclinical studies. *Parasitology*. 2018;145:443–52. <https://doi.org/10.1017/S003118201700169X>

18. Senanayake SA, Abeyewicreme W, Dotson EM, Karunaweera ND. Characteristics of *Phlebotomine* sandflies in selected areas of Sri Lanka. *Southeast Asian J Trop Med Public Health*. 2015;46:994-1004.
19. Gajapathy K, Peiris LBS, Goodacre SL, Silva A, Jude PJ, Surendran SN. Molecular identification of potential leishmaniasis vector species within the *Phlebotomus (Euphlebotomus) argentipes* species complex in Sri Lanka. *Parasit Vectors*. 2013;6:302. <https://doi.org/10.1186/1756-3305-6-302>
20. Ilango K. A taxonomic reassessment of the *Phlebotomus argentipes* species complex (Diptera: Psychodidae: Phlebotominae). *J Med Entomol*. 2010;47:1-15. <https://doi.org/10.1093/jmedent/47.1.1>
21. Ranasinghe S, Wickremasinghe R, Munasinghe A, Hulangamuwa S, Sivanantharajah S, Seneviratne K, et al. Cross-sectional study to assess risk factors for leishmaniasis in an endemic region in Sri Lanka. *Am J Trop Med Hyg*. 2013;89:742-9. <https://doi.org/10.4269/ajtmh.12-0640>
22. Kariyawasam KK, Edirisuriya CS, Senerath U, Hensmen D, Siriwardana HV, Karunaweera ND. Characterisation of cutaneous leishmaniasis in Matara district, southern Sri Lanka: evidence for case clustering. *Pathog Glob Health*. 2015;109:336-43. <https://doi.org/10.1179/2047773215Y.0000000032>
23. Siriwardana HVYD, Karunanayake P, Goonerathne L, Karunaweera ND. Emergence of visceral leishmaniasis in Sri Lanka: a newly established health threat. *Pathog Glob Health*. 2017;111:317-26. <https://doi.org/10.1080/20477724.2017.1361564>
24. Galgamuwa LS, Dharmaratne SD, Iddawela D. Leishmaniasis in Sri Lanka: spatial distribution and seasonal variations from 2009 to 2016. *Parasit Vectors* 2018;11:60. <http://dx.doi.org/10.1186/s13071-018-2647-5>
25. Ranawaka RR, Abeygunasekara PH, Weerakoon HS. Correlation of clinical, parasitological and histopathological diagnosis of cutaneous leishmaniasis in an endemic region in Sri Lanka. *Ceylon Med J*. 2012;57:149-52. <https://doi.org/10.4038/cmj.v57i4.5082>
26. Getis A, Ord JK. The analysis of spatial association by use of distance statistics. *Geogr Anal*. 1992;24:189-206. <https://doi.org/10.1111/j.1538-4632.1992.tb00261.x>
27. Ord JK, Getis A. Local spatial autocorrelation statistics: distributional issues and an application. *Geogr Anal*. 1995;27:286-306. <https://doi.org/10.1111/j.1538-4632.1995.tb00912.x>
28. Getis A. Interactive modeling using second-order analysis. *Environ Plann A*. 1984;16:173-83. <https://doi.org/10.1068/a160173>
29. Chapman LAC, Morgan ALK, Adams ER, Bern C, Medley GF, Hollingsworth TD. Age trends in asymptomatic and symptomatic *Leishmania donovani* infection in the Indian subcontinent: A review and analysis of data from diagnostic and epidemiological studies. *PLoS Negl Trop Dis*. 2018;12:e0006803. <https://doi.org/10.1371/journal.pntd.0006803>
30. Naotunne TD, Rajakulendran S, Abeywickreme W, Kulasiri CD, Perera J, Premaratne UN, et al. Cutaneous leishmaniasis in Sri Lanka. An imported disease linked to the Middle East and African employment boom. *Trop Geogr Med*. 1990;42:72-4.
31. Nawaratna SS, Weilgama DJ, Wijekoon CJ, Dissanayake M, Rajapaksha K. Cutaneous leishmaniasis, Sri Lanka. *Emerg Infect Dis*. 2007;13:1068-70. <https://doi.org/10.3201/eid1307.060773>
32. Sandanayaka R, Kahawita I, Gamage A, Siribaddana S, Agampodi S. Emergence of cutaneous leishmaniasis in Polonnaruwa, Sri Lanka 2008-2011. *Trop Med Int Health*. 2014;19:140-5. <https://doi.org/10.1111/tmi.12232>
33. El-Safi SH, Peters W. Studies on the leishmaniasis in the Sudan. 1. Epidemic of cutaneous leishmaniasis in Khartoum. *Trans R Soc Trop Med Hyg*. 1991;85:44-7. [https://doi.org/10.1016/0035-9203\(91\)90151-N](https://doi.org/10.1016/0035-9203(91)90151-N)
34. Kweku MA, Odoom S, Puplampu N, Desewu K, Nuako GK, Gyan B, et al. An outbreak of suspected cutaneous leishmaniasis in Ghana: lessons learnt and preparation for future outbreaks. *Glob Health Action*. 2011;4:5527. <https://doi.org/10.3402/gha.v4i0.5527>
35. Rosypal AC, Tripp S, Kinlaw C, Hailemariam S, Tidwell RR, Lindsay DS, et al. Surveillance for antibodies to *Leishmania* spp. in dogs from Sri Lanka. *J Parasitol*. 2010;96:230-1. <https://doi.org/10.1645/GE-2288>
36. Nawaratna SSK, Weilgama DJ, Rajapaksha K. Cutaneous leishmaniasis in Sri Lanka: a study of possible animal reservoirs. *Int J Infect Dis*. 2009;13:513-7. <https://doi.org/10.1016/j.ijid.2008.08.023>
37. Siriwardana HV, Noyes HA, Beeching NJ, Chance ML, Karunaweera ND, Bates PA. *Leishmania donovani* and cutaneous leishmaniasis, Sri Lanka. *Emerg Infect Dis*. 2007;13:476-8. <https://doi.org/10.3201/eid1303.060242>
38. Karunaweera ND, Galappaththy GN, Wirth DF. On the road to eliminate malaria in Sri Lanka: lessons from history, challenges, gaps in knowledge and research needs. *Malar J*. 2014;13:59. <https://doi.org/10.1186/1475-2875-13-59>
39. Kishore K, Kumar V, Kesari S, Dinesh DS, Kumar AJ, Das P, et al. Vector control in leishmaniasis. *Indian J Med Res*. 2006;123:467-72.
40. Morrison AC, Ferro C, Morales A, Tesh RB, Wilson ML. Dispersal of the sand fly *Lutzomyia longipalpis* (Diptera: Psychodidae) at an endemic focus of visceral leishmaniasis in Colombia. *J Med Entomol*. 1993;30:427-35. <https://doi.org/10.1093/jmedent/30.2.427>
41. Orshan L, Elbaz S, Ben-Ari Y, Akad F, Afik O, Ben-Avi I, et al. Distribution and dispersal of *Phlebotomus papatasi* (Diptera: Psychodidae) in a zoonotic cutaneous leishmaniasis focus, the Northern Negev, Israel. *PLoS Negl Trop Dis*. 2016;10:e0004819. <https://doi.org/10.1371/journal.pntd.0004819>
42. Nandha B, Srinivasan R, Jambulingam P. Cutaneous leishmaniasis: knowledge, attitude and practices of the inhabitants of the Kani forest tribal settlements of Tiruvananthapuram district, Kerala, India. *Health Educ Res*. 2014;29:1049-57. <https://doi.org/10.1093/her/cyu064>
43. Rajapaksa US, Ihalamulla RL, Udagedera C, Karunaweera ND. Cutaneous leishmaniasis in southern Sri Lanka. *Trans R Soc Trop Med Hyg*. 2007;101:799-803. <https://doi.org/10.1016/j.trstmh.2006.05.013>
44. Refai WF, Madarasingha NP, Sumanasena B, Weerasingha S, De Silva A, Fernando R, et al. Efficacy, Safety and cost-effectiveness of thermotherapy in the treatment of *Leishmania donovani*-induced cutaneous leishmaniasis: a randomized controlled clinical trial. *Am J Trop Med Hyg*. 2017;97:1120-6. <https://doi.org/10.4269/ajtmh.16-0879>

Address for correspondence: Nadira D. Karunaweera, University of Colombo, Department of Parasitology, Faculty of Medicine, 25 Kynsey Rd, Colombo 0008, Sri Lanka; email: nadira@parasit.cmb.ac.lk

Candidatus Mycoplasma haemohominis in Human, Japan

Norimichi Hattori,¹ Makoto Kuroda,¹ Harutaka Katano,¹ Takahiro Takuma,¹ Takayoshi Ito,¹ Nana Arai, Ryo Yanai, Tsuyoshi Sekizuka, Sho Ishii, Yoko Miura, Takahiro Tokunaga, Hiroyuki Watanabe, Norihiro Nomura, Junichi Eguchi, Hideki Hasegawa, Tsuyoshi Nakamaki, Takaji Wakita, Yoshihito Niki

Hemotropic mycoplasmas are common pathogens in animals, but it remains unclear what role these pathogens play in human infections. We report clinical and biologic characterization of *Candidatus* Mycoplasma haemohominis infection in a 42-year-old man in Japan. The patient had severe hemophagocytic syndrome 1 month after an accidental needlestick injury. Metagenomic deep sequencing identified *Candidatus* M. haemohominis and determined its draft genome for an isolate from serum of the patient. A high copy number of the *Candidatus* M. haemohominis genome was detected in serum and bone marrow samples. Electron microscopy examination showed morphologic characteristics of *Candidatus* M. haemohominis. Levofloxacin monotherapy induced resistance caused by a gyrase A gene mutation in the quinolone resistance-determining region, but a combination treatment with moxifloxacin and minocycline was effective. We identified *Candidatus* M. haemohominis in a patient who had life-threatening symptoms related to multiple organ infection. Human infection with this mycoplasma might occur more frequently than has been generally recognized.

Hemotropic mycoplasmas (or hemoplasmas; size <math><1\ \mu\text{m}</math>) are unculturable, cell wall-deficient, gram-negative bacteria that parasitize on the surface of the erythrocytes of numerous domestic and wild animals, such as cats, dogs, rodents, swine, cattle, sheep, bears, and bats (1–4). These pathogens can cause pyrexia, hemolytic anemia, and icterus (1,5). There have been few studies on molecular characterization of hemoplasmas to confirm infections in

humans (6–11), possibly because hemoplasmas are unculturable and are liable to be overlooked by physicians (11,12). Steer et al. found that the putative species *Mycoplasma* species *Candidatus* Mycoplasma haemohominis can infect humans and cause hemolytic anemia and pyrexia (9). However, only a partial sequence of *Candidatus* M. haemohominis (GenBank accession no. GU562825) could be confirmed (9). Moreover, clinical manifestations of *Candidatus* M. haemohominis infections in humans have not been well characterized.

We identified and characterized *Candidatus* M. haemohominis infections in a patient with pyrexia of unknown origin. The patient had various life-threatening symptoms that were not limited to hemolytic anemia and was infected with this bacterium after an accidental needlestick injury. We also analyzed the genome of *Candidatus* M. haemohominis isolated from specimens obtained from the patient. This study was approved by the Institutional Review Board of Showa University (Tokyo, Japan) and the National Institute of Infectious Diseases (Tokyo). We obtained informed consent from the patient.

Case-Patient

The case-patient was a 42-year-old man (physician) who had no unusual medical history and no recent overseas travel history. The patient was admitted to Showa University School of Medicine Hospital (Tokyo, Japan) because of pyrexia, anemia, and liver dysfunction. One month before admission, he had accidentally pricked his finger when performing needle biopsy of the liver for 1 inpatient, who was admitted to this hospital because of cryptogenic liver injury and anemia after traveling overseas. Two weeks after the needlestick injury, the case-patient had pyrexia and whole-body erythema with pruritus (Figure 1). His rash disappeared after 3 days. However, he was

Author affiliations: Showa University School of Medicine, Tokyo, Japan (N. Hattori, T. Takuma, N. Arai, R. Yanai, S. Ishii, Y. Miura, T. Tokunaga, T. Nakamaki, Y. Niki); National Institute of Infectious Diseases, Tokyo (M. Kuroda, H. Katano, T. Sekizuka, H. Hasegawa, T. Wakita); Showa University Koto Toyosu Hospital, Tokyo (T. Ito, H. Watanabe, N. Nomura, J. Eguchi)

DOI: <https://doi.org/10.3201/eid2601.190983>

¹These authors contributed equally to this article.

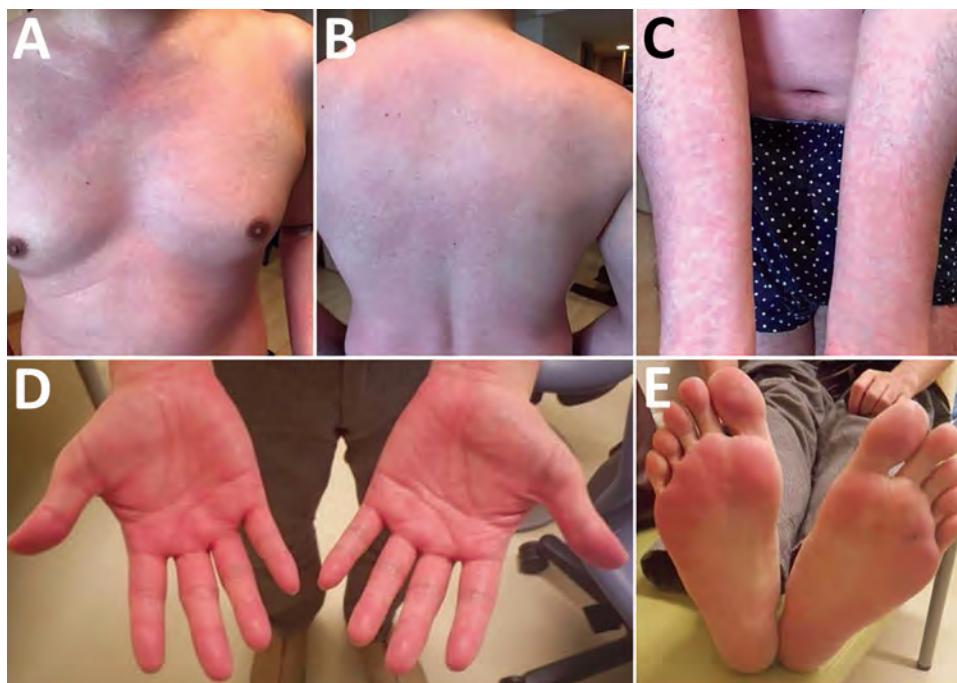


Figure 1. Whole-body erythema and pruritus in a 42-year-old man infected with *Candidatus Mycoplasma haemohominis*, Japan. Images show general erythema and pruritus covering >80% of the body surface area. A) Chest, B) back, C) arms, D) hands, E) feet.

admitted to the hospital because lymphadenopathy, hepatosplenomegaly, and pyrexia developed.

We provide the clinical course for the case-patient (Figure 2). At admission, the case-patient had relative bradycardia (96 beats/min) and a body temperature of 39.5°C. Laboratory results showed an increase in levels of aspartate aminotransferase (274 U/L, reference range 10–40 U/L), lactate dehydrogenase (664 U/L, reference range 120–245 U/L), ferritin (8,748 ng/mL, reference range 20–400 ng/mL), soluble interleukin-2 receptor (8,791 U/mL, reference range 122–496 U/mL), and C-reactive protein (8.45 mg/dL, reference range 0.00–0.20 mg/dL). A complete blood count showed anemia (hemoglobin concentration 11.9 g/dL, reference range 13.6–18.3 g/L), but the leukocyte count (4.8×10^3 cells/ μ L, reference range 3.5 – 9.0×10^3 cells/ μ L) and platelet count (16.5×10^4 / μ L, reference range 14.0 – 37.9×10^4 cells/ μ L) were within reference intervals. A Coombs test result was negative, but low haptoglobin concentrations (<8 mg/dL, range 30–200 mg/dL) were found.

Test results were negative for hepatitis A, B, C, and E viruses; measles virus; rubella virus; parvovirus; and HIV. Results of antinuclear antibody (titer 1:80) and smooth muscle antibody (titer 1:40) tests were positive, but test results for antimitochondrial M2 antibody, mitochondrial antibody, double-stranded DNA antibody, and lupus anticoagulant were negative. Epstein-Barr virus (EBV) DNA load determined by PCR was 3.0×10^2 copies/mL. However, Southern blot hybridization did not detect clonality of EBV-infected cells. Levels of herpes simplex virus, human herpesvirus

6 and 8, varicella zoster virus, and cytomegalovirus were below reference values, as determined by PCR. The serum IgG level was 3,967 mg/dL (reference range 800–1,750 mg/dL). However, the result of a serum-free light chain test was within the reference limit.

Our initial diagnosis was hemophagocytic syndrome (HPS) related to undetermined disease, and the patient was given steroid pulse therapy (1,000 mg) on day 1. Because his symptoms persisted, he was given etoposide (100 mg/m²), cyclosporine (5 mg/kg), and dexamethasone (20 mg) on day 7. After this treatment was started, whole-genome sequencing of a peripheral blood sample detected a novel hemotropic *Mycoplasma* sp.

On day 11, we found a prolonged activated partial thromboplastin time (50.6 s, reference range 25–45 s), normal prothrombin time (12.6 s), low coagulation factor VIII activity (1.8%, reference range 78%–165%), and low von Willebrand factor (VWF) activity (ristocetin cofactor <10%, reference range 50%–150%). Moreover, serum levels of fibrinogen, antithrombin III, and disintegrin and metalloproteinase with thrombospondin type 1 motif, member 13 were within reference ranges. Microscopic examination of a Giemsa-stained blood smear showed coccoid forms on the erythrocyte surface (Figure 3, panel A). A bone marrow aspirate showed hemophagocytosis and increased levels of reactive plasma cells (19.5%) (Figure 3, panels B, C). On day 12, hypoglycemia (glucose level <5 mg/dL, reference range, 61–139 mg/dL) was noted in blood from the collection tube used for

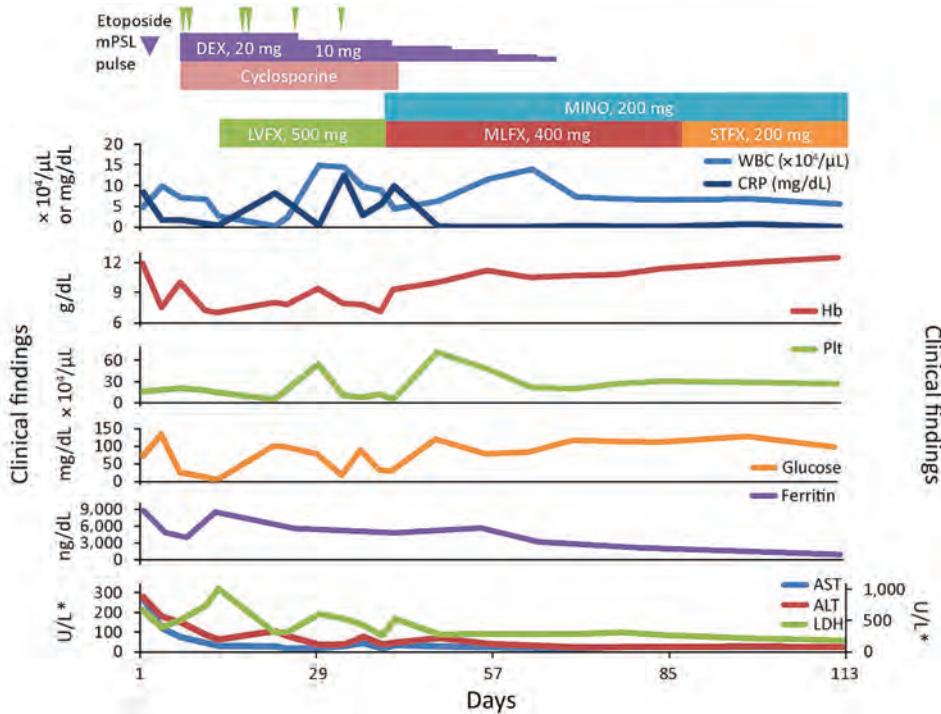


Figure 2. Clinical course for a 42-year-old man infected with *Candidatus Mycoplasma haemohominis*, Japan. *For ALT, AST, and LDH, left y-axis is for AST and ALT and right y-axis is for LDH. ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; DEX, dexamethasone; Hb, hemoglobin; LDH, lactate dehydrogenase; LVFX, levofloxacin; MINO, minocycline; MLFX, moxifloxacin; mPSL, methylprednisolone; PLT, platelets; STFX, sitafloxacin; WBC, white blood cells.

serologic tests, but the glucose level was within the reference range for blood in a container that contained citric acid with NaF.

On day 13, the case-patient received a diagnosis of *Candidatus M. haemohominis* infection-associated HPS and was given levofloxacin. Hypoglycemia improved after administration of levofloxacin. However, pyrexia and anemia developed, and coccoid forms on erythrocytes in peripheral blood and bone marrow were again observed on day 37. On day 46, he was given moxifloxacin and minocycline because of suspected bacterial resistance to levofloxacin. After this treatment was initiated, his symptoms, such as pyrexia, anemia, and hypoglycemia, promptly resolved, and the patient was discharged on day 61. One year after discontinuation of treatment, the patient remained well, and no *Candidatus M. haemohominis* DNA was detected in his serum.

Materials and Methods

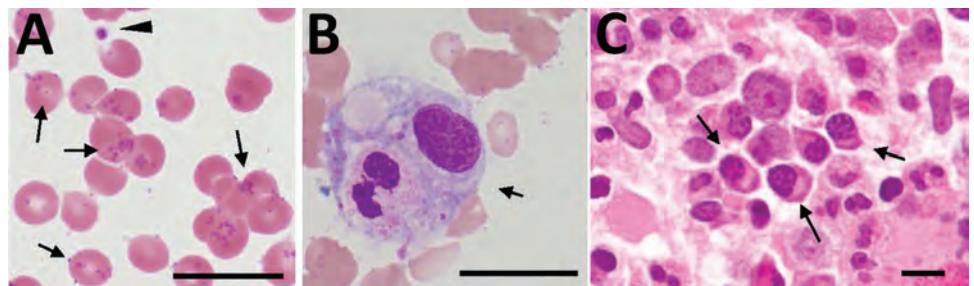
High-Throughput Unbiased RNA Sequencing

We purified total RNA from a patient serum sample by using the RNeasy Mini Kit (QIAGEN, <https://www.qiagen.com>). We prepared RNA-Seq libraries by using the ScriptSeq version 2 RNA-Seq Library Preparation Kit (Illumina, <https://www.illumina.com>) and sequenced these libraries as single-end 151-mers by using the NextSeq 500 sequencer (Illumina) (13,14).

Whole-Genome Analysis

We performed metagenomic short-read DNA sequencing by using NextSeq 500 (Illumina) with DNA extracted from serum specimens. We excluded human-related short reads by using a Burrows-Wheeler Mapping Program with default parameters against human genome sequences (GRCh38.p13) (15).

Figure 3. Distribution of *Candidatus Mycoplasma haemohominis* in a 42-year-old man, Japan. A) Peripheral blood smear showing coccoid forms. Small basophilic bodies are present on the surface of and outside erythrocytes (arrows). Arrowhead indicates a platelet. Giemsa stained. B) Hemophagocytosis (arrow) in bone marrow aspirate. Giemsa stained. C) Bone marrow biopsy specimen showing infiltration of plasma cells (arrows). Hematoxylin and eosin stained. Scale bars indicate 20 μm.



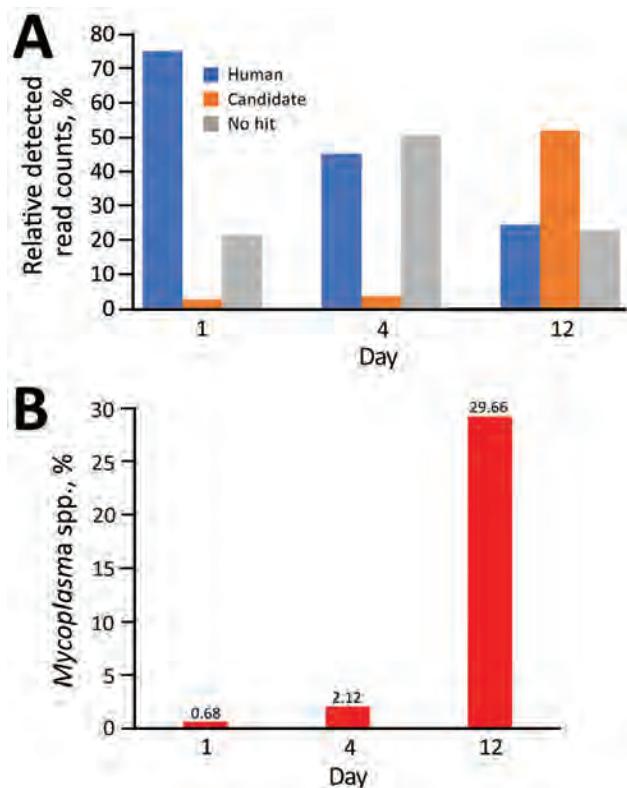


Figure 4. Prediction of presence of *Candidatus Mycoplasma haemohominis* in serum samples of a 42-year-old man, Japan. A) Relative percentage of candidate bacteria. B) Percentage of *Mycoplasma* spp. detected.

We obtained a draft genome sequence of *Candidatus M. haemohominis* by de novo assembly using metagenomic DNA-Seq short reads. First, we excluded human genomic DNA sequences ($\approx 58.3\%$) by using read-mapping analysis, followed by de novo assembly with the remaining short reads. The total number of contigs was 703, and total length was ≈ 1.42 Mb, suggesting that whole contigs included bacterial and human-related DNA sequences. Therefore, to extract the bacterial sequences, we considered read depth and coverage, % GC of each contig, and a blastn search (<https://blast.ncbi.nlm.nih.gov>). A total of 23 contigs showed a marked read depth coverage (average $\times 5,500$) and 30% GC content, although blastn search analysis showed that 4 contigs were assigned to the *Mycoplasma* genome and 19 contigs were assigned to unknown sequences.

Electron Microscopy

We used negative staining for serum samples. Small aliquots of serum samples were absorbed onto glow-discharged, 300-mesh, heavy-duty carbon-coated copper Cu grids (Veco Grids; Nisshin EM, <http://nisshin-em.co.jp>) for 2 min, and excess liquid was blotted

with Whatman filter paper (GE Healthcare, <https://www.gehealthcare.com>). We then washed grids twice with Milli-Q water (<http://emdmillipore.com>) and negatively stained them with 2% uranyl acetate. We observed specimens by using an H7700 transmission electron microscope (Hitachi, <https://www.hitachi.com>) at 80 kV and $\times 10,000$ magnification.

We fixed tissue samples with 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at room temperature, postfixed these samples in 1% osmium tetroxide, and embedded them in Epon resin. We stained ultrathin sections with uranyl acetate and lead citrate and observed them under a transmission electron microscope (HT7700; Hitachi) at 80 kV.

In Situ Hybridization

We used 16S and 23S rRNA genes for in situ hybridization analysis. We amplified a target fragment by using PCR with digoxigenin-11-dUTP and MHaemohominis1F (5'-AATTAACGCTGATGGCATGC-3') and MHaemohominis600R (5'-TCCTACCGTATTC-TAGACGGAC-3') primers. We purified a PCR amplicon by using a PCR Purification Kit (QIAGEN) and denatured it by heat shock before hybridization. We treated deparaffinized slides with 0.3% H_2O_2 /methanol for 30 min and 0.2 mol/L HCl for 20 min, then incubated slides with proteinase K (3–10 μ g/mL) for 30 min at 37°C. After prehybridization, we hybridized slides with 2 pmol/L of denatured probe per slide in hybridization buffer (20% formamide; 5 \times saline-sodium citrate [SSC], 5 \times Denhardt solution; 50 mmol/L HEPES buffer [pH 7.0], and 40 μ g/mL salmon sperm DNA) overnight at 42°C. We washed the slides twice with 2 \times SSC at 50°C for 15 min and twice with 0.2 \times SSC at 50°C for 15 min, added anti-digoxigenin monoclonal antibody (Sigma, <https://www.sigmaaldrich.com>), and incubated the slides for 45 min. We then amplified signals by using a Gen-Point Kit (Dako Agilent, <https://www.agilent.com>) and detected these signals by using 3,3'-diaminobenzidine as a chromogen.

Real-Time PCR

We used a DNA fragment of the 16S rRNA gene of *Candidatus M. haemohominis* in a Taqman real-time PCR (16). We then performed PCR amplification in 25- μ L reaction mixtures containing QuantiTect Probe PCR Master Mixture (QIAGEN), 0.4 μ mol/L of each primer, 0.2 μ mol/L of TaqMan probe, and 100 ng of isolated DNA. PCR conditions were 95°C for 15 min, followed by 45 cycles of 94°C for 15 s and 60°C for 1 min in a Mx3005P Apparatus (Dako Agilent).

Sequence Data

We deposited metagenomic short-read sequences for DNA-Seq in the DDBJ (BioProject PRJDB7871; BioSample SAMD00156464; DRR accession no. DRR164892). We deposited the draft, annotated genome sequence of *Candidatus M. haemohominis* SWG34-3 in the DNA Data Bank of Japan (accession nos. SAMD00156495 and BIMN01000001-23). The 16S rRNA sequence was deposited under accession no. MHSWG343_r0010. The predicted gyrase A gene

nucleotide sequence was deposited under accession no. MHSWG343_02220, and the coding sequence was deposited under accession no. GCE63237.1.

Results

Identification and Whole-Genome Sequencing of *Candidatus M. haemohominis*

Metagenomic deep RNA sequence analysis strongly suggested that rather than other pathogens or virus

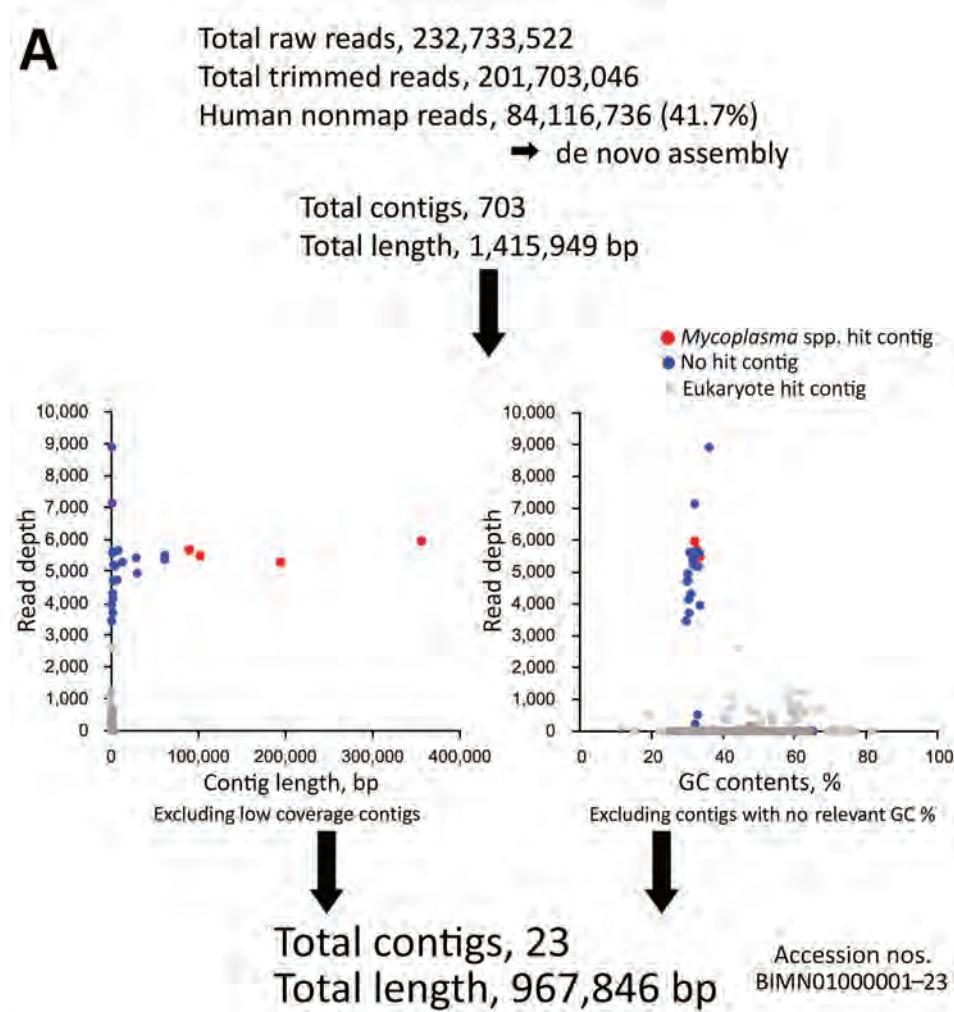
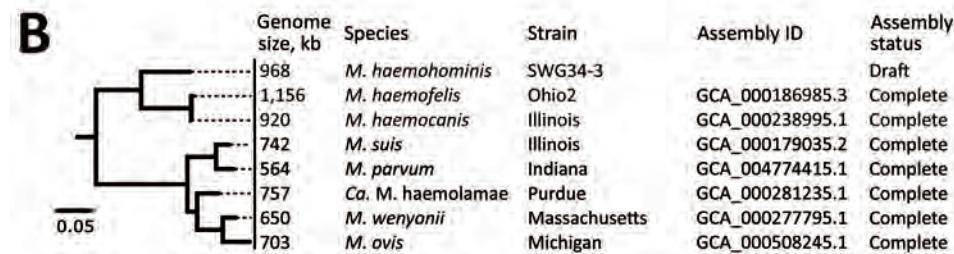


Figure 5. Analysis for *Candidatus Mycoplasma haemohominis* in serum of a 42-year-old man, Japan. A) Prediction that de novo assemblies contained bacteria and human DNA sequences. Bacteria-related sequences were identified by using read depth, % GC, and blastn (<https://blast.ncbi.nlm.nih.gov>) search results. Read depth indicates how many times next-generation sequencing confirmed the sequence at each nucleotide position. B) Phylogenetic tree of 16S rRNA genes of *Mycoplasma* spp. The tree was constructed by using FastTree version 2.1.10 (<http://www.microbesonline.org>). Scale bar indicates nucleotide substitutions per site. ID, identification.



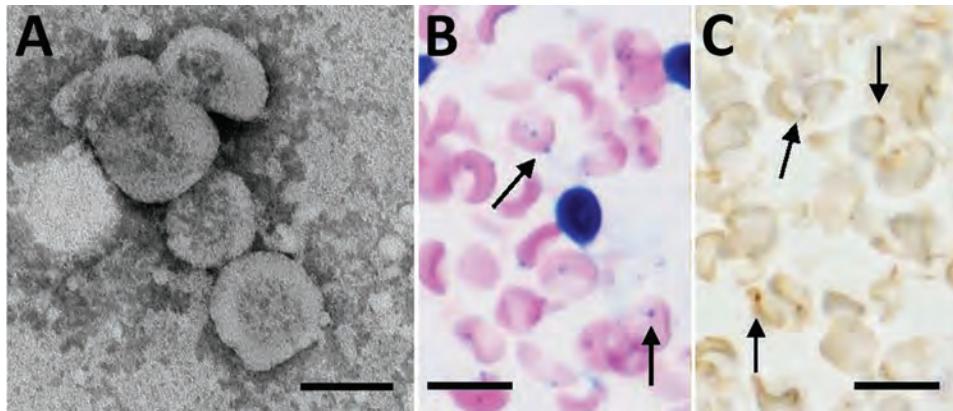


Figure 6. Morphologic features of *Candidatus Mycoplasma haemohominis* isolated from a serum sample of a 42-year-old man, Japan. A) Spheres are bacterial particles with a diameter of 300–600 nm. Negative stained; scale bar indicates 200 nm. B) Bacteria on the surface of erythrocytes (arrows) (Giemsa stained). C) In situ hybridization showing bacteria on the surface of macrophages (arrows). Scale bars indicate 10 μ m.

infections, the *Mycoplasma* spp. could be associated with signs and symptoms of the case-patient. RNA-Seq short reads related to the *Mycoplasma* spp. were increasingly detected in the serum of the case-patient (Figure 4). A total of 41.7% of human-unmapped reads were extracted and then subjected to de novo assembly to identify the mycoplasma draft genome (23 contigs, 967,846 bp) (Figure 5, panel A). The draft genome sequence identified a potential pathogen that could be *Candidatus M. haemohominis* on the basis of 16S rRNA sequence homology. Also, this pathogen showed similarity with closely related species, such as *M. haemofelis* and *M. haemocanis* (Figure 5, panel B).

Negative-staining and electron microscopy of *Candidatus M. haemohominis* particles identified ribosomes, DNA, and soluble RNA in the cytoplasm (Figure 6, panel A). In situ hybridization identified *Candidatus M. haemohominis* on the surface of erythrocytes (Figure 6, panel B) and in the cytoplasm of macrophages in bone marrow (Figure 6, panel C).

Monitoring and Treatment for *Candidatus M. haemohominis* Infection

We determined the level of *Candidatus M. haemohominis* DNA in serum by using real-time PCR

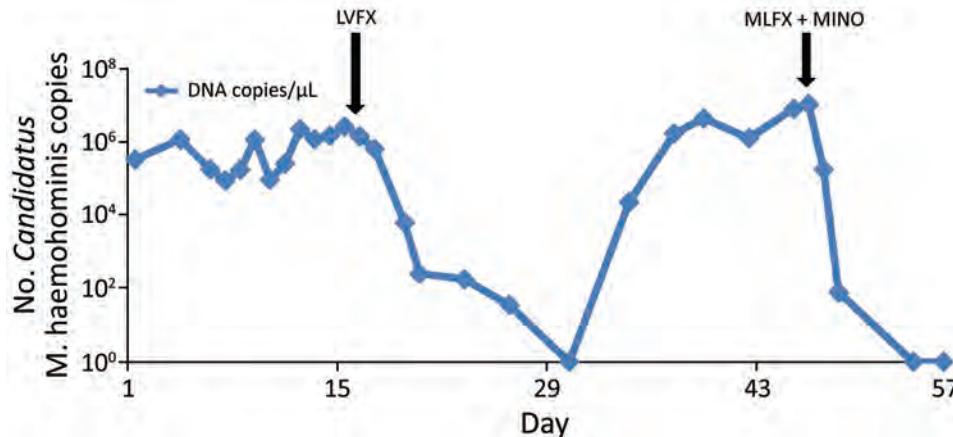


Figure 7. Copy number of the *Candidatus Mycoplasma haemohominis* genome in 1- μ L serum samples from a 42-year-old man, Japan. Copy number was determined by using a real-time PCR. LVFX, levofloxacin; MINO, minocycline; MLFX, moxifloxacin.

(Figure 7). A high copy number for *Candidatus M. haemohominis* DNA was detected 18 days after the accidental needlestick injury. The bacterial load in serum decreased below the detection limit (<10 copies/reaction) 14 days after the patient was given levofloxacin, but again increased. Metagenomic analysis also indicated a high detection rate for *Mycoplasma* spp. reads in serum before and after treatment with levofloxacin. Read-mapping analysis showed that only 2-nt mutations were identified in samples after treatment with levofloxacin. Moreover, 1 of 2 mutations was a nonsynonymous mutation in the quinolone resistance-determining regions (QRDR) of the DNA gyrase subunit A GyrA (i.e., Gly95Cys, corresponding to aa 81 by numbering for *Escherichia coli*) (Figure 8). The bacterial load promptly decreased after combination therapy with moxifloxacin and minomycin. We also identified *Candidatus M. haemohominis* by using real-time PCR and in situ hybridization with samples from a liver biopsy of the patient.

Discussion

Infection with *Candidatus M. haemohominis* needs to be distinguished from other hemoplasmas in

Sample	Total reads	Median read depth for <i>Candidatus M. haemohominis</i>	Region coverage, %	Nucleotide variations (Contig 1/173,202)	Treatment with levofloxacin
Day 9	139,962,665	5,454	99.95	G (GGT, Gly95*)	Before
Day 46	138,862,010	10,935	99.94	T (TGT, Cys95*)	After

* Corresponds to 81 aa in *Escherichia coli* numbering.

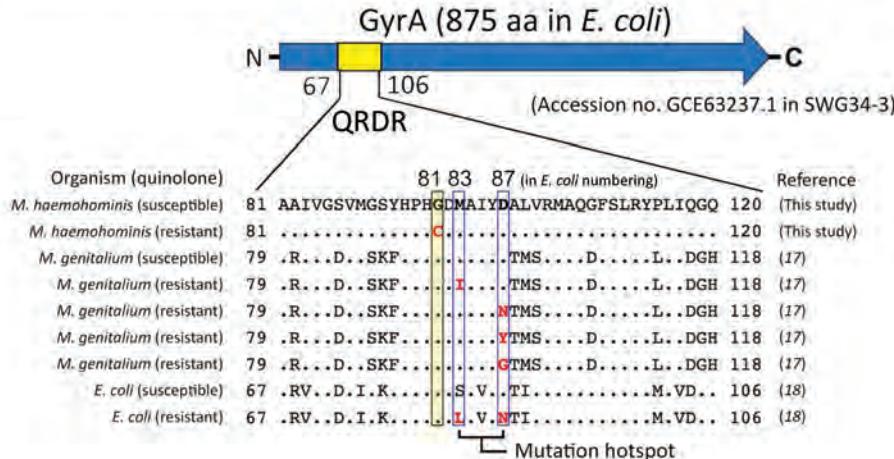


Figure 8. Detection of a *gyrA* mutation in the QRDR of *Candidatus Mycoplasma haemohominis* genome isolated from a 42-year-old man, Japan, who was given levofloxacin. Read-mapping analysis identified a nonsynonymous amino acid substitution in *GyrA* for quinolone-resistant *Candidatus M. haemohominis* from a serum sample. Bottom panel shows a schematic of the *GyrA* amino acid sequence and alignment with those of other QRDRs. Dots indicate identical amino acids corresponding to the *Candidatus M. haemohominis* sequence. Red indicates nucleotide or amino acid mutations. Arrow indicates direction of transcription. QRDR, quinolone resistance-determining region.

terms of clinical symptoms. As described previously, infection with possible hemoplasmas results in hemolytic anemia and pyrexia (6–11). Our results showed that infection with *Candidatus M. haemohominis* can cause various life-threatening symptoms in humans, such as HPS, liver damage, and bleeding.

We identified *Candidatus M. haemohominis* in the case-patient and clarified the clinical features of *Candidatus M. haemohominis* infection as follows. First, *Candidatus M. haemohominis* infection might cause HPS. Second, this infection might cause reactive plasmacytosis. Third, the increase in plasma cells might induce antibody production and hypergammaglobulinemia. Fourth, infected patients might have coccoid bacterial forms on erythrocyte surfaces and pseudohypoglycemia in vitro. Fifth, this infection might also be accompanied by mild to severe hemorrhagic episodes.

We suggest that our results will help in diagnoses of *Candidatus M. haemohominis* infections, enabling early therapeutic intervention that might cure patients with these infections. *Candidatus M. haemohominis* patients are highly susceptible to misdiagnosis with other diseases, such as collagen diseases (e.g., systemic lupus erythematosus) and virus infections (e.g., severe fever with thrombocytopenia syndrome and EBV-associated HPS), because of the wide range of complications.

Generally, antimicrobial drug therapy with fluoroquinolones or tetracyclines is effective against

hemoplasmas in animals. Combination therapy (9) or sequential treatment (19) with these drugs can be more effective against hemoplasmas because a single agent is often insufficient for consistent elimination of bacteremia (19). Moreover, detection of mutations in QRDR of gyrase A (Gly95Cys) suggested that *Candidatus M. haemohominis* is resistant to older fluoroquinolones (e.g., levofloxacin) (20,21). In contrast, newer fluoroquinolones, such as moxifloxacin or sitafloxacin, and tetracyclines, are effective against mycoplasmas harboring this mutation (21).

Laboratory tests for our case-patient showed low blood glucose levels (<5 mg/dL, reference range 61–139 mg/dL) but related no clinical signs. Hemoplasma-associated hypoglycemia in the absence of any associated clinical signs has been described in various animal species (22). This phenomenon is proportional to the severity of bacteremia and depends on the hemoplasma species (22). Because it is possible that glucose would be useful only for carbohydrate metabolism in the hemoplasma species (23), this finding might cause attachment in the epierythrocytic environment, which is rich in glucose, and result in alternative energy source pathways that become redundant.

We observed moderate to severe hemorrhagic episodes for this case-patient. Although the pathogenesis of bleeding in patients infected with *Candidatus M. haemohominis* is not completely understood, a dramatic decrease in coagulation factor

VIII and VWF activities was observed in this case-patient, which is similar to that observed in patients with acquired von Willebrand syndrome (24,25). Given the hypergammaglobulinemia and increased plasma cell levels observed in this case-patient and other patients, the possible mechanisms of infection are adsorption of vWF onto plasma cells or activated platelets apart from the presence of vWF-specific antibodies and inhibitors (24,25).

In conclusion, we identified and characterized *Candidatus M. haemohominis* infection in a human. Although our study has limitations, our results provide useful knowledge about this infection. Other patients infected with *Candidatus M. haemohominis* may have died before a confirmed diagnosis was made. Thus, *Candidatus M. haemohominis* infection might occur more frequently than generally recognized. Further epidemiologic investigations of infection with *Candidatus M. haemohominis* in humans and of intermediate host(s) for this pathogen would clarify the extent of human infection and possible transmission routes.

Acknowledgments

We thank the case-patient for participating in this study, the clinical staff who supported and cared for this patient, and Yuko Sato and Michiyo Kataoka for providing technical assistance with immunohistochemical analysis, in situ hybridization, and electron microscopy.

This study was supported by the Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development (grants JP19fk0108048, JP19fk0108103, and JP19fk0108104).

N.H., T.T. (Takahiro Takuma), T.I., M.K., T.S., H.K., H.H., T.N., T.W., and Y.N. designed the study and interpreted the data; M.K. performed metagenomic analysis; T.S. performed bioinformatics; H.K. and H.H. performed pathologic analysis; N.A., H.W., N.N., J.E., R.Y., S.I., Y.M., and T.T. (Takahiro Tokunaga) provided assistance in the design of the study and characterized patient samples; N.H., M.K., H.K., T.S., and H.H. wrote the manuscript; and T.N., T.W., and Y.N. critically reviewed the manuscript. All authors approved the final version of the manuscript submitted for publication.

About the Author

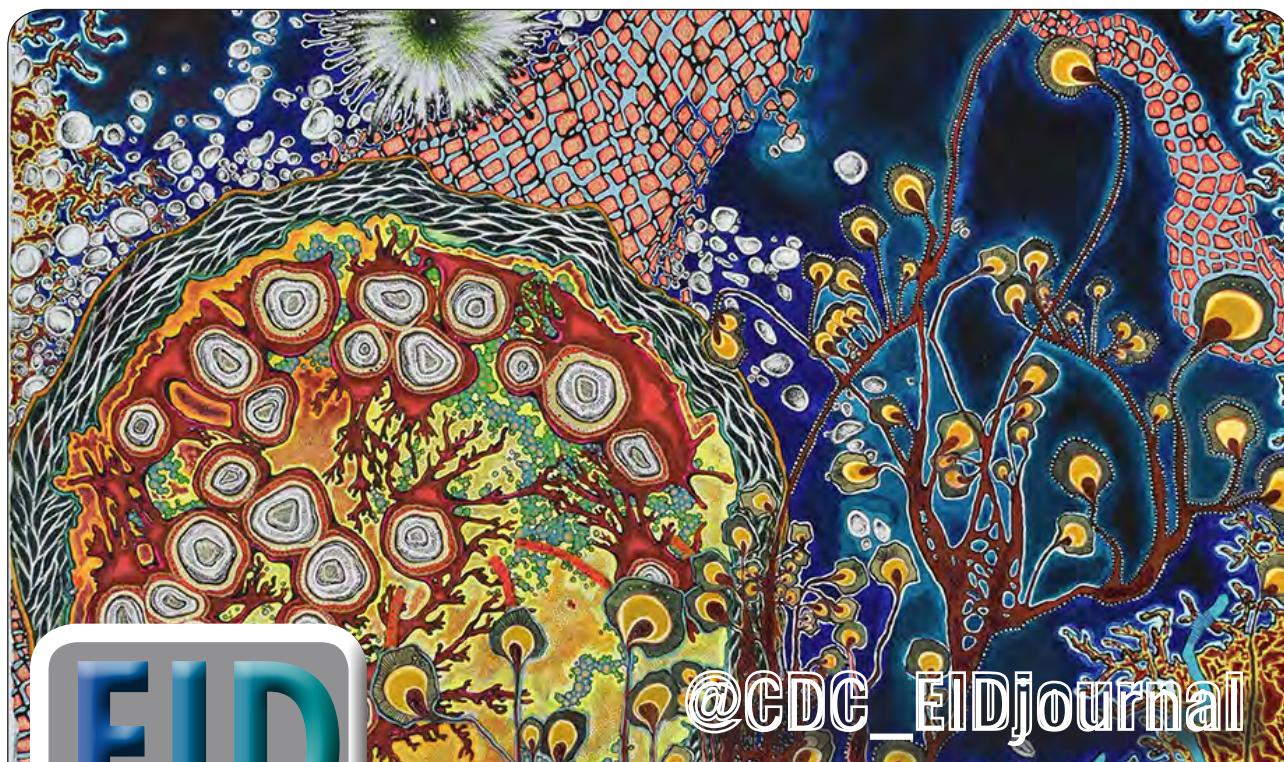
Dr. Hattori is a research scientist at Showa University School of Medicine, Tokyo, Japan. His primary research interest is hematology.

References

- Messick JB. Hemotropic mycoplasmas (hemoplasmas): a review and new insights into pathogenic potential. *Vet Clin Pathol.* 2004;33:2-13. <https://doi.org/10.1111/j.1939-165X.2004.tb00342.x>
- Harasawa R, Fujita H, Kadosaka T, Ando S, Rikihisa Y. Proposal for '*Candidatus Mycoplasma haemomuris* subsp. musculi' in mice, and '*Candidatus Mycoplasma haemomuris* subsp. rattii' in rats. *Int J Syst Evol Microbiol.* 2015;65:734-7. <https://doi.org/10.1099/ijs.0.069856-0>
- Iso T, Suzuki J, Sasaoka F, Sashida H, Watanabe Y, Fujihara M, et al. Hemotropic mycoplasma infection in wild black bears (*Ursus thibetanus japonicus*). *Vet Microbiol.* 2013;163:184-9. <https://doi.org/10.1016/j.vetmic.2012.12.016>
- Millán J, López-Roig M, Delicado V, Serra-Cobo J, Esperón F. Widespread infection with hemotropic mycoplasmas in bats in Spain, including a hemoplasma closely related to "*Candidatus Mycoplasma hemohominis*". *Comp Immunol Microbiol Infect Dis.* 2015;39:9-12. <https://doi.org/10.1016/j.cimid.2015.01.002>
- Sykes JE. Feline hemotropic mycoplasmas. *Vet Clin North Am Small Anim Pract.* 2010;40:1157-70. <https://doi.org/10.1016/j.cvsm.2010.07.003>
- dos Santos AP, dos Santos RP, Biondo AW, Dora JM, Goldani LZ, de Oliveira ST, et al. Hemoplasma infection in HIV-positive patient, Brazil. *Emerg Infect Dis.* 2008;14:1922-4. <https://doi.org/10.3201/eid1412.080964>
- Yuan CL, Liang AB, Yao CB, Yang ZB, Zhu JG, Cui L, et al. Prevalence of *Mycoplasma suis* (*Eperythrozoon suis*) infection in swine and swine-farm workers in Shanghai, China. *Am J Vet Res.* 2009;70:890-4. <https://doi.org/10.2460/ajvr.70.7.890>
- Sykes JE, Lindsay LL, Maggi RG, Breitschwerdt EB. Human coinfection with *Bartonella henselae* and two hemotropic mycoplasma variants resembling *Mycoplasma ovis*. *J Clin Microbiol.* 2010;48:3782-5. <https://doi.org/10.1128/JCM.01029-10>
- Steer JA, Tasker S, Barker EN, Jensen J, Mitchell J, Stocki T, et al. A novel hemotropic *Mycoplasma* (hemoplasma) in a patient with hemolytic anemia and pyrexia. *Clin Infect Dis.* 2011;53:e147-51. <https://doi.org/10.1093/cid/cir666>
- Maggi RG, Mascarelli PE, Havenga LN, Naidoo V, Breitschwerdt EB. Co-infection with *Anaplasma platys*, *Bartonella henselae* and *Candidatus Mycoplasma haematoparvum* in a veterinarian. *Parasit Vectors.* 2013;6:103. <https://doi.org/10.1186/1756-3305-6-103>
- Maggi RG, Compton SM, Trull CL, Mascarelli PE, Mozayani BR, Breitschwerdt EB. Infection with hemotropic *Mycoplasma* species in patients with or without extensive arthropod or animal contact. *J Clin Microbiol.* 2013;51:3237-41. <https://doi.org/10.1128/JCM.01125-13>
- Yang D, Tai X, Qiu Y, Yun S. Prevalence of *Eperythrozoon* spp. infection and congenital eperythrozoonosis in humans in Inner Mongolia, China. *Epidemiol Infect.* 2000;125:421-6. <https://doi.org/10.1017/S0950268899004392>
- Takeuchi F, Sekizuka T, Yamashita A, Ogasawara Y, Mizuta K, Kuroda M. MePIC, metagenomic pathogen identification for clinical specimens. *Jpn J Infect Dis.* 2014;67:62-5. <https://doi.org/10.7883/yoken.67.62>
- Huson DH, Beier S, Flade I, Górski A, El-Hadidi M, Mitra S, et al. MEGAN community edition: interactive exploration and analysis of large-scale microbiome sequencing data. *PLOS Comput Biol.* 2016;12:e1004957. <https://doi.org/10.1371/journal.pcbi.1004957>
- Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2010;26:589-95. <https://doi.org/10.1093/bioinformatics/btp698>

16. Tasker S, Peters IR, Mumford AD, Day MJ, Gruffydd-Jones TJ, Day S, et al. Investigation of human haemotropic *Mycoplasma* infections using a novel generic haemoplasma qPCR assay on blood samples and blood smears. *J Med Microbiol*. 2010;59:1285-92. <https://doi.org/10.1099/jmm.0.021691-0>
17. Murray GL, Bradshaw CS, Bissessor M, Danielewski J, Garland SM, Jensen JS, et al. Increased macrolide and fluoroquinolone resistance in *Mycoplasma genitalium*. *Emerg Infect Dis*. 2017;23:809-12. <https://doi.org/10.3201/eid2305.161745>
18. Fricke WF, Wright MS, Lindell AH, Harkins DM, Baker-Austin C, Ravel J, et al. Insights into the environmental resistance gene pool from the genome sequence of the multidrug-resistant environmental isolate *Escherichia coli* SMS-3-5. *J Bacteriol*. 2008;190:6779-94. <https://doi.org/10.1128/JB.00661-08>
19. Novacco M, Sugiarto S, Willi B, Baumann J, Spiri AM, Oestmann A, et al. Consecutive antibiotic treatment with doxycycline and marbofloxacin clears bacteremia in *Mycoplasma haemofelis*-infected cats. *Vet Microbiol*. 2018;217:112-20. <https://doi.org/10.1016/j.vetmic.2018.03.006>
20. Hamasuna R, Le PT, Kutsuna S, Furubayashi K, Matsumoto M, Ohmagari N, et al. Mutations in *ParC* and *GyrA* of moxifloxacin-resistant and susceptible *Mycoplasma genitalium* strains. *PLoS One*. 2018;13:e0198355. <https://doi.org/10.1371/journal.pone.0198355>
21. Weigel LM, Steward CD, Tenover FC. *gyrA* mutations associated with fluoroquinolone resistance in eight species of *Enterobacteriaceae*. *Antimicrob Agents Chemother*. 1998;42:2661-7. <https://doi.org/10.1128/AAC.42.10.2661>
22. Tasker S, Peters IR, Papisoulitis K, Cue SM, Willi B, Hofmann-Lehmann R, et al. Description of outcomes of experimental infection with feline haemoplasmas: copy numbers, haematology, Coombs' testing and blood glucose concentrations. *Vet Microbiol*. 2009;139:323-32. <https://doi.org/10.1016/j.vetmic.2009.06.028>
23. Barker EN, Darby AC, Helps CR, Peters IR, Heesom KJ, Arthur CJ, et al. Molecular characterization of the uncultivable hemotropic bacterium *Mycoplasma haemofelis*. *Vet Res (Faisalabad)*. 2011;42:83. <https://doi.org/10.1186/1297-9716-42-83>
24. Mohri H. Acquired von Willebrand syndrome: features and management. *Am J Hematol*. 2006;81:616-23. <https://doi.org/10.1002/ajh.20455>
25. Tiede A, Rand JH, Budde U, Ganser A, Federici AB. How I treat the acquired von Willebrand syndrome. *Blood*. 2011;117:6777-85. <https://doi.org/10.1182/blood-2010-11-297580>

Address for correspondence: Norimichi Hattori, Division of Hematology, Department of Medicine, Showa University School of Medicine, 1-5-8 Hatanodai, Shinagawa-Ku, Tokyo 142-8666, Japan; email: nhattor@med.showa-u.ac.jp



@CDC_EIDjournal

Want to stay updated on the latest news in Emerging Infectious Diseases? Let us connect you to the world of global health. Discover groundbreaking research studies, pictures, podcasts, and more by following us on Twitter at @CDC_EIDjournal.

Nutritional Care for Patients with Ebola Virus Disease

Mija Ververs, Magi Gabra¹

During the Ebola virus disease (EVD) outbreak of 2014–2016 in West Africa, practitioners faced challenges providing nutritional care for patients in Ebola treatment units (ETUs). The current EVD outbreak in the Democratic Republic of the Congo demonstrates the need to understand lessons learned from previous outbreaks and to update nutritional guidelines. We conducted a literature review to identify articles that included nutrition as an integral part of supportive care. We found little information on the specific nutritional care or practical challenges within an ETU. This review showed that nutritional care for EVD patients is poorly described, and therefore the optimal composition and implementation of nutritional care remain unknown. We recommend that researchers and practitioners share specific and practical details of their experiences in providing nutritional support within ETUs to expand the knowledge base and ultimately improve the nutritional care for an increasingly prevalent patient population.

In 2014, the World Health Organization (WHO), the United Nations Children’s Fund (UNICEF), and the World Food Programme (WFP) produced interim guidelines with recommendations for providing nutritional support to patients in Ebola treatment units (ETUs) (1). These guidelines were based on existing WHO evidence-based guidance adapted to the Ebola crisis, in addition to a rapid literature review on Ebola virus disease (EVD) and nutritional management of hemorrhagic fevers. The document aimed to address key clinical problems for EVD patients, their nutritional needs, and optimal nutritional care, including the practical aspects of providing nutritional support within treatment centers.

In the context of the current Ebola virus outbreak in the Democratic Republic of the Congo (DRC), we summarize existing practices and research findings on nutritional care for EVD patients in ETUs and compare them, when relevant, with

2014 WHO/UNICEF/WFP guidelines. More specifically, we aim to identify gaps to guide future practices and research, ultimately leading to improved nutritional guidelines.

Methods

We conducted a literature search using the MEDLINE database (through PubMed and OVID search engines), Global Health, and Scopus, and used Google Scholar to search gray literature. Medical Subject Headings terms used included “hemorrhagic fever, Ebola,” “Ebola virus,” and “nutrition.” We also searched on the following terms: diet, vitamin, malnutrition, breastfeed, nutrients, fortified, micronutrient, caloric, calories, soup, porridge, cereal, legume, sugar, and dextrose. The search identified articles relating to Ebola virus and nutrition published from January 1, 2014, through August 30, 2019. We did not apply language restrictions, and for search engines that allowed it, we included only articles about studies with human subjects. We screened only the first 120 results from Google Scholar because of decreasing relevance of articles. We downloaded and managed articles through EndNote X9 (Clarivate Analytics, <https://endnote.com>).

Results

We identified a total of 429 articles (Figure); 268 articles remained after deduplication. We excluded 240 articles after screening for information on nutrition or feeding during care for Ebola patients; content that caused exclusion included animal and bushmeat consumption in relation to Ebola transmission, testing and safety of breast milk in seropositive patients, food availability, malnutrition, agricultural stability before and during Ebola outbreaks, pharmacologic experiments for Ebola treatment, and molecular studies on the pathogenesis of Ebola virus. We completed full-text review on the remaining 28 articles. We excluded 5 non-English articles and added 1 more article identified from gray literature, resulting in review of 24 articles.

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: <https://doi.org/10.3201/eid2601.191024>

¹The authors contributed equally to this article.

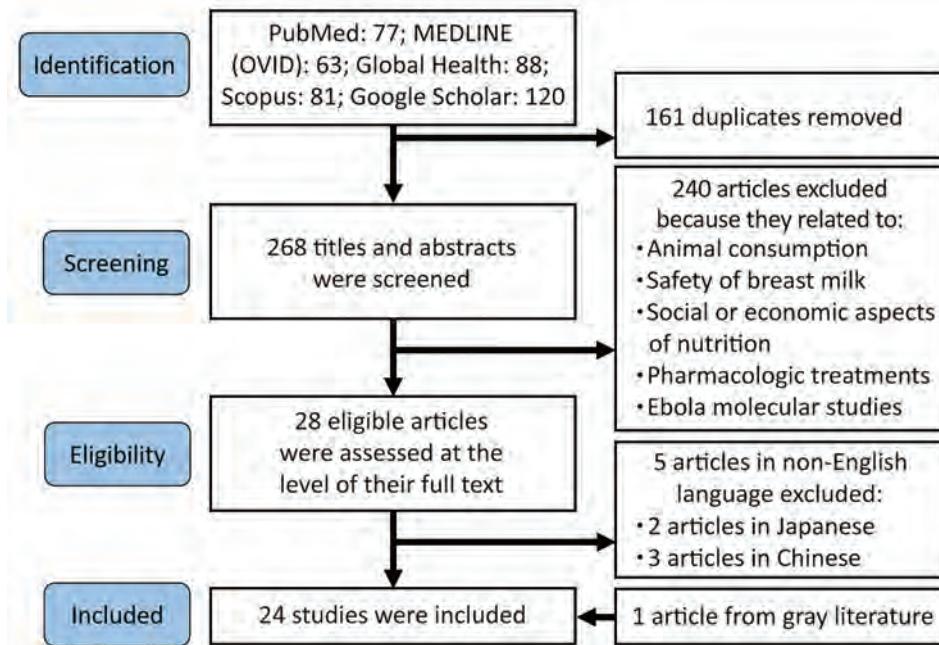


Figure. Flowchart summarizing literature search and selection process for review of nutritional care for patients with Ebola virus disease.

Most of the articles ($n = 14$) on nutritional support during Ebola treatment were case studies, cohort studies, or management recommendations focused on the clinical features, care provided, and outcomes observed (2–15). Four articles focused solely on patient care provided in the United States and Europe (5,6,8,14). Six described patients in West Africa during the 2014 outbreak: 3 in Sierra Leone (3,4,11), 1 in the Democratic Republic of the Congo (9), 1 in Liberia (2), and 1 in both Liberia and Sierra Leone (15). One particular study was a prospective cohort study on the effects of supplemental vitamin A on disease mortality rate (15). Two articles were field manuals, one an emergency interim country guidance for clinical management (16) and the other a list of essential medications for Ebola patient care (17). One article provided evidence-based guidelines for patient care using the Grading of Recommendations Assessment, Development, and Evaluation (GRADE) methodology (18). One was a systematic review of current trends of Ebola virus management (19). Two articles were analyses of policies and programming regarding infant feeding (20) and nutrition (21). An editorial on nutritional management of patients with Ebola virus disease (22) and a comment published on the overall changes in care for Ebola virus disease (23) were included, as well as a personal report of a nurse’s experience in the field while working in an ETU (24). The final item was a letter from a nutrition advisor during the Ebola outbreak (25), which we identified from gray literature.

Despite the initially large number of papers with the key search term “nutrition” related to EVD case management, few explicitly described the details of the delivered nutritional support. Most clinical management papers delineated supportive care for dehydration and electrolytes through oral rehydration solutions (ORS) or intravenous fluid administration and did not refer to nutrition. Two studies and a retrospective review concluded that dehydration was associated with worse outcomes and increased mortality rates in patients (7,9,11). Both Leligdowicz et al. (7) and Smit et al. (11) included electrolyte imbalance as an additional risk factor for poor outcomes. Smit et al. found that poor nutritional status was also associated with increased mortality (11). The systematic review by Sivnandy et al. noted that nutritional care should include a “good amount of protein supplements” but did not specify a recommended amount or type of protein (19).

The evidence-based recommendations produced by Lamontagne et al. supported the importance of hydration in supportive care and monitoring serum biochemistry for electrolyte repletion (23). In creating the recommendations, a multidisciplinary panel met to analyze data on supportive care and voted to create a list of evidence-based guidelines. The panel strongly recommended administering ORS in an adequate amount, rather than using nonstandardized rehydration. The panel also strongly recommended that serum biochemistry (e.g., testing of electrolytes, glucose) should be made available, but the group did not produce any statements on nutrition (18).

Clinical Cases

Johnson et al. (5) and Uyeki et al. (14) described total parenteral nutrition (TPN) in patient care in the United States and Europe. Patients treated in the biocontainment unit at the University of Nebraska Medical Center had a “moderate state of protein malnutrition” at admission, but protein malnutrition was not defined (5). A nutritional therapist was consulted for both cases and TPN was started, but further details were not provided. In another case study, a patient treated in Hamburg, Germany, did not tolerate enteral nutrition, and so the treatment team initiated TPN (6). When the patient showed improvement, enteral nutrition was reinitiated with a low-fiber standard formula; the authors did not provide details of the diet. A physician turned patient hydrated himself with a commercial sports rehydration beverage and a sugary drink mix before seeking treatment at Emory University Hospital in Atlanta, Georgia, USA, where he was given protein drinks and multivitamins. No information on the content of the protein drink or the frequency of use was provided (8). Similarly, a case study of 581 patients in an ETU in Freetown, Sierra Leone, documented the use of 1 capsule of ImmunoBoost nutrition supplement (Novopharm Formulations, <http://www.novopharm1.com>) per day and ORS and juice drinks consumed freely (3). However, the authors did not describe treatment rationale or composition.

In a recent study, Aluisio et al. assessed oral vitamin A supplementation in 424 patients admitted to ETUs in Sierra Leone and Liberia in 2014–2015 (15). Mortality rate was significantly lower among patients who had received 200,000–400,000 IU of vitamin A in the first 48 hours of admission, compared with those who had not received vitamin A at admission (relative Risk = 0.77, 95% CI 0.59–0.99; $p = 0.041$). An editorial published in the *Asian Pacific Journal of Tropical Disease* provided specific recommendations on the energy, protein, fat, and micronutrient components for the nutritional management of EVD patients (22). The authors recommended the following: achieving a protein requirement of 1.2 g/kg of ideal bodyweight, 55%–60% of total energy needs via carbohydrates, provision of soft foods to avoid gastrointestinal irritation, and avoidance of trans-fatty acids. It is important to note that there were no references to clinical research or case studies of patients with EVD to support these recommendations.

A nurse’s account of her experience working in an ETU in Liberia provided some insight on actual feeding practices (24). Wilson reported that a locally popular artificial juice powder composed of mostly

sugar and vitamin C (Foster Clark, <http://fosterclark.com>) was occasionally mixed with the ORS to increase palatability. In addition, patients received 3 meals, individually packaged in plastic bags, daily. Patients who were well enough often consumed food purchased or cooked by family or loved ones.

Pediatric Nutrition

Trehan et al. published 2 papers with extensively detailed nutritional care specifically for pediatric patients with Ebola virus disease (12,13). Recommendations included, though were not limited to, assessing nutritional status using mid-upper arm circumference, providing ample ORS and therapeutic milks (F-75, F-100; Nutriset, <https://www.nutriset.fr>), and serving ready-to-use therapeutic foods (RUTF) in biscuit and paste form. To improve hydration and intake, flavored ORS solutions were recommended if available, although drinks with added sugar were not recommended because they may exacerbate diarrhea. RUTF pastes and biscuits were 2 examples of energy-dense foods with complete micronutrient and macronutrient composition that retain taste and cleanliness for extended periods and are easily consumed by weak patients. The authors recommended the prioritization of RUTF over local foods in the acute phase of the illness. Instructions were included on how to prepare RUTF to produce a semi-solid porridge for easier consumption and described the minimum quantity of RUTF (for children <15 years varying from 2 to 5 sachets with RUTF paste or 3 to 8 RUTF biscuits). For infants <6 months of age, the authors recommended ready-to-use infant formula. For children >6 months of age who were only able to tolerate liquids, the authors recommended therapeutic milks over dairy milk or commercial infant formulas because of the increased nutritional content. Although it was more nutritionally complete than F-75, F-100 therapeutic milk was not endorsed for all pediatric patients because its higher osmotic load compared with that of F-75 may cause increased diarrhea. The authors recommended that formulas be refrigerated and prepared multiple times a day to avoid spoiling.

A nutrition advisor described infant feeding methods to reduce mother-to-infant transmission (25). Because feeding utensils and human-to-human contact carried an increased risk for transmission, the author reported the use of a syringe attached to a feeding tube to safely feed infants. Brandt et al. reviewed infant feeding policies and programming during the 2014–2016 Ebola outbreak and highlighted the inconsistencies of the messaging around breastfeeding (20). They recommended including infant and young

child feeding experts in outbreak response and creating consistent, appropriate, and tailored messages regarding breastfeeding and infant nutrition in the early phase of the response.

Perception of Nutrition Response

One descriptive qualitative study of key informants analyzed community perceptions of the nutrition-related response in Guinea (21). That study had 2 main objectives: the first was to determine how the Ebola outbreak affected infant and child nutrition on a community level, and the second was to gauge stakeholders' perception on the acceptability and effectiveness of the nutrition response, including the WHO/UNICEF/WFP 2014 interim guidelines for nutritional care. A consistent theme across informants was the lack of emphasis on nutrition by health professionals and community members during the Ebola outbreak. Key informants also noted limitations around the use of the WHO/UNICEF/WFP interim guidelines; some informants were unaware of the guidelines or questioned their usefulness, and some reported finding the guidelines useful but difficult to implement. The authors recommended that nutrition be a core component of response and integrated into all aspects of care, treatment, and recovery.

Discussion

In this literature review, we summarize existing practices and research findings on nutritional care for EVD patients. A wealth of literature exists on the clinical management and supportive care, but our review found only a limited number of publications on specific nutritional care, and they often lacked detailed descriptions of the actual nutritional care provided. The results could lead to the conclusion that the role of nutritional care in ETUs is perceived to have limited importance. However, the importance of good nutrition in fighting infection is widely known, and several researchers emphasized the need for good nutritional care for EVD patients (11–14,17,21,22). In addition, it is well established that adequate nutrition is essential in the management of critical illness or sepsis (26,27). Our review showed that nutritional care is poorly described and therefore the optimal composition and implementation of care remains unknown. One noticeable finding was the use of total parenteral nutrition (TPN) only in high-resource settings. It is unclear whether TPN is preferred over enteral nutrition and whether the role of enteral nutrition for patients' survival is different in high- or low- resource settings.

We intended to compare actual nutritional care in ETUs with the 2014 WHO/UNICEF/WFP interim

guidelines. However, various care descriptions from our literature review preceded the availability of these guidelines (2,3,5,6,8,9,14,17,22,24). This restricted our ability to compare recommended and actual practices. Only 3 works published after November 2014 commented on using the guidelines or reported any successes or challenges with attempting to follow them (12,13,21).

WHO recommends that EVD patients should be provided with a minimum recommended daily allowance (RDA) of nutrients through normal traditional or fortified foods (1,16) or micronutrient powders (28). It also states that, until further evidence is available, excess use of any micronutrient for EVD patients is not recommended, unless correcting for a specific micronutrient loss (e.g., treating hypokalemia). However, in many EVD-affected countries in sub-Saharan Africa, malnutrition, including micronutrient deficiencies, is widely prevalent (29,30), and 1 RDA of, for example, vitamins would not sufficiently address existing suboptimal levels in EVD patients.

To improve nutritional care for EVD patients, more documentation is needed on nutritional care in ETUs. We recommend that researchers and practitioners share specific and practical details of their experiences in providing nutritional support within ETUs to further facilitate the scientific base and ultimately improve the nutritional care for an increasingly prevalent patient population. In addition, research is necessary to determine whether specific macronutrients or micronutrients improve treatment outcomes in ETUs and elucidate their mechanism of action. For instance, the roles of albumin (14), selenium (31,32), and electrolytes (33,34) deserve further exploration, and the recognized importance of vitamin A in mounting an immune response to infectious diseases urgently merits further studies. It is also worth investigating whether anthropometric changes in patients admitted to ETUs relate to outcomes of illness and death. In addition, we see a need to examine whether the recommendation of providing 1 RDA is sufficient for managing patients with nutrient losses secondary to EVD-induced enteropathy, complicated by an underlying suboptimal nutritional status. We further underline the need for immediate research on breast milk and transmissibility of EVD (35). Last, we propose to not only examine whether specific nutritional care can raise patient survival rates, but to assess how it may contribute to symptom relief in the critically ill. Although adequate nutrition cannot cure patients with EVD, maintaining an optimal nutritional status could improve their response to treatment.

This study was performed as regular work of the Centers for Disease Control and Prevention (M.V.) and as student work (M.G.). It used no additional funding.

References

- World Health Organization/UNICEF/World Food Programme. Interim guideline: nutritional care of children and adults with Ebola virus disease in treatment centres. 2014 [cited 2019 May 31]. https://apps.who.int/iris/bitstream/handle/10665/137425/9789241508056_eng.pdf
- Wong KK, Perdue CL, Malia J, Kenney JL, Peng S, Gwathney JK, et al.; Monrovia Medical Unit. Supportive care of the first 2 Ebola virus disease patients at the Monrovia Medical Unit. *Clin Infect Dis*. 2015;61:e47–51. <https://doi.org/10.1093/cid/civ420>
- Ansumana R, Jacobsen KH, Sahr F, Idris M, Bangura H, Boie-Jalloh M, et al. Ebola in Freetown area, Sierra Leone—a case study of 581 patients. *N Engl J Med*. 2015;372:587–8. <https://doi.org/10.1056/NEJMc1413685>
- Damkjær M, Rudolf F, Mishra S, Young A, Storgaard MJ. Clinical features and outcome of Ebola virus disease in pediatric patients: a retrospective case series. *J Pediatr*. 2017;182:378–81.e1. <https://doi.org/10.1016/j.jpeds.2016.11.034>
- Johnson DW, Sullivan JN, Piquette CA, Hewlett AL, Bailey KL, Smith PW, et al. Lessons learned: critical care management of patients with Ebola in the United States. *Crit Care Med*. 2015;43:1157–64. <https://doi.org/10.1097/CCM.0000000000000935>
- Kreuels B, Wichmann D, Emmerich P, Schmidt-Chanasit J, de Heer G, Kluge S, et al. A case of severe Ebola virus infection complicated by gram-negative septicemia. *N Engl J Med*. 2014;371:2394–401. <https://doi.org/10.1056/NEJMoa1411677>
- Leligdowicz A, Fischer WA II, Uyeki TM, Fletcher TE, Adhikari NK, Portella G, et al. Ebola virus disease and critical illness. *Crit Care*. 2016;20:217. <https://doi.org/10.1186/s13054-016-1325-2>
- Lyon GM, Mehta AK, Varkey JB, Brantly K, Plyler L, McElroy AK, et al.; Emory Serious Communicable Diseases Unit. Clinical care of two patients with Ebola virus disease in the United States. *N Engl J Med*. 2014;371:2402–9. <https://doi.org/10.1056/NEJMoa1409838>
- Nanclares C, Kapetshi J, Lionetto F, de la Rosa O, Tamfun JJ, Alia M, et al. Ebola virus disease, Democratic Republic of the Congo, 2014. *Emerg Infect Dis*. 2016;22:1579–86. <https://doi.org/10.3201/eid2209.160354>
- Richards GA, Baker T, Amin P; Council of the World Federation of Societies of Intensive and Critical Care Medicine. Ebola virus disease: report from the task force on tropical diseases by the World Federation of Societies of Intensive and Critical Care Medicine. *J Crit Care*. 2018;43:352–5. <https://doi.org/10.1016/j.jcrrc.2017.11.002>
- Smit MA, Michelow IC, Glavis-Bloom J, Wolfman V, Levine AC. Characteristics and outcomes of pediatric patients with Ebola virus disease admitted to treatment units in Liberia and Sierra Leone: a retrospective cohort study. *Clin Infect Dis*. 2017;64:243–9. <https://doi.org/10.1093/cid/ciw725>
- Trehan I, De Silva SC. Management of Ebola virus disease in children. *Infect Dis Clin North Am*. 2018;32:201–14. <https://doi.org/10.1016/j.idc.2017.10.010>
- Trehan I, Kelly T, Marsh RH, George PM, Callahan CW. Moving towards a more aggressive and comprehensive model of care for children with Ebola. *J Pediatr*. 2016;170:28–33.e7. <https://doi.org/10.1016/j.jpeds.2015.11.054>
- Uyeki TM, Mehta AK, Davey RT Jr, Liddell AM, Wolf T, Vetter P, et al.; Working Group of the U.S.–European Clinical Network on Clinical Management of Ebola Virus Disease Patients in the U.S. and Europe. Clinical management of Ebola virus disease in the United States and Europe. *N Engl J Med*. 2016;374:636–46. <https://doi.org/10.1056/NEJMoa1504874>
- Aluisio AR, Perera SM, Yam D, Garbern S, Peters JL, Abel L, et al. Vitamin A supplementation was associated with reduced mortality in patients with Ebola virus disease during the West African outbreak. *J Nutr*. 2019;149:1757–65. <https://doi.org/10.1093/jn/nxz142>
- World Health Organization. Clinical management of patients with viral haemorrhagic fever. A pocket guide for the front-line health worker. 2016 [cited 2019 Oct 28]. <https://www.who.int/csr/resources/publications/clinical-management-patients>
- Pierce WF, Ready SD, Chapman JT, Kulick C, Shields A, Wang J, et al. Essential medications for patients with suspected or confirmed Ebola virus disease in resource-limited environments. *Mil Med*. 2017;182:e2006–16. <https://doi.org/10.7205/MILMED-D-17-00010>
- Lamontagne F, Fowler RA, Adhikari NK, Murthy S, Brett-Major DM, Jacobs M, et al. Evidence-based guidelines for supportive care of patients with Ebola virus disease. *Lancet*. 2018;391:700–8. [https://doi.org/10.1016/S0140-6736\(17\)31795-6](https://doi.org/10.1016/S0140-6736(17)31795-6)
- Sivanandy P, Sin SH, Ching OY, Rajasekar D, Woon GS, Chiew HH, et al. Current trends in the management of Ebola virus disease—an updated systematic review. *Asian Pac J Trop Dis*. 2016;6:589–95. [https://doi.org/10.1016/S2222-1808\(16\)61091-5](https://doi.org/10.1016/S2222-1808(16)61091-5)
- Brandt A, Serrano Oria Ó, Kallon M, Bazzano AN. Infant feeding policy and programming during the 2014–2015 Ebola virus disease outbreak in Sierra Leone. *Glob Health Sci Pract*. 2017;5:507–15. <https://doi.org/10.9745/GHSP-D-16-00387>
- Kodish SR, Rohner F, Beaucliere J-M, Daffe M, Ag Ayoya M, Wirth JP, et al. Implications of the Ebola virus disease outbreak in Guinea: qualitative findings to inform future health and nutrition-related responses. *PLoS One*. 2018;13:e0202468. <https://doi.org/10.1371/journal.pone.0202468>
- Chaiyasit K, Wiwanitkit V. Nutritional management in Ebola haemorrhagic fever. *Asian Pac J Trop Dis*. 2015;5(Suppl. 1):S11–2. [https://doi.org/10.1016/S2222-1808\(15\)60846-5](https://doi.org/10.1016/S2222-1808(15)60846-5)
- Lamontagne F, Clément C, Kojan R, Godin M, Kabuni P, Fowler RA. The evolution of supportive care for Ebola virus disease. *Lancet*. 2019;393:620–1. [https://doi.org/10.1016/S0140-6736\(19\)30242-9](https://doi.org/10.1016/S0140-6736(19)30242-9)
- Wilson D. CE: inside an Ebola treatment unit: a nurse’s report. *Am J Nurs*. 2015;115:28–38. <https://doi.org/10.1097/01.NAJ.0000475288.30664.70>
- Serrano Oria Ó. Letters. *Field Exchange*. 2015;50:94–5. <https://www.ennonline.net/attachments/2401/FEX-50-Web-Final.pdf>
- McClave SA, Taylor BE, Martindale RG, Warren MM, Johnson DR, Braunschweig C, et al.; Society of Critical Care Medicine; American Society for Parenteral and Enteral Nutrition. Guidelines for the provision and assessment of nutrition support therapy in the adult critically ill patient. *JPEN J Parenter Enteral Nutr*. 2016;40:159–211. <https://doi.org/10.1177/0148607115621863>
- Mehta NM, Skillman HE, Irving SY, Coss-Bu JA, Vermilyea S, Farrington EA, et al. Guidelines for the provision and assessment of nutrition support therapy in the pediatric

critically ill patient. JPEN J Parenter Enteral Nutr. 2017;41:706-42. <https://doi.org/10.1177/0148607117711387>

28. World Health Organization. Optimized supportive care for Ebola virus disease: clinical management standard operating procedures. 2019 [cited 2019 Sep 30]. <https://www.who.int/publications-detail/optimized-supportive-care-for-ebola-virus-disease>
29. Food and Agriculture Organization of the United Nations; International Fund for Agricultural Development; United Nations Children's Fund; World Food Programme; World Health Organization. The state of food security and nutrition in the world 2019: safeguarding against economic slowdowns and downturns. Rome: Food and Agriculture Organization; 2019 [cited 2019 Nov 12]. <https://www.wfp.org/publications/2019-state-food-security-and-nutrition-world-sofi-safeguarding-against-economic>
30. Ritchie H, Roser M. Micronutrient deficiency. 2017 [cited 2019 Oct 3]. <https://ourworldindata.org/micronutrient-deficiency>
31. Taylor EW, Ruzicka JA, Premadasa L, Zhao L. Cellular selenoprotein mRNA tethering via antisense interactions with Ebola and HIV-1 mRNAs may impact host selenium biochemistry. Curr Top Med Chem. 2016;16:1530-5. <https://doi.org/10.2174/1568026615666150915121633>
32. Chiappelli F, Bakhordarian A, Thames AD, Du AM, Jan AL, Nahcivan M, et al. Ebola: translational science considerations. J Transl Med. 2015;13:11. <https://doi.org/10.1186/s12967-014-0362-3>
33. Hunt L, Gupta-Wright A, Simms V, Tamba F, Knott V, Tamba K, et al. Clinical presentation, biochemical, and haematological parameters and their association with outcome in patients with Ebola virus disease: an observational cohort study. Lancet Infect Dis. 2015;15:1292-9. [https://doi.org/10.1016/S1473-3099\(15\)00144-9](https://doi.org/10.1016/S1473-3099(15)00144-9)
34. van Griensven J, Bah EI, Haba N, Delamou A, Camara BS, Olivier KJ, et al.; Ebola-Tx Consortium. Electrolyte and metabolic disturbances in Ebola patients during a clinical trial, Guinea, 2015. Emerg Infect Dis. 2016;22. <https://doi.org/10.3201/eid2212.161136>
35. Ververs M, Arya A. Ebola virus disease and breastfeeding: time for attention. Lancet. 2019;394:825. [https://doi.org/10.1016/S0140-6736\(19\)32005-7](https://doi.org/10.1016/S0140-6736(19)32005-7)

Address for correspondence: Mija Ververs, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop E-22, Atlanta, GA 30329-4027, USA; email: mververs@cdc.gov

Emerging Infectious Diseases Spotlight Topics

etymologia

Antimicrobial resistance • Ebola • Etymologia
 Food safety • HIV-AIDS • Influenza
 Lyme disease • Malaria • MERS • Pneumonia
 Rabies • Tuberculosis • Ticks • Zika

<https://wwwnc.cdc.gov/eid/page/spotlight-topics>

EID's spotlight topics highlight the latest articles and information on emerging infectious disease topics in our global community.

Paid Leave and Access to Telework as Work Attendance Determinants during Acute Respiratory Illness, United States, 2017–2018¹

Faruque Ahmed,¹ Sara Kim, Mary Patricia Nowalk, Jennifer P. King, Jeffrey J. VanWormer, Manjusha Gaglani, Richard K. Zimmerman, Todd Bear, Michael L. Jackson, Lisa A. Jackson, Emily Martin, Caroline Cheng, Brendan Flannery, Jessie R. Chung, Amra Uzicanin

Medscape **ACTIVITY** EDUCATION

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

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

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

All other clinicians completing this activity will be issued a certificate of participation. To participate in this journal CME activity: (1) review the learning objectives and author disclosures; (2) study the education content; (3) take the post-test with a 75% minimum passing score and complete the evaluation at <http://www.medscape.org/journal/eid>; and (4) view/print certificate. For CME questions, see page 190.

Release date: December 18, 2019; Expiration date: December 18, 2020

Learning Objectives

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

- Describe work attendance during acute respiratory illness (ARI) and associated characteristics among workers ages 19 to 64 years with medically attended ARI or influenza during the 2017–2018 influenza season, according to a survey study
- Identify determinants of work attendance during the first 3 days after ARI onset among workers ages 19 to 64 years with medically attended ARI or influenza during the 2017–2018 influenza season, according to a survey study
- Determine clinical implications of work attendance during the first 3 days after ARI onset among workers ages 19 to 64 years with medically attended ARI or influenza during the 2017–2018 influenza season, according to a survey study

CME Editor

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

CME Author

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

Authors

Disclosure: Faruque Ahmed, PhD; Sara S. Kim, MPH; Jennifer King, MPH; Jeffrey J. VanWormer, PhD; Manjusha Gaglani, MBBS; Richard K. Zimmerman, MD, MPH; Todd M. Bear, PhD; Michael Jackson, PhD; Caroline Cheng, MS; Brendan Flannery, PhD; Jessie R. Chung, MPH; and Amra Uzicanin, MD, MPH, have disclosed no relevant financial relationships. Mary Patricia Nowalk, PhD, has disclosed the following relevant financial relationships: received grants for clinical research from Merck & Co., Inc. Lisa A. Jackson, MD, MPH, has disclosed the following relevant financial relationships: received grants for clinical research from Pfizer Inc. Emily Martin, MPH, PhD, has disclosed the following relevant financial relationships: served as an advisor or consultant for Pfizer Inc.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (F. Ahmed, S. Kim, B. Flannery, J.R. Chung, A. Uzicanin); University of Pittsburgh, Pittsburgh, Pennsylvania, USA (M.P. Nowalk, R.K. Zimmerman, T. Bear); Marshfield Clinic Research Institute, Marshfield, Wisconsin, USA (J.P. King, J.J. VanWormer); Texas A&M University, Temple, Texas, USA (M. Gaglani); Kaiser Permanente Washington Health Research Institute, Seattle, Washington, USA (M.L. Jackson,

L.A. Jackson); University of Michigan, Ann Arbor, Michigan, USA (E. Martin, C. Cheng)

DOI: <https://doi.org/10.3201/eid2601.190743>

¹Preliminary results from this study were presented at the International Conference on Emerging Infectious Diseases, August 27–29, 2018, Atlanta, Georgia, USA.

We assessed determinants of work attendance during the first 3 days after onset of acute respiratory illness (ARI) among workers 19–64 years of age who had medically attended ARI or influenza during the 2017–2018 influenza season. The total number of days worked included days worked at the usual workplace and days teleworked. Access to paid leave was associated with fewer days worked overall and at the usual workplace during illness. Participants who indicated that employees were discouraged from coming to work with influenza-like symptoms were less likely to attend their usual workplace. Compared with workers without a telework option, those with telework access worked more days during illness overall, but there was no difference in days worked at the usual workplace. Both paid leave benefits and business practices that actively encourage employees to stay home while sick are necessary to reduce the transmission of ARI and influenza in workplaces.

The annual economic burden of influenza in the United States, depending on the severity of the influenza season, ranges from \$15 billion to \$64 billion, of which lost productivity accounts for a substantial proportion (1). The annual economic burden of noninfluenza viral respiratory tract infections is estimated to be \$40 billion (2). As a result of absenteeism and diminished work capacity, employees with medically attended influenza can expect to lose 69% of their usual workplace productivity and employees with noninfluenza acute respiratory illness (ARI) can expect to lose 58% of their usual workplace productivity during the week after symptom onset (3). With about two thirds of the US adult population participating in the labor force (4), workplace contacts can play a major role in the transmission of influenza (5). Influenza vaccination can reduce illness and work absenteeism associated with influenza (6), but fewer than one third of US adults 18–64 years of age were vaccinated in the 2017–2018 influenza season (7).

Respiratory etiquette, regular hand hygiene, and staying home for ≥ 24 hours after fever subsides can help slow the spread of seasonal and pandemic influenza (8). For employed adults, staying home when ill usually entails taking sick days or working from home. During a respiratory illness, some employees may have a telework option, whereby they are permitted to perform their usual work functions while staying at home (without having to use paid time off or sick leave benefits). Telework may be a good mitigation strategy during an influenza pandemic if ill persons work remotely and avoid exposing coworkers during the contagious period (9). About 24% of employed persons in the United States telework

regularly, varying from 8% in production occupations to 34% in managerial and professional occupations (10). Teleworking options also tend to track closely with education; 13% of workers with less than a high school diploma report being able to telework, compared with 37% of those with a bachelor's degree or higher (10).

Approximately 74% of US civilian workers receive paid sick leave and 75% receive paid vacation leave benefits (11). However, the effect of access to telework and paid leave benefits on staying away from the workplace during influenza illness is largely unknown (5). This study assessed the association between access to telework and paid leave benefits and short-term work attendance in employed adults during a medically attended ARI or influenza episode.

Methods

Study Population

Study enrollees were patients seeking care for an ARI with cough within 7 days of illness onset during November 1, 2017–April 19, 2018 (the 2017–18 influenza season), at outpatient facilities affiliated with sites participating in the US Influenza Vaccine Effectiveness Network. The sites are in Ann Arbor and Detroit, Michigan; Pittsburgh, Pennsylvania; Temple, Texas; Seattle, Washington; and Marshfield, Wisconsin, USA. The study methods have been published previously (12,13). The institutional review boards at the sites approved the study. Study participants provided informed consent.

Data Collection

Data collected at the enrollment visit included sex, race/ethnicity, education, general health before illness, number of children <12 years of age living in household, date of illness onset, and symptoms (fever/feverishness, sore throat) (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-0743-App1.pdf>). Data extracted from electronic medical records included age, medical conditions associated with increased risk of influenza complications (based on medical encounters associated with International Classification of Diseases codes in the year before enrollment) (12), and influenza vaccination. Nasal and oropharyngeal swab specimens were collected at the enrollment visit; all persons were tested for influenza viruses using real-time reverse transcription PCR (rRT-PCR).

Adults 19–64 years of age were asked to complete a survey 7–14 days after enrollment. The follow-up survey for the 2017–18 influenza season included

questions about the following: hours expected to work in a typical week, hours usually worked from home (telework, telecommute, or remote work), receipt of any paid leave that could be used for an illness (e.g., sick leave, personal time off, vacation leave), whether they worked the day before illness, and work attendance during the first 3 days of illness (including number of days worked at the usual workplace and number of days worked from home) (Appendix Table 1). Participants were also asked about recovery from illness, return to normal activities (e.g., work, exercise, housework/chores), type of employee, type of position, and number of employees in the company/organization. Workers were asked to rate their level of agreement with 3 statements about their place of work using a Likert scale (Appendix Table 1). Responses were dichotomized as “agree” or “not agree”; “strongly agree” and “agree” responses were categorized as “agree.”

Definitions

Study participants who reported that they regularly worked from home ≥ 1 hour in a typical week were classified as having access to telework (habitual teleworkers). Because persons who worked from home for only a few hours a week may not telework ≥ 1 full day a week, we performed an analysis based on the hours teleworked (none, < 8 hours, or ≥ 8 hours) to assess the robustness of the findings (14). Persons who reported that they received any paid leave that could be used for an illness (e.g., paid sick leave, vacation leave, or personal time off) were classified as having paid leave benefit (15). Part-time workers were those working ≥ 20 but < 35 hours; full-time was defined as ≥ 35 hours/week (10). We computed the total number of days worked in the first 3 days of illness by summing the number of days worked at the usual workplace and the number of days worked from home. We defined laboratory-confirmed influenza as a positive rRT-PCR test for influenza A or B from a nasal or oropharyngeal swab specimen.

Inclusion and Exclusion Criteria

If an adult 19–64 years of age enrolled in the study ≥ 2 times because of multiple episodes of ARI during the influenza season, we included the first enrollment (Figure). Participants who completed the follow-up survey > 14 days after enrollment were excluded to minimize recall bias (16). Participants were also excluded if they were unemployed, self-employed, owned their own business, worked solely from home, or were employed < 20 hours/week. Only responses that added up to 3 days for the question on work

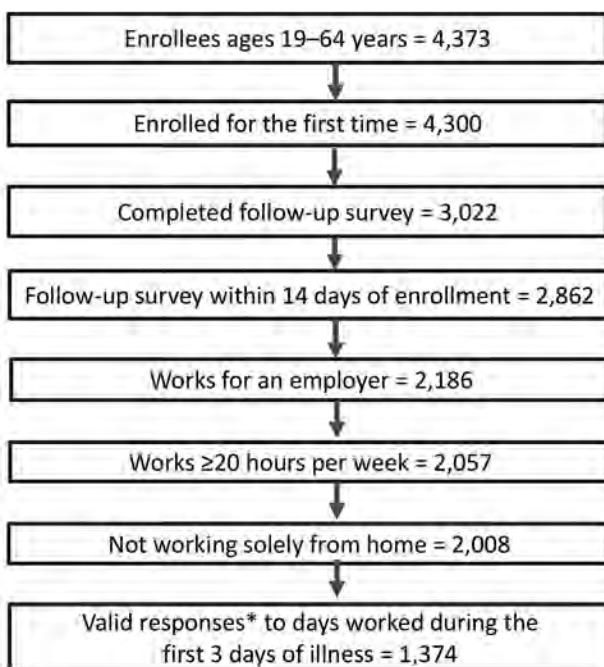


Figure. Total enrolled and number of persons included in analyses of work attendance during the first 3 days of acute respiratory illness or influenza, United States, 2017–18 influenza season.

*Valid responses are those that added up to 3 days for the question on work attendance during the first 3 days of illness (see Appendix Table 2, <https://wwwnc.cdc.gov/EID/article/26/1/19-0743-App1.pdf>).

attendance during the first 3 days of illness were considered valid and used for the final analysis. Examples of valid and invalid responses are provided in Appendix Table 2.

Statistical Analysis

We computed the median days to return to normal activities and the median days to recovery from illness using the Kaplan-Meier method to take into account that a substantial proportion of participants had not returned to normal activities (13%) or recovered from their illness (32%) at the time of follow-up. We used a χ^2 test to assess differences between categories, Student *t*-test for means, and Wilcoxon rank-sum tests to assess differences between medians. We ran zero-inflated Poisson regression, which accounts for excess zeroes, using PROC GENMOD in SAS version 9.4 (SAS Institute, <https://www.sas.com>) to compute ratios of days worked adjusted for potential confounding variables (17). The dependent variable in the regression models was the number of days worked during the first 3 days of illness (i.e., 0, 1, 2, or 3 days). In addition to running models using the total days worked during the first 3 days of illness, an indicator of productivity, as the dependent variable,

we ran models using days worked at the usual workplace during the first 3 days of illness, a measure of the potential for virus transmission to co-workers, as the dependent variable. The exposure variables were access to telework (0 = no, 1 = yes) and access to paid leave (0 = no, 1 = yes). We used a forward selection process. Because age, sex, and socioeconomic status have been identified as confounders (18), we retained age, sex, and education (proxy for socioeconomic status) in the models. Statistical significance was set at $\alpha = 0.05$ (2-tailed).

Results

A total of 4,300 adults 19–64 years of age were enrolled across the study sites. Overall, 2,862 (67%) adults completed the follow-up survey within 14 days of enrollment (Figure), and 2,008 workers met the employment criteria. Of these, 1,374 (68%) had valid responses for the question on work attendance during the first 3 days of illness and were included in the analysis. The proportion of adults with valid responses was 85% (757/894) for the Washington and Wisconsin sites and 55% (617/1,114) for the other 3 sites. The proportion with valid responses was higher for non-Hispanic whites and other races compared with non-Hispanic blacks and Hispanics (71% vs. 54%; $p < 0.001$) and for hourly workers compared with non-hourly workers (71% vs. 66%; $p < 0.05$). Valid responses were also higher for persons who received influenza vaccination (72% vs. 65%; $p < 0.01$) and those with paid leave benefits (70% vs. 64%; $p < 0.05$). The proportion with valid responses did not differ by age, sex, education, access to telework, and other variables (data not shown).

Among the 1,374 adults with valid responses, the median age was 42 years, 64% were female, and 82% were non-Hispanic white. The median hours expected to work in a typical week was 40 (5th, 95th percentile: 25, 52). Thirty-six percent of participants had laboratory-confirmed influenza; patients with influenza were more likely to report fever (84%) than patients who tested negative for influenza (53%) ($p < 0.001$), whereas the proportions reporting sore throat were similar (77% vs. 81%). In accordance with the criteria for enrollment in the study, all participants' symptoms included cough.

The median time from illness onset to enrollment was 3 days (5th, 95th percentile: 1, 7), and the median interval from enrollment to follow-up was 7 days (5th, 95th percentile: 6, 12). Based on the Kaplan-Meier method, the median interval from illness onset to return to normal activities was 7 days, and the median interval from illness onset to recovery from

illness was 11 days. During the first 3 days of illness, 539 (39%) reported that they did not work at all. The mean number of total days worked during the first 3 days of illness was 1.14 days (range 0–3 days), the mean number of days absent from work because of illness was 1.06 days (range 0–3 days), and the mean number of days not worked because of having a scheduled day off or any other reason was 0.80 days (range 0–3 days).

Data on access to telework were available for 1,362 adults and data on paid leave benefits for 1,356 adults; 198 (15%) reported having access to telework, and 1,074 (79%) received paid leave benefits. Among persons with access to telework, the median hours usually teleworked was 8 hours/week (5th, 95th percentile: 2, 30 hours/week). Adults who reported access to telework and paid leave were significantly different from those who reported no access by having higher education levels ($p < 0.001$), working full time ($p < 0.01$), and being salaried employees ($p < 0.001$) (Table 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-0743-T1.htm>). Furthermore, adults with access to telework and paid leave were more likely to be encouraged by their employer to go home if they had influenza-like symptoms at work ($p < 0.01$); these employees also had greater control over taking days off from work for illnesses ($p < 0.001$) (Table 1).

The proportion of adults who worked the day before illness was similar for those with access to telework compared with those without access, as well as for those with paid leave benefits compared with those without (Table 1). Among adults who worked the day before illness, telework was more common among those with access to telework (habitual teleworkers) than for those who were not habitual teleworkers (14% vs. 1%, $p < 0.001$).

During the first 3 days of illness, the proportion who did not work at all was 28% (55/198) for those with access to telework compared with 41% (477/1,164) for those without telework access ($p < 0.001$). The mean of the total days worked was greater for adults with access to telework than for adults without access to telework (mean 1.46 vs. 1.09 days; $p < 0.001$) (Table 2). This difference was attributable to more days teleworking while ill, as there was no difference in the mean number of days worked at the usual workplace while ill (Table 2). Adults without access to telework took more time off because of illness (mean 1.10 vs. 0.80 days; $p < 0.001$). In contrast, adults with access to paid leave showed no differences in the mean total days worked during acute illness or mean days worked at the usual workplace, compared with those among persons without access to paid leave (Table 2).

RESEARCH

Table 2. Work attendance during the first 3 days of illness among adults with medically attended acute respiratory illness or influenza, United States, 2017–18 influenza season*

Work attendance	Mean no. days worked			
	Access to telework		Paid leave benefits	
	Yes, n = 198	No†, n = 1,164	Yes, n = 1,074	No, n = 282
Worked	1.46‡	1.09	1.15	1.09
Usual workplace	1.05	1.07	1.07	1.05
Teleworked	0.41‡	0.02	0.08	0.04
Did not work	1.54‡	1.91	1.85	1.91
Felt ill	0.80‡	1.10	1.03	1.17
Day off	0.64	0.72	0.72	0.66
Other reasons	0.11	0.10	0.10	0.07

*Days worked or not worked ranged from 0 to 3 days. Boldface indicates statistical significance.

†Among 1,164 persons with no telework access (i.e., did not habitually telework), 15 persons reported that they worked from home for ≥1 d during the first 3 d of illness.

‡p<0.001.

The results of zero-inflated Poisson regression analyses showed that participants who had access to paid leave were significantly less likely to work during the first 3 days of illness (adjusted ratio of days worked 0.81, 95% CI 0.68–0.96) or to work at their usual workplace (adjusted ratio of days worked 0.81, 95% CI 0.67–0.96) (Table 3). Persons who worked in an organization in which employees were discouraged from coming to work if they had influenza-like symptoms were also significantly less likely to work during the first 3 days of illness (adjusted ratio 0.86, 95% CI 0.76–0.97) or to work at their usual workplace (adjusted ratio 0.85, 95% CI 0.74–0.96) (Table 3). In contrast, persons with access to telework were significantly more likely to work during the first 3 days of illness (adjusted ratio 1.25, 95% CI 1.07–1.46) (Table 3). However, access to telework was not associated with the number of days worked at the usual workplace (adjusted ratio 0.98, 95% CI 0.82–1.17) (Table 3).

The findings were similar among workers with laboratory-confirmed influenza (Table 4; Appendix Tables 3–5). Results were similar for sites with higher

proportions of valid responses (Washington and Wisconsin) and lower proportions (Michigan, Pennsylvania, and Texas sites) (Appendix Table 6). The analysis by hours teleworked showed similar findings (Appendix Table 7).

Discussion

Among working adults who sought medical care for an ARI from 5 sites across the country, we found that 79% had access to paid leave and 15% were able to telework. Our study results show that both paid leave benefits and business practices that actively encourage employees to stay home when ill may be necessary to keep sick employees away from the workplace. Access to telework, where feasible, helps retain some work productivity.

Because infectiousness of adults with influenza is greatest during the first 3 days of illness (19,20), preventing workplace attendance of ill persons during the first several days of illness might be most necessary for reducing workplace-based transmission. In previous research, a greater proportion of workers

Table 3. Adjusted analysis to assess the association with days worked during the first 3 days of illness among adults with medically attended acute respiratory illness or influenza, United States, 2017–18 influenza season*

Characteristic	Total days worked, n = 1,306	Days worked at the usual workplace, n = 1,306
Access to telework		
No	Referent	Referent
Yes	1.25 (1.07–1.46)‡	0.98 (0.82–1.17)
Access to paid leave		
No	Referent	Referent
Yes	0.81 (0.68–0.96)‡	0.81 (0.67–0.96)‡
Discouraged from coming to work with influenza-like symptoms		
Not agree	Referent	Referent
Agree	0.86 (0.76–0.97)‡	0.85 (0.74–0.96)‡

*Values are adjusted ratios of days worked (95% CI). Boldface indicates statistical significance. Total days worked represents the sum of days worked at the usual workplace and days teleworked during the first 3 days of illness. The dependent variable in the zero-inflated Poisson regressions was days worked during the first 3 days of illness (i.e., 0, 1, 2, or 3 d). The final models contained the following independent variables: access to telework; access to paid leave; employees are discouraged from coming to work when they have flulike symptoms; age; sex; education; fever; worked the day before illness; having a lot of control over taking days off for illnesses; full-time worker; and employee type. The variable “employees are encouraged to go home if they have influenza-like symptoms at work” was excluded from the models because it was highly correlated with the variable “employees are discouraged from coming to work when they have influenza-like symptoms” (Spearman correlation coefficient 0.76; p<0.001); the latter variable has more relevance for reducing virus transmission in the workplace (not coming to work at all vs. coming to work with influenza-like symptoms and then told to go home). Sixty-eight records were excluded because of missing values.

‡p<0.01.

‡p<0.05.

Table 4. Adjusted analysis to assess the association with days worked during the first 3 days of illness, United States, 2017–18 influenza season, by laboratory-confirmed influenza*

Characteristic	Total days worked		Days worked at the usual workplace	
	Influenza positive, n = 464	Influenza negative, n = 839	Influenza positive, n = 464	Influenza negative, n = 839
Access to telework				
No	Referent	Referent	Referent	Referent
Yes	1.46 (1.09–1.96)†	1.19 (0.99–1.43)	1.15 (0.83–1.60)	0.92 (0.75–1.14)
Access to paid leave				
No	Referent	Referent	Referent	Referent
Yes	0.81 (0.57–1.14)	0.82 (0.68–1.00)	0.79 (0.55–1.12)	0.83 (0.68–1.02)
Discouraged from coming to work with influenza-like symptoms				
Not agree	Referent	Referent	Referent	Referent
Agree	0.71 (0.55–0.91)‡	0.92 (0.80–1.06)	0.72 (0.55–0.94)†	0.90 (0.78–1.05)

*Data are presented as adjusted ratios of days worked (95% confidence interval), unless otherwise indicated. Boldface indicates statistical significance. The dependent variable in the zero-inflated Poisson regressions was the number of days worked during the first 3 days of illness. The final models contained the following independent variables: access to telework; access to paid leave; employees are discouraged from coming to work when they have influenza-like symptoms; age; sex; education; fever; worked the day before illness; having a lot of control over taking days off for illnesses; full-time worker; and employee type. Sixty-eight records were excluded because of missing values, and an additional 3 records were excluded because laboratory confirmation of influenza by real-time reverse transcription PCR was not available.

†p<0.05.

‡p<0.01.

reported going to work always or most of the time when they have a cold or influenza, compared with those experiencing more serious illnesses, injuries, or major physical problems (21). Reasons for working while experiencing influenza-like illness (ILI) include still being able to perform job duties, not feeling bad enough to miss work, not thinking their illness is contagious or could make other persons sick, and professional obligation to co-workers (22). We have documented that workplace cultures that encourage employees to refrain from coming to work when ill may play a crucial role in keeping workers away from the workplace when sick. In this study, persons with access to paid leave worked fewer days overall and at the usual workplace while ill. Two previous studies reported that access to paid sick days was associated with staying home for medically confirmed ILI or influenza (23,24), and 1 study found no association between having paid sick leave benefits and staying home from work because of ILI (25). These 3 studies did not assess telework.

We found that workers with access to telework used this benefit to work more total days while ill than those without it. Access to telework may enable persons to work from home on a day that they might otherwise have to take a sick day to comply with the “stay home when sick” recommendation. Availability of telework options is therefore possibly beneficial from the employer’s perspective in terms of reduced sick leave usage and preserved productivity. However, we observed little difference between workers who have access to telework and those who do not regarding the number of days worked at the usual workplace while sick. This finding suggests that just having telework policies in place may not

be sufficient to keep workers with access to telework from going to their workplace while sick. More effort is needed to encourage sick workers with telework access to work from home instead of at their usual workplace. In contrast to our findings with regard to telework, a previous study of workers in 3 large US companies (a national retail chain, a transportation company, and a durable goods manufacturing company) during the 2007–08 influenza season reported that workers who could telework had a 30% lower rate of attending work at their usual worksite when they had severe ILI symptoms (26). However, the authors acknowledged that their study was based on a convenience sample of only 3 employers, which limited the generalizability of their findings.

The 2017–18 influenza season, during which influenza A(H3N2) viruses predominated, was a high-severity season with widespread influenza activity across the country for an extended period (27). The influenza A(H3N2) strain typically causes more severe symptoms than the influenza A(H1N1) strain (28). The health-related workplace absenteeism rate in the 2017–18 influenza season was higher than the average rate of the previous 5 seasons (29). It is unknown whether the findings of this study would be similar in a less-severe influenza season. However, our results were similar for influenza-negative ARI cases, which are usually less severe than influenza cases. Thus, it seems likely that the findings would be similar in less-severe influenza seasons.

Our study has some limitations. First, almost one third of eligible adults were excluded from the analysis because of invalid responses regarding work attendance during the first 3 days of illness. However, similar results were seen for the Washington and

Wisconsin sites, which had higher rates of valid responses than the other 3 sites. Second, we assessed work attendance during the first 3 days of illness. Further research on work attendance during the subsequent days of illness may be helpful. Third, our study was conducted among workers with medically attended ARI. The findings may not be generalizable to workers with non-medically attended ARI, which tends to be less severe (30). Fourth, our study indicates that employees with access to telework worked more days overall than those without telework access. We did not, however, assess actual levels of workplace productivity or measures of output. Adults may have reduced work performance if they worked, whether on-site or remotely from home, while not feeling well (13,31). Fifth, our definition of paid leave included both paid sick days and paid vacation days. Because paid vacation leave may be less flexible than paid sick leave for taking time off on short notice for an unexpected illness (15), more research is needed to assess the effect of paid sick days on work attendance among persons with ARI or influenza. Finally, although we adjusted for potential confounding variables, an observational study such as ours cannot rule out the possibility that unmeasured variables (e.g., occupation) may have distorted the results. However, the proportion who worked the day before illness, which represents baseline measurement of the outcome, was similar between the intervention and control groups (e.g., telework access versus no telework access), indicating that the groups were initially comparable with a lower likelihood of the presence of confounding variables (32).

The desired public health result of employee access to paid leave and telework is an increased ability to comply with the public health recommendation to stay home when ill, which helps decrease risk of disease transmission in the workplace. Ideally, staying home when ill with a respiratory infectious disease should eventually become commonplace behavior or even a social norm. Having access to paid leave is likely a critical enabling factor that reduces financial barriers to staying away from work when ill (33–35). Organizational policies that are conducive to providing paid leave are therefore critically needed, but almost equally crucial are supportive business practices that actively encourage employees to stay home when sick. Therefore, both broader macro-level policy interventions and stimulation of business culture change at a micro level of individual work organizations, possibly even individual teams, may be necessary to help reduce the transmission of ARI and seasonal or pandemic influenza in workplaces.

This work was supported by the Centers for Disease Control and Prevention. The University of Pittsburgh was also supported by the National Institutes of Health (grant no. UL1 TR001857).

About the Author

Dr. Ahmed is a senior epidemiologist at the Division of Global Migration and Quarantine, National Center for Emerging and Zoonotic Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia, USA. His research interests include prevention and control of seasonal and pandemic influenza.

References

1. Biggerstaff M, Chen J, Rolfes M, O'Halloran A, Garg S, Zhou F, et al. The economic burden of influenza in the United States since 2009. Options X for the Control of Influenza Conference; Singapore; August 28–September 1, 2019.
2. Fendrick AM, Monto AS, Nightengale B, Sarnes M. The economic burden of non-influenza-related viral respiratory tract infection in the United States. *Arch Intern Med*. 2003;163:487–94. <https://doi.org/10.1001/archinte.163.4.487>
3. Van Wormer JJ, King JP, Gajewski A, McLean HQ, Belongia EA. Influenza and workplace productivity loss in working adults. *J Occup Environ Med*. 2017;59:1135–9. <https://doi.org/10.1097/JOM.0000000000001120>
4. Bureau of Labor Statistics. The Employment Situation—August 2017 [cited 2019 Mar 3]. https://www.bls.gov/news.release/archives/empisit_09012017.htm
5. Edwards CH, Tomba GS, de Blasio BF. Influenza in workplaces: transmission, workers' adherence to sick leave advice and European sick leave recommendations. *Eur J Public Health*. 2016;26:478–85. <https://doi.org/10.1093/eurpub/ckw031>
6. Grohskopf LA, Sokolow LZ, Broder KR, Olsen SJ, Karron RA, Jernigan DB, et al. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices—United States, 2016–17 influenza season. *MMWR Recomm Rep*. 2016;65:1–54. <https://doi.org/10.15585/mmwr.rr6505a1>
7. Centers for Disease Control and Prevention. Estimates of influenza vaccination coverage among adults—United States, 2017–18 flu season [cited 2018 Dec 7]. <https://www.cdc.gov/flu/fluview/coverage-1718estimates.htm>
8. Qualls N, Levitt A, Kanade N, Wright-Jegade N, Dopson S, Biggerstaff M, et al.; CDC Community Mitigation Guidelines Work Group. Community mitigation guidelines to prevent pandemic influenza—United States, 2017. *MMWR Recomm Rep*. 2017;66:1–34. <https://doi.org/10.15585/mmwr.rr6601a1>
9. Ahmed F, Zviedrite N, Uzicanin A. Effectiveness of workplace social distancing measures in reducing influenza transmission: a systematic review. *BMC Public Health*. 2018;18:518. <https://doi.org/10.1186/s12889-018-5446-1>
10. Bureau of Labor Statistics. American Time Use Survey—2018 results [cited 2019 Jun 26]. <https://www.bls.gov/news.release/pdf/atus.pdf>
11. Bureau of Labor Statistics. Employee benefits in the United States—March 2018 [cited 2019 Jan 11]. <https://www.bls.gov/news.release/pdf/ebs2.pdf>
12. Jackson ML, Chung JR, Jackson LA, Phillips CH, Benoit J, Monto AS, et al. Influenza vaccine effectiveness in the United States during the 2015–2016 season. *N Engl J Med*. 2017;377:534–43. <https://doi.org/10.1056/NEJMoa1700153>

13. Petrie JG, Cheng C, Malosh RE, VanWormer JJ, Flannery B, Zimmerman RK, et al. Illness severity and work productivity loss among working adults with medically attended acute respiratory illnesses: US Influenza Vaccine Effectiveness Network 2012–2013. *Clin Infect Dis*. 2016;62:448–55.
14. Mateyka PJ, Rapino MA, Landivar LC. Home-based workers in the United States: 2010 [cited 2019 Jun 26]. <https://www.census.gov/prod/2012pubs/p70-132.pdf>
15. Clemans-Cope L, Perry CD, Kenney GM, Pelletier JE, Pantell MS. Access to and use of paid sick leave among low-income families with children. *Pediatrics*. 2008;122:e480–6. <https://doi.org/10.1542/peds.2007-3294>
16. Stewart WF, Ricci JA, Leotta C. Health-related lost productive time (LPT): recall interval and bias in LPT estimates. *J Occup Environ Med*. 2004;46(Suppl):S12–22. <https://doi.org/10.1097/01.jom.0000126685.59954.55>
17. UCLA Institute for Digital Research and Education. Zero-inflated Poisson regression: SAS data analysis examples [cited 2019 Jun 28]. <https://stats.idre.ucla.edu/sas/dae/zero-inflatedpoisson-regression/>
18. Hensing G.; Swedish Council on Technology Assessment in Health Care (SBU). Chapter 4. Methodological aspects in sickness-absence research. *Scand J Public Health Suppl*. 2004;63(63_suppl):44–8. <https://doi.org/10.1080/14034950410021844>
19. Carrat F, Vergu E, Ferguson NM, Lemaître M, Cauchemez S, Leach S, et al. Time lines of infection and disease in human influenza: a review of volunteer challenge studies. *Am J Epidemiol*. 2008;167:775–85. <https://doi.org/10.1093/aje/kwm375>
20. Becker NG, Wang D. Can antiviral drugs contain pandemic influenza transmission? *PLoS One*. 2011;6:e17764. <https://doi.org/10.1371/journal.pone.0017764>
21. National Public Radio. The workplace and health. May 2016 [cited 2019 Jan 10]. <https://www.npr.org/documents/2016/jul/workplace-health-poll.pdf>
22. Chiu S, Black CL, Yue X, Greby SM, Laney AS, Campbell AP, et al. Working with influenza-like illness: presenteeism among US health care personnel during the 2014–2015 influenza season. *Am J Infect Control*. 2017;45:1254–8. <https://doi.org/10.1016/j.ajic.2017.04.008>
23. Piper K, Youk A, James AE III, Kumar S. Paid sick days and stay-at-home behavior for influenza. *PLoS One*. 2017;12:e0170698. <https://doi.org/10.1371/journal.pone.0170698>
24. Asfaw A, Rosa R, Pana-Cryan R. Potential economic benefits of paid sick leave in reducing absenteeism related to the spread of influenza-like illness. *J Occup Environ Med*. 2017;59:822–9. <https://doi.org/10.1097/JOM.0000000000001076>
25. Zhai Y, Santibanez TA, Kahn KE, Black CL, de Perio MA. Paid sick leave benefits, influenza vaccination, and taking sick days due to influenza-like illness among U.S. workers. *Vaccine*. 2018;36:7316–23. <https://doi.org/10.1016/j.vaccine.2018.10.039>
26. Rousculp MD, Johnston SS, Palmer LA, Chu BC, Mahadevia PJ, Nichol KL. Attending work while sick: implication of flexible sick leave policies. *J Occup Environ Med*. 2010;52:1009–13. <https://doi.org/10.1097/JOM.0b013e3181f43844>
27. Garten R, Blanton L, Elal AIA, Alabi N, Barnes J, Biggerstaff M, et al. Update: influenza activity in the United States during the 2017–18 season and composition of the 2018–19 influenza vaccine. *MMWR Morb Mortal Wkly Rep*. 2018;67:634–42. <https://doi.org/10.15585/mmwr.mm6722a4>
28. Hayward AC, Fragaszy EB, Bermingham A, Wang L, Copas A, Edmunds WJ, et al.; Flu Watch Group. Comparative community burden and severity of seasonal and pandemic influenza: results of the Flu Watch cohort study. *Lancet Respir Med*. 2014;2:445–54. [https://doi.org/10.1016/S2213-2600\(14\)70034-7](https://doi.org/10.1016/S2213-2600(14)70034-7)
29. Groenewold MR, Burrer SL, Ahmed F, Uzicanin A, Luckhaupt SE. Health-related workplace absenteeism among full-time workers – United States, 2017–18 influenza season. *MMWR Morb Mortal Wkly Rep*. 2019;68:577–82. <https://doi.org/10.15585/mmwr.mm6826a1>
30. Fragaszy EB, Warren-Gash C, White PJ, Zambon M, Edmunds WJ, Nguyen-Van-Tam JS, et al.; Flu Watch Group. Effects of seasonal and pandemic influenza on health-related quality of life, work and school absence in England: results from the Flu Watch cohort study. *Influenza Other Respir Viruses*. 2018;12:171–82. <https://doi.org/10.1111/irv.12506>
31. Palmer LA, Rousculp MD, Johnston SS, Mahadevia PJ, Nichol KL. Effect of influenza-like illness and other wintertime respiratory illnesses on worker productivity: The child and household influenza-illness and employee function (CHIEF) study. *Vaccine*. 2010;28:5049–56. <https://doi.org/10.1016/j.vaccine.2010.05.011>
32. Harris AD, Bradham DD, Baumgarten M, Zuckerman IH, Fink JC, Perencevich EN. The use and interpretation of quasi-experimental studies in infectious diseases. *Clin Infect Dis*. 2004;38:1586–91. <https://doi.org/10.1086/420936>
33. Blake KD, Blendon RJ, Viswanath K. Employment and compliance with pandemic influenza mitigation recommendations. *Emerg Infect Dis*. 2010;16:212–8. <https://doi.org/10.3201/eid1602.090638>
34. Blendon RJ, Koonin LM, Benson JM, Cetron MS, Pollard WE, Mitchell EW, et al. Public response to community mitigation measures for pandemic influenza. *Emerg Infect Dis*. 2008;14:778–86. <https://doi.org/10.3201/eid1405.071437>
35. Bleser WK, Miranda PY, Salmon DA. Child influenza vaccination and adult work loss: reduced sick leave use only in adults with paid sick leave. *Am J Prev Med*. 2019;56:251–61. <https://doi.org/10.1016/j.amepre.2018.09.013>

Address for correspondence: Faruque Ahmed, Centers for Disease Control and Prevention, 1600 Clifton Road NE, Mailstop V18-2, Atlanta, GA 30329-4027, USA; email: fahmed@cdc.gov

Preclinical Detection of Prions in Blood of Nonhuman Primates Infected with Variant Creutzfeldt-Jakob Disease

Luis Concha-Marambio,¹ Marcelo A. Chacon, Claudio Soto

Variant Creutzfeldt-Jakob disease (vCJD) is caused by prion infection with bovine spongiform encephalopathy and can be transmitted by blood transfusion. Protein misfolding cyclic amplification (PMCA) can detect prions in blood from vCJD patients with 100% sensitivity and specificity. To determine whether PMCA enables prion detection in blood during the preclinical stage of infection, we performed a blind study using blood samples longitudinally collected from 28 control macaques and 3 macaques peripherally infected with vCJD. Our results demonstrate that PMCA consistently detected prions in blood during the entire preclinical stage in all infected macaques, without false positives from noninfected animals, when using the optimized conditions for amplification of macaque prions. Strikingly, prions were detected as early as 2 months postinoculation (>750 days before disease onset). These findings suggest that PMCA has the potential to detect vCJD prions in blood from asymptomatic carriers during the preclinical phase of the disease.

Prion diseases are rare and fatal neurodegenerative diseases transmitted by infectious proteinaceous agents called prions, which are composed of a disease-associated misfolded version (PrP^{Sc}) of the normally expressed prion protein (PrP^C) (1–3). Prion diseases affect humans and various species of mammals, including cattle, sheep and goats, and cervids (4). In humans, Creutzfeldt-Jakob disease (CJD) is the most common prion disease and can be sporadic (sCJD), familial (fCJD), or iatrogenically transmitted (iCJD). In the 1990s, a new variant of CJD (vCJD) was described in the United Kingdom (5); this variant is a result of interspecies transmission of bovine spongiform encephalopathy (BSE) prions from cattle to

humans (6–8). Unlike classical CJD, vCJD presents an extensive peripheral deposition, with demonstrated PrP^{Sc} accumulation in various peripheral tissues, particularly lymphoreticular tissues (spleen, appendix, and tonsil) (9–11). vCJD has been suggested to be transmitted among humans by transfusion of nonleukodepleted erythrocytes or purified protein factors from plasma (12,13). A study performed in transgenic mice models to compare the risk for primary and secondary transmission of vCJD showed that, although transmission of BSE to humans is probably restricted by the presence of a major species barrier, secondary transmission between humans has a substantially reduced barrier (14). Moreover, this study showed that all humans, irrespective of PrP codon-129 genotype, could be susceptible to secondary transmission of vCJD through routes such as blood transfusion. A lengthy preclinical disease is predicted by these models, which may represent a risk for further disease transmission (14).

Detection of prions in blood has been hampered because of the unconventional nature of prions (absence of nucleic acids) and the minute amount of them circulating in blood, making them difficult to detect even by bioassay in transgenic mice (15). Extraction protocols to enrich PrP^{Sc} from blood have been developed and coupled to antibody detection methods (16), but sensitivity was only 70% for end-stage disease blood samples (17). In contrast with conventional biochemical methods, we developed a detection platform for self-replicating PrP^{Sc} called protein misfolding cyclic amplification (PMCA) (18). During PMCA, small amounts of infectious PrP^{Sc} aggregates convert PrP^C into PrP^{Sc}, producing larger protein aggregates that are fragmented into many smaller nucleating seeds for the continued *in vitro* conversion of PrP^C into PrP^{Sc} (18–20). This elongation/

Author affiliations: University of Texas, Houston, Texas, USA (L. Concha-Marambio, M.A. Chacon, C. Soto); Universidad de los Andes, Santiago, Chile (L. Concha-Marambio, C. Soto)

DOI: <https://doi.org/10.3201/eid2601.181423>

¹Current affiliation: Amprion, Inc., San Diego, California, USA.

fragmentation process is performed cyclically to exponentially amplify PrP^{Sc}, facilitating its detection. PMCA can amplify vCJD prions from brain homogenate (BH) diluted 10⁻¹⁰- to 10⁻¹¹-fold, reaching a 10–100 billion-fold amplification (21). This level of amplification has allowed detection of prions in blood and urine samples from vCJD patients (21,22), reaching sensitivities and specificities approaching 100% in experiments confirmed in various laboratories (23,24).

It is unclear how early prions can be detected in the blood of infected persons at the preclinical stage of the disease. In this study, we analyzed the preclinical detection of prions in blood samples from macaques (*Macaca fascicularis*) experimentally infected with the vCJD agent as an animal model for infected asymptomatic human carriers.

Materials and Methods

Nonhuman Primate Experimental Infection and Longitudinal Blood Collection

Experimental inoculation of macaques and collection of blood materials was done at the Food and Drug Administration (FDA) laboratory (Silver Spring, Maryland, USA) as previously described (25). In brief, macaque-adapted vCJD (m-vCJD) was generated by intracerebral injection with BH from a confirmed vCJD patient. A 10% BH solution from the terminally ill macaque was used for intraperitoneal (2 mL) and intravenous (1 mL) inoculation into the 3 macaques used in this study. Blood samples were collected every 2 months for the first year and every month for the rest of the experiment. Samples were collected in either citrate phosphate dextrose buffer or EDTA. Part of the blood was separated to prepare plasma, buffy coat (BC), and erythrocyte components. We received panels of deidentified samples for blind experiments (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/18-1423-App1.pdf>).

Processing of Blood Samples

We previously described a sarkosyl precipitation method to extract vCJD prions from blood and remove interferences in the PMCA assay (21). In brief, we incubated 250 or 500 μ L of blood or blood fractions with an equal volume of 20% sarkosyl for 10 min at room temperature. We then ultracentrifuged the mixture at 100,000 $\times g$ for 1 h at 4°C. After washing the pellet, we resuspended it in PMCA substrate for subsequent amplification and detection. Although this procedure might be difficult to implement for routine testing of blood samples, we have previously shown that processing and centrifugation

may be overcome by working with a smaller volume of blood samples (21).

PMCA Protocol

The PMCA protocol for amplification of human prions has been described elsewhere (20–22), although some modifications were made for the amplification of preclinical samples (described later). As PMCA substrate, we used 10% BH from transgenic mice expressing human PrP^C with methionine/methionine at codon 129 (TgHu129M). These mice express PrP at 16-fold the levels of expression of endogenous protein. We prepared BH in conversion buffer (PBS supplemented with 150 mmol/L NaCl and 1% TritonX-100) with protease inhibitors (complete, EDTA-free; Roche, <https://www.roche.com>). After performing homogenization, we removed debris by centrifugation at 800 $\times g$ at 4°C for 1 min. We vortexed, aliquoted, and stored the supernatant at –80°C until use. We supplemented the homogenate with 0.05% digitonin and 12 mmol/L EDTA; in some cases we also added 100 μ g/mL of heparin as indicated. We placed samples in 0.2 mL PCR tubes (Eppendorf, <https://www.eppendorf.com>) containing 3 polytetrafluoroethylene beads (Hoover Precision Bioproducts, <http://www.hooverprecision.com>) and sonicated them for 30 s every 30 min in a microplate sonicator (QSonica Q700, <https://www.sonicator.com>), using a titanium horn. When we amplified blood and blood fractions, the first round of PMCA included 144 cycles followed by subsequent rounds of 96 cycles, unless otherwise specified. After a PMCA round, we started a new PMCA round by adding 10 μ L of each sample to new PCR tubes containing 3 beads and 90 μ L of fresh substrate. When analyzing BC samples, we made a pseudo-passage of the first round, in which we added 90 μ L of fresh substrate to 100 μ L from the first round with no dilution of the material, to reduce the viscosity of the solution by adding more reaction mixture containing substrate. We used diluted samples of vCJD BH as positive controls. We prepared this material from the frontal cortex of a human with pathology-confirmed vCJD.

Proteinase K Digestion and Western Blotting

After PMCA, we digested all samples using proteinase K (PK) at a concentration of 50 μ g/mL for 1 h at 37°C. We stopped PK digestion by boiling the sample at 100°C for 10 min after mixing with NuPage or Novex sample loading buffer (NuPage Bis-Tris gels with MES buffer and Novex Tris-Glycine gels with Tris-SDS buffers). We transferred proteins onto nitrocellulose membranes (0.45 μ m; Amersham Biosciences, <https://www.gelifesciences.com>) and probed them with

monoclonal antibody 6D11 (1:20,000) for 1 h at room temperature, while we used secondary anti-mouse antibody (Sigma, <https://www.sigmaaldrich.com>) at 1:3,000 dilution and incubated for 1 h. We used ECL chemiluminescent reagent (Amersham) and a Chemidoc imaging system (BioRad, <https://www.bio-rad.com>) to develop and capture the images.

Results

m-vCJD Prion Conversion of Human PrP^C into PrP^{Sc} in PMCA

We previously showed that PMCA can efficiently amplify vCJD prions using TgHu-PrP^C substrate (21). Because the sequence of macaque and human PrP has 9 aa differences, we first evaluated whether human PrP^C could be converted into PrP^{Sc} in PMCA by m-vCJD prions. Therefore, we prepared 10-fold serial dilutions of BH from the 3 macaques peripherally infected with the vCJD agent and analyzed them with 3 rounds of PMCA, alongside similar dilutions of BH from a human with confirmed vCJD (Figure 1). In the first round of PMCA, the detection limit in the macaque BHs was 10^{-4} to 10^{-5} , whereas human vCJD was detectable up to a dilution of 10^{-6} . In the second round, we detected vCJD and m-vCJD BH at 10^{-9} to 10^{-10} dilutions, and in the third round the detection limit decreased to 10^{-10} or 10^{-11} depending on the macaque. In summary, 3 rounds of PMCA were necessary to amplify prions in m-vCJD BH dilutions to similar levels as prions in human vCJD BH dilutions, albeit with a reduced conversion efficiency in the first round.

Detection of m-vCJD Prions by PMCA from Blood Collected at Final Bleed

We wanted to determine whether endogenous m-vCJD prions in blood could be detected using

the current PMCA conditions. We processed whole blood, plasma, BC, and erythrocyte samples by using sarkosyl precipitation and analyzed them with 4 rounds of PMCA (Figure 2). Similar to our previous report in humans, prions in the m-vCJD whole blood samples were detected by PMCA in the second round, whereas m-vCJD erythrocytes displayed lower amplification with 1 of 3 samples remaining negative after 4 PMCA rounds. Prions in m-vCJD plasma and BC samples were readily detectable by the second round in 2 of 3 infected macaques. As expected, whole blood, blood fractions, and BH from a control macaque were all negative after 4 rounds of PMCA.

To determine the reproducibility and stability of these samples, we analyzed a panel of 50 deidentified plasma and whole blood samples from infected and control macaques that were subjected to 1–6 freeze/thaw cycles. Using plasma, we detected 12 of 12 samples from infected macaques, whereas only 9 of 12 whole blood samples were found positive (data not shown). Next, we analyzed an additional panel of 93 blinded plasma samples from 28 control and 2 m-vCJD infected macaques, including terminal bleed samples and samples collected 1 month before clinical signs (Figure 3; Appendix Table 1). The panel included 96 samples, but 3 tubes were partially or totally open upon delivery and were excluded from the study, while keeping identification numbers provided by FDA for the rest of the blinded samples (1–96). After 4 rounds of PMCA, we detected prions in 3 of 3 replicates from 2 m-vCJD samples collected at the final bleed; all the controls were negative in all 3 replicates, except for 1 macaque that was negative in 2 of 3 replicates (Appendix Table 1). However, under these conditions, we were unable to detect m-vCJD preclinical samples. Therefore, the sensitivity of this PMCA setting was not sufficient for preclinical detection of m-vCJD prions in blood.

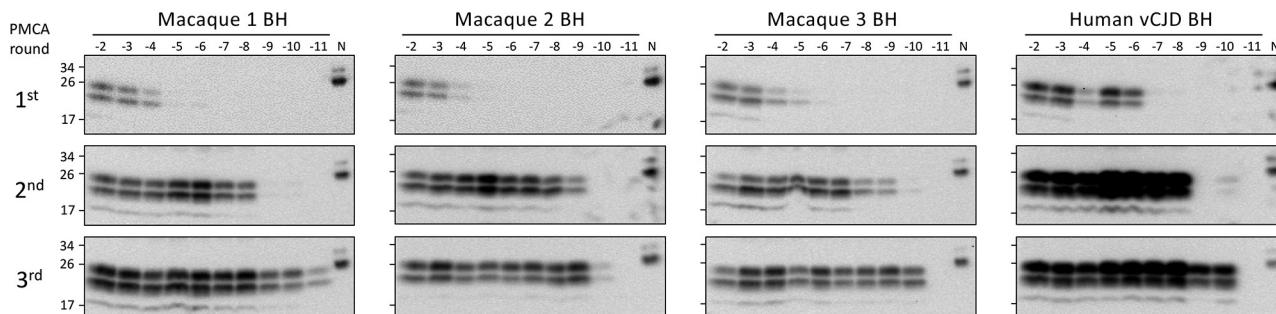


Figure 1. Amplification of macaque-adapted vCJD prions by PMCA. BH from 3 macaques peripherally infected with macaque-adapted vCJD was serially diluted and amplified by 3 rounds of PMCA, using BH from transgenic mice expressing human normally expressed prion protein with methionine at codon 129 (TgHu129M) as substrate. Human BH from a vCJD patient was analyzed as positive control. After completion of the 3 rounds of PMCA, samples were digested with 50 μ g/mL of proteinase K and analyzed by Novex SDS-PAGE (<https://www.thermofisher.com>). N refers to transgenic mouse normal BH without proteinase K treatment, which was used as a migration control. BH, brain homogenate; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.

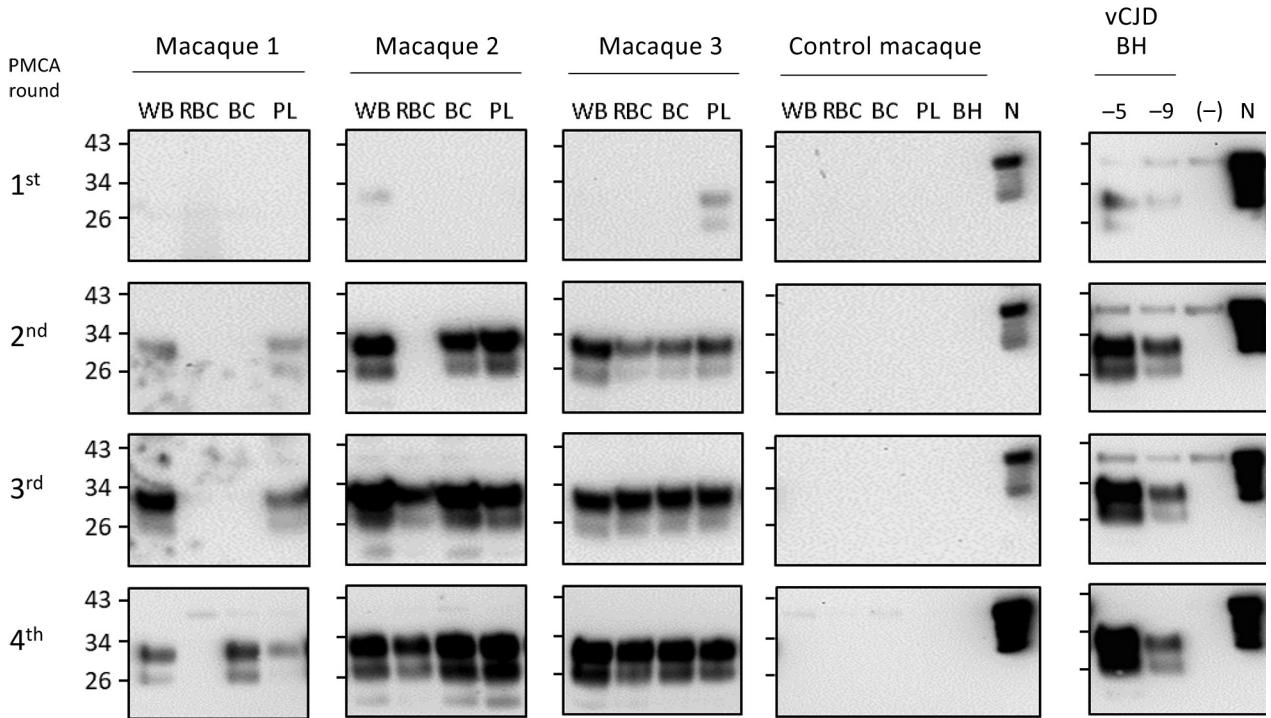


Figure 2. Detection of macaque-adapted vCJD prions in blood and blood fractions of macaques collected at the final bleed. WB, RBC, BC, and PL collected at the terminal bleed of 3 macaques infected with macaque-adapted vCJD and 1 noninfected control were processed and analyzed by 4 PMCA rounds. Dilutions of vCJD BH of 10^{-5} (-5) and 10^{-9} (-9) were analyzed as positive controls; an unseeded reaction (-) was used as a negative control. After completion of the 4 rounds of PMCA, samples were digested with 50 $\mu\text{g}/\text{mL}$ of proteinase K and analyzed by Western blot. N refers to transgenic mouse normal BH without proteinase K treatment, which was used as a migration control. BC, buffy coat; BH, brain homogenate; PL, plasma; PMCA, protein misfolding cyclic amplification; RBC, erythrocytes; vCJD, variant Creutzfeldt-Jakob disease; WB, whole blood.

Standardization of PMCA for Preclinical Detection of Prions in Blood Samples

The limiting factor for prion amplification using blood samples was probably the conversion inefficiency during the first PMCA round. During this round, m-vCJD prions needed to overcome a small species barrier and natural PMCA inhibitors remaining from blood. Because heparin has been shown to boost the *in vitro* replication of human prions (26), we studied the effect of heparin on the replication of brain m-vCJD prions, using human PrP^C as substrate (Figure 4, panel A). Addition of heparin to the PMCA substrate (hep-substrate) enhanced the replication of m-vCJD prions by 3 orders of magnitude in a single round of 96 cycles. Moreover, the amount of PrP^{res} detected after PK digestion by Western blot was clearly higher with hep-substrate, which also decreases the chance of false negatives. Therefore, we used enhanced PMCA with hep-substrate for detection of m-vCJD prions in blood and blood fractions collected at the final bleed. This modification allowed detection in most samples after only 1 round of PMCA (data not shown). However, we could detect prions

in only 1 of 3 preclinical plasma samples, although the positive sample required only 1 PMCA round of 144 cycles (data not shown). The amount of PrP^{Sc} in blood at the preclinical stage of the disease is probably very low; thus, we increased the sample volume from 100 μL to 500 μL and compared the detection of prions in preclinical m-vCJD plasma and BC (Figure 4, panel B). Three rounds of PMCA allowed detection of prions in all 3 preclinical m-vCJD BC samples, whereas only 2 m-vCJD plasma samples were positive after 4 PMCA rounds. This is not entirely surprising, because it has been extensively reported that the largest concentration of prions in blood is located in the BC fraction (27,28). Therefore, given the expected higher concentration of prions in BC and availability of samples, we decided to use BC for the experiments of preclinical detection.

Prion Detection with High Specificity and Sensitivity Throughout Preclinical Stage

Using enhanced-PMCA and BC samples, we performed a comprehensive study of PrP^{Sc} detection in longitudinally collected blood samples from

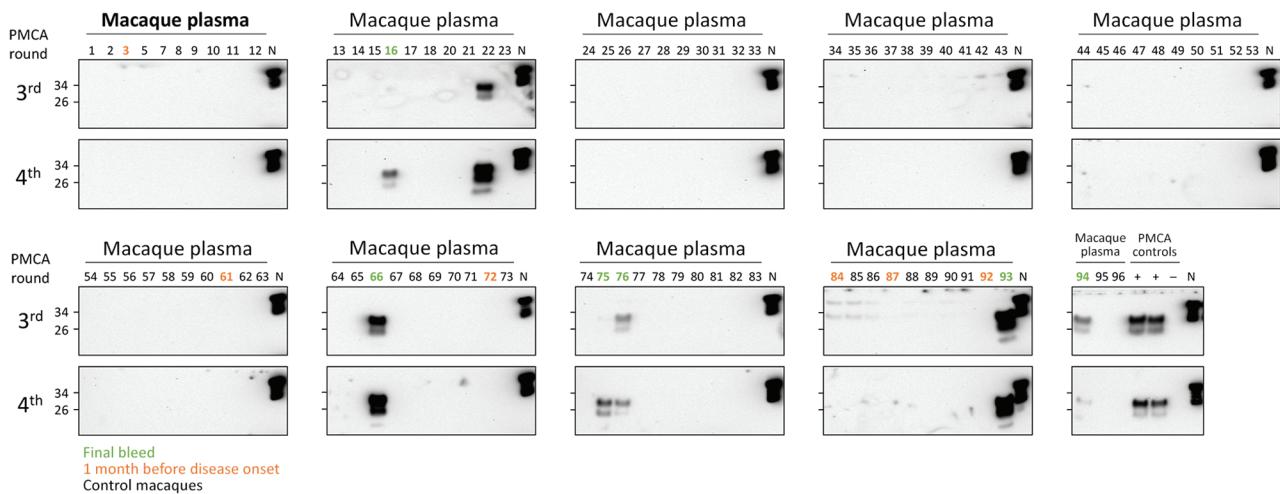


Figure 3. PMCA analysis of deidentified plasma samples from macaques infected with macaque-adapted vCJD and control macaques. Plasma samples from 2 infected (M1 and M3) and 28 control macaques were sarkosyl precipitated and analyzed by 4 rounds of PMCA. This panel of samples included 6 plasma samples collected at the final bleed (M1, #16, #75, #76; M3, #66, #93, #94), 6 plasma samples collected 1 month before disease onset (M1, #72, #84, #92; M3, #3, #61, #87), and 81 plasma samples from control macaques (93 samples total). Tubes with samples #4, #6, and #19 were partially or totally open upon arrival and were not analyzed. Dilutions of vCJD BH of 10^{-5} and 10^{-9} were used as a positive control; the negative control was the unseeded reaction. After completion of the 4 rounds of PMCA, samples from the third and fourth rounds were digested with 50 $\mu\text{g}/\text{mL}$ of proteinase K and then analyzed by Western blot. N refers to transgenic mouse normal BH without proteinase K treatment used as a migration control. BH, brain homogenate; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.

macaques peripherally infected with vCJD. The goal was to estimate sensitivity and specificity, as well as the earliest time point in which prions can be consistently detected in blood. Using 5 rounds of enhanced PMCA, we analyzed 140 blinded BC samples either collected from 23 uninfected macaques (Appendix Table 2) or the 3 m-vCJD challenged macaques throughout the entire length of infection; we analyzed 29 samples collected from 3 macaques in duplicate or quadruplicate (Appendix Tables 3–5). BC samples were heavily contaminated with erythrocytes, making them highly viscous. In turn, the pellets from these samples were consistently larger than the previous BC samples, which resulted in many PMCA reactions forming a paste that could not be pipetted to seed the second PMCA round. To work around this issue, we further modified the PMCA protocol by incorporating a pseudo-passage, in which we added 90 μL of hep-substrate to the first round of PMCA and performed amplification cycles for 2 more days. Subsequently, we performed 4 regular PMCA rounds; samples from the fourth and fifth rounds were PK digested and analyzed by Western blot (Figure 5). Of the 140 BC samples, we found 69 positives by PMCA from the 72 m-vCJD positive samples, whereas all the 68 controls were found negative. When we grouped all the replicates of each collected sample, our results showed that all collected samples from m-vCJD

macaques have ≥ 1 positive signal. The m-vCJD BC replicates that were negative (empty circles in Figure 6) did not correlate with earlier preclinical times, suggesting that these replicates were negative because of the quality of the sample rather than a particularly low concentration of prions that was below the detection limit. It is noteworthy that the 3 negative samples were labeled as having qualitative differences before analyzing the results (2 out of 3 were unusually viscous during sarkosyl precipitation, and the other had a unique colorless pellet). Because the specificity of PMCA is extremely high and we expect the quantity of PrP^{Sc} in blood at the preclinical phase of the disease to be low, we defined the collected samples as positive if ≥ 1 replicates are positive. Therefore, we consistently detected prions from all 3 macaques throughout the entire incubation period, starting from the first blood collection at 65 days postinoculation (dpi) until the final bleed. Thus, preclinical detection was achieved 759 days before onset (dbo) for macaques 1 and 2 and 644 dbo for macaque 3 (Figure 6). Considering all 140 samples separately, the detection of m-vCJD prions in BC samples from the preclinical panel reached a sensitivity of 95.8% (95% CI 88.3–99.1%) and a specificity of 100% (95% CI 94.7–100%). However, when grouping the replicates of each collected sample, sensitivity and specificity were 100%.

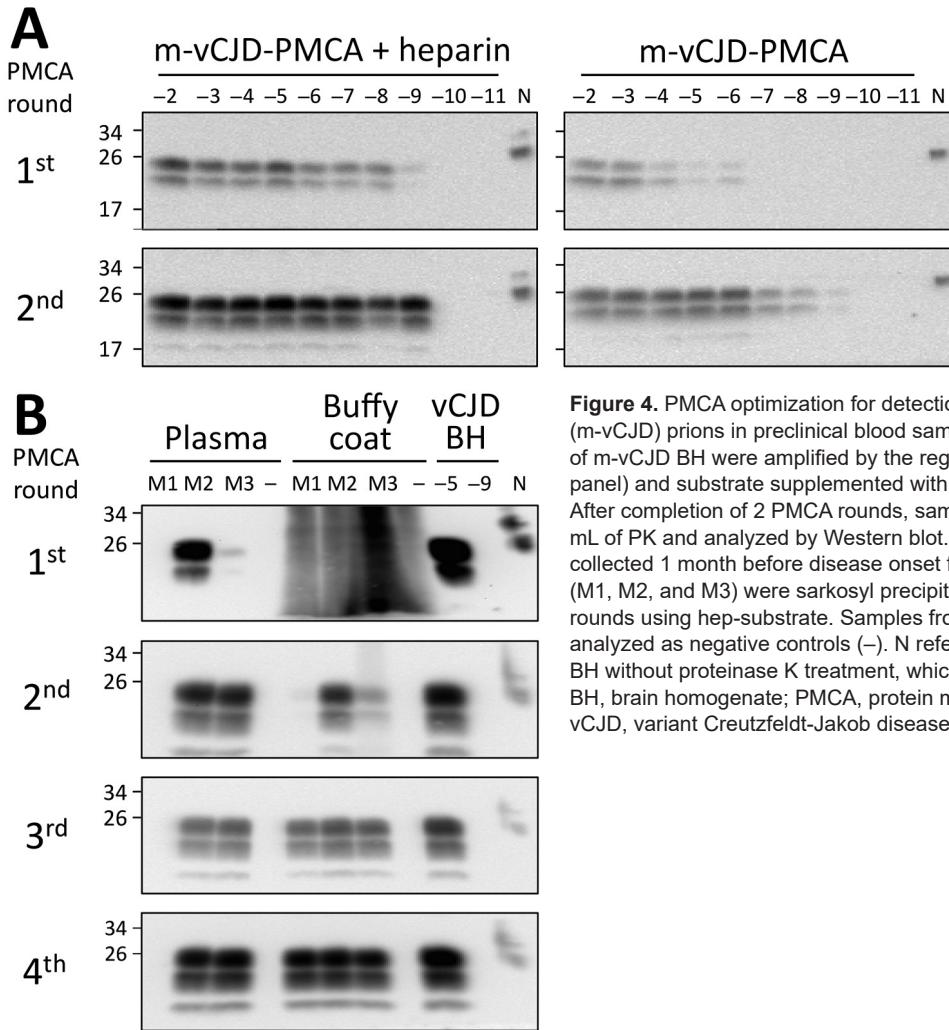


Figure 4. PMCA optimization for detection of macaque-adapted vCJD (m-vCJD) prions in preclinical blood samples. A) Tenfold serial dilutions of m-vCJD BH were amplified by the regular PMCA substrate (right panel) and substrate supplemented with 100 µg/mL heparin (left panel). After completion of 2 PMCA rounds, samples were digested with 50 µg/mL of PK and analyzed by Western blot. B) PL and BC (500 µL) samples collected 1 month before disease onset from 3 m-vCJD infected macaques (M1, M2, and M3) were sarkosyl precipitated and analyzed by 4 PMCA rounds using hep-substrate. Samples from noninfected macaques were analyzed as negative controls (-). N refers to transgenic mouse normal BH without proteinase K treatment, which was used as a migration control. BH, brain homogenate; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.

Prions in Very Early Stages as Endogenously Generated m-vCJD Prions, Not Part of Inoculum

Given the peripheral infection route used in this bioassay and the very early detection achieved by PMCA, there was a possibility of PMCA detecting the inoculum. This result is unlikely, however, because we have shown previously in rodent models that the half-life of radiolabeled PrP^{Sc} in blood is 3.24 h, and <1% of the injected dose remains detectable after 24 h (29,30). If the clearance rate is the same in macaques, the injected material should be eliminated from blood at a rate of 2 logs per day. Therefore, after 6 days the amount of inoculum remaining in blood would be equivalent to a 10⁻¹² dilution of m-vCJD BH. Thus, it is highly unlikely that the early detection is explained by the presence of the inoculum in blood 2 months after injection. Nevertheless, to shed light on this issue, we analyzed the second and third rounds of the PMCA-positive preclinical BC samples by Western blot (Figure 7),

considering that we previously established that the number of rounds needed to detect a sample as positive correlates with the amount of prions in the sample (31). In macaque 1, all samples were negative in the second round of PMCA. In the third round, samples collected 65 dpi were negative, whereas consistent positive signals started appearing 128 dpi (639 dbo). In macaques 2 and 3, BC samples collected closest to the inoculation time were negative in the second round of PMCA, whereas consistent detection started at 368 dpi (456 dbo) for macaque 2 and 429 dpi (280 dbo) for macaque 3. These results show that samples collected closer to the inoculation time were negative in initial PMCA rounds, indicating lower quantity of PrP^{Sc}. Conversely, samples collected at later times were positive in the second round, suggesting higher amounts of PrP^{Sc} in blood. These data allude to a buildup of prions in blood during the incubation period, suggesting that we are not detecting the inoculum.

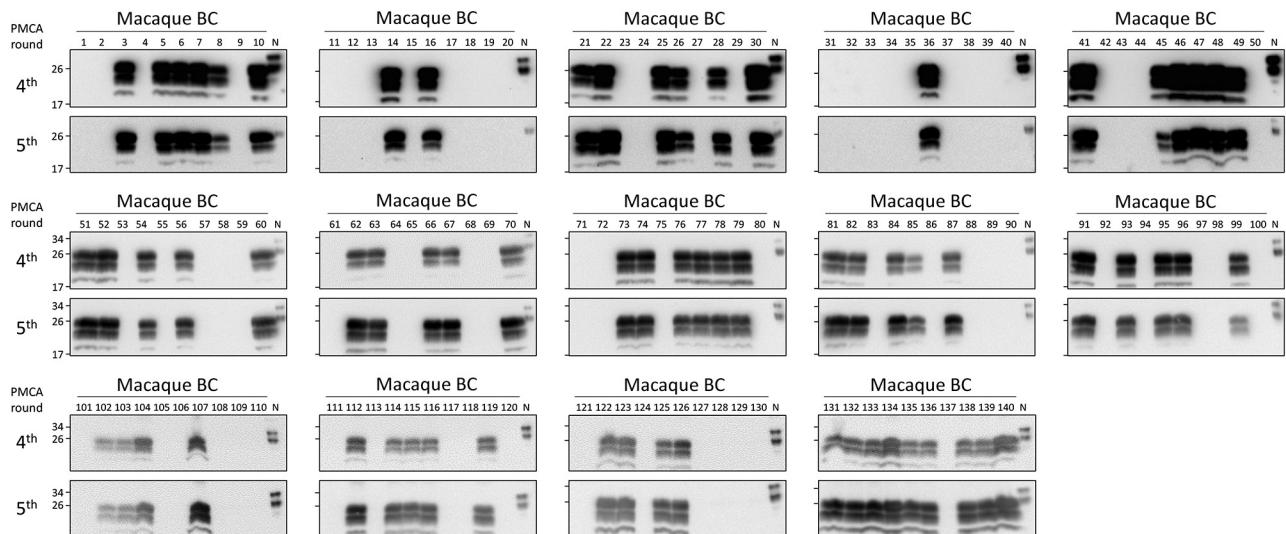


Figure 5. Preclinical detection of macaque-adapted vCJD prions in BC of peripherally infected macaques. A total of 140 deidentified samples (500 μ L each) were sarkosyl precipitated and analyzed by 5 rounds of PMCA. After amplification, samples from the fourth and fifth rounds were digested with 50 μ g/mL of PK and analyzed by Western blot. N refers to transgenic mouse normal BH without proteinase K treatment, which was used as a migration control. BH, brain homogenate; m-vCJD, macaque-adapted vCJD; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.

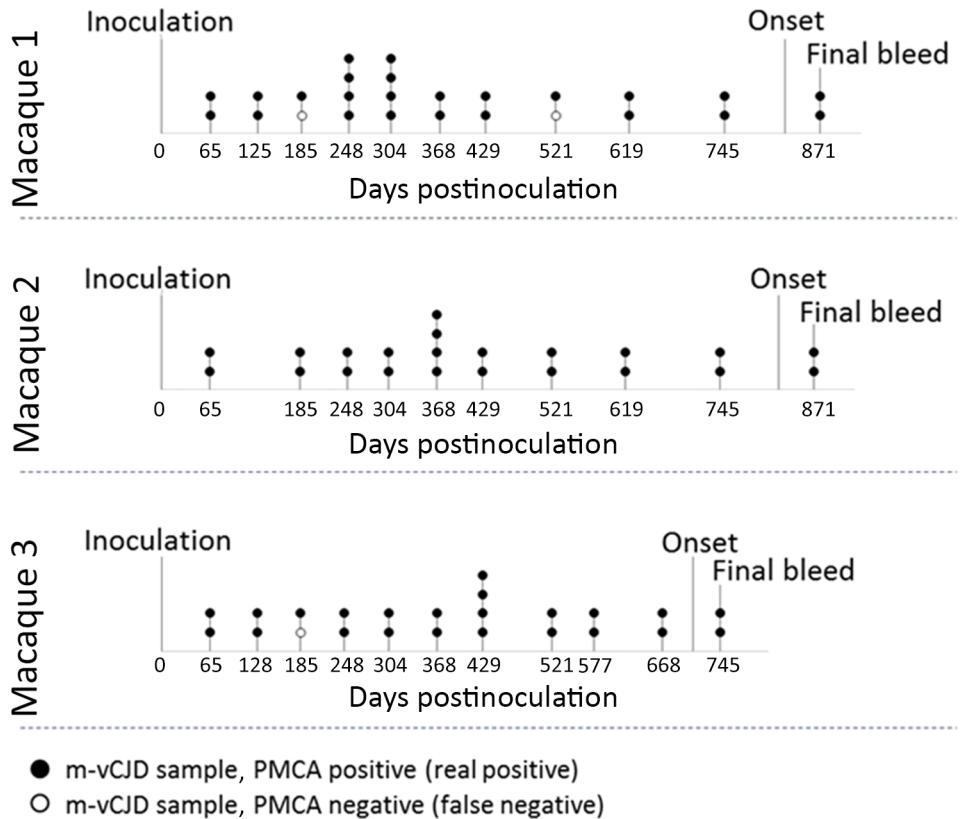
Discussion

The future of the vCJD epidemic is still uncertain, with recent estimations of 1 carrier of prion infection in every 2,000 persons who lived in the United Kingdom during the BSE epidemic (32). However, in a recent update from the same group, prions were detected in a cohort of persons born after the BSE epidemic, suggesting that the number of silent carriers of prions might be higher than originally anticipated. Before 2017, the population at risk was believed to be restricted to persons carrying Met-Met at codon 129 in the *PRNP* gene, because all clinical vCJD cases occurred in 129 methionine homozygotes (129MM). However, the confirmation of the first patient heterozygous for the *PRNP* codon 129 (129MV) has altered that perspective (33). Therefore, 89% of the UK population (42% 129MM and 47% 129MV) exposed to BSE are potential carriers who could harbor prions in their peripheral organs and blood. The likely transmission of vCJD through blood components has been reported and represents a risk for iatrogenic transmission of this disease (11–13,34). Although the policies implemented to control the BSE epidemic have contributed to the decline of vCJD cases (35), the number of persons silently carrying infectious prions in their peripheral organs and fluids is unknown. Therefore, a highly sensitive and specific detection method for prions is needed to screen the blood supply to ensure its safety.

The recent demonstration that PMCA enables the accurate detection of prions in blood of confirmed vCJD

patients was a major milestone in the quest for a vCJD blood test. Independent studies from us and another group obtained a 100% specificity and sensitivity for vCJD prion detection in blood during the clinical stage of the disease (21,24). Given that the incubation period for some human prion diseases can be >50 years (36), a test to screen blood needs to detect prions as early as possible during the asymptomatic stage of the disease. To analyze the efficacy of PMCA for preclinical detection of vCJD prions in blood, we used samples longitudinally collected throughout the incubation period from nonhuman primates that were deliberately infected with vCJD prions. The study included samples collected >2 years before the first neurologic symptoms and as little as 2 months after animals were infected. Considering each replicate individually, sensitivity of the assay was 96% and specificity of the assay was 100%. Considering individual animals (samples collected in duplicates and quadruplicates), sensitivity and specificity both reached 100%. Macaque 1 showed lower levels of PrP^{Sc} in blood (Figures 2; 4, panel B; 7), despite the indistinguishable disease progression described by McDowell et al. between macaques 1 and 2 (25). The difference in PrP^{Sc} levels in blood is probably the result of intrinsic animal-to-animal variability, but it could be an indication that PrP^{Sc} levels in blood and brain are independent, given that all 3 macaques showed similar levels of PrP^{Sc} in the brain. It could also suggest differences in the clearance of PrP^{Sc} from brain to blood, perhaps indicating changes in blood-brain

Figure 6. Schematic representation of the animals and samples used in study of preclinical detection of prions in blood of nonhuman primates infected with vCJD. The 72 m-vCJD samples previously analyzed by PMCA (Figure 5) were collected throughout the whole incubation period, starting 65 dpi until the final bleed. The first blood collection at 65 days postinoculation represents 759 (M1 and M2) and 644 (M3) days before the onset of the first neurologic signs. The 72 m-vCJD BC samples included 28 duplicates (represented as 2 circles in the timeline) and 4 quadruplicates (represented as 4 circles in the timeline). Open circles represent m-vCJD BC samples that were PMCA negative; dark circles represent m-vCJD BC samples that were PMCA positive. BH, brain homogenate; m-vCJD, macaque-adapted vCJD; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.



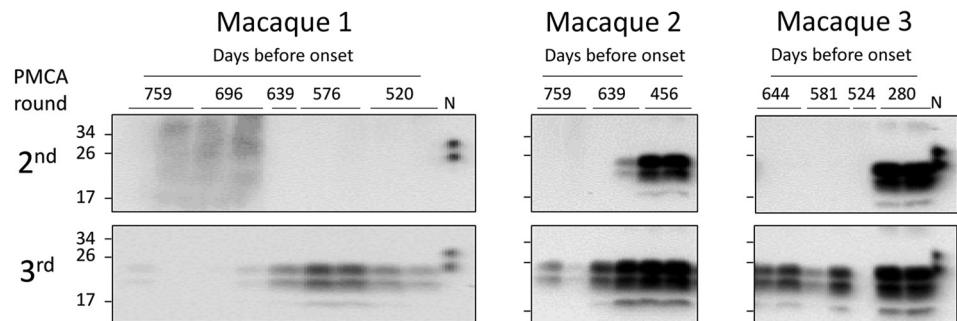
barrier tightness. The differences may also suggest different levels of peripheral prion replication. At this time we do not have enough information to distinguish among these possibilities.

Our results confirm and extend a previous report by Lacroux et al., who used a similar model in macaques infected with vCJD in which they detected prions 960 and 990 days before onset of the disease in 2 macaques, which showed clinical signs 43–46 months postinoculation (23). Given that we used an animal model experimentally infected with vCJD-BH,

we cannot necessarily conclude that similar detection levels will be obtained in human samples. In addition, PrP^{Sc} detection does not necessarily indicate that the material would be infectious in vivo, considering that PMCA is orders of magnitude more sensitive than the infectivity bioassay (31). This finding raises a difficult ethical issue of how to deal with persons who return a PMCA-positive blood test, especially considering that no treatment is available for this disease.

Unfortunately, few human blood samples collected before vCJD developed in donors are available

Figure 7. Detection of m-vCJD prions by PMCA in macaques during early stages of disease. These prions were probably endogenously generated rather than present in the inoculum. The second and third rounds of the PMCA-positive preclinical buffy coat samples were digested with 50 µg/mL of proteinase K and analyzed by Western blot. Samples were arranged from the earliest preclinical on the left to the closest to disease onset on the right. N refers to transgenic mouse normal BH without proteinase K treatment, which was used as a migration control. BH, brain homogenate; m-vCJD, macaque-adapted vCJD; PMCA, protein misfolding cyclic amplification; vCJD, variant Creutzfeldt-Jakob disease.



to test PMCA for preclinical detection in humans. In a previous study, researchers analyzed such samples from 2 donors and found them positive for PMCA (24). Overall, our results suggest that PMCA has the potential to be used as a screening method to improve the safety of the blood supply and perhaps as a tool to determine the prevalence of prion carriers in countries at high risk for vCJD (e.g., United Kingdom and France). Future studies should aim to confirm the high sensitivity and specificity of the assay using many human control samples and an alternative model for preclinical detection in blood, such as sheep transfused with blood from BSE-infected sheep. It will also be crucial to test all available samples from persons affected by vCJD who donated blood before the disease appeared. Finally, it is necessary to highlight that the principles behind PMCA may be also used to detect misfolded protein aggregates responsible for common neurodegenerative diseases, such as Alzheimer's and Parkinson's diseases, which also self-propagate by a prion-like seeding mechanism (37,38). We and others have shown that seeding amplification assays can be implemented to detect misfolded aggregates composed of amyloid- β , tau, and α -synuclein in human biologic fluids (39–44), suggesting that PMCA represents a platform technology for highly sensitive detection of misfolded proteins.

Acknowledgments

We thank Luisa Gregori and David Asher for kindly providing us the blood reference materials for the validation of prion detection tests and for the critical review of the manuscript. We are also grateful to Glenn Telling for providing a colony of transgenic mice expressing human Pr^{PC}. Finally, we thank Charles Mays for help editing this manuscript.

This study was supported in part by grants from the National Institute of Health (P01AI106705, P01AI077774, R42NS079060, and SB1NS079060) to C.S.

About the Author

Dr. Concha-Marambio is the senior scientist at Amprion Inc., in San Diego, CA, USA. His research is focused on the development of biochemical techniques detecting prion-like neurodegenerative diseases.

References

1. Prusiner SB. Molecular biology of prion diseases. *Science*. 1991;252:1515–22. <https://doi.org/10.1126/science.1675487>
2. Soto C. Prion hypothesis: the end of the controversy? *Trends Biochem Sci*. 2011;36:151–8. <https://doi.org/10.1016/j.tibs.2010.11.001>
3. Colby DW, Prusiner SB. Prions. *Cold Spring Harb Perspect Biol*. 2011;3:a006833. <https://doi.org/10.1101/cshperspect.a006833>
4. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci*. 2001;24:519–50. <https://doi.org/10.1146/annurev.neuro.24.1.519>
5. Will RG, Ironside JW, Zeidler M, Estibeiro K, Cousens SN, Smith PG, et al. A new variant of Creutzfeldt-Jakob disease in the UK. *Lancet*. 1996;347:921–5. [https://doi.org/10.1016/S0140-6736\(96\)91412-9](https://doi.org/10.1016/S0140-6736(96)91412-9)
6. Hill AF, Desbruslais M, Joiner S, Sidle KC, Gowland I, Collinge J, et al. The same prion strain causes vCJD and BSE. *Nature*. 1997;389:448–50. <https://doi.org/10.1038/38925>
7. Collinge J, Sidle KC, Meads J, Ironside J, Hill AF. Molecular analysis of prion strain variation and the aetiology of “new variant” CJD. *Nature*. 1996;383:685–90. <https://doi.org/10.1038/383685a0>
8. Bruce ME, Will RG, Ironside JW, McConnell I, Drummond D, Suttie A, et al. Transmissions to mice indicate that “new variant” CJD is caused by the BSE agent. *Nature*. 1997;389:498–501. <https://doi.org/10.1038/39057>
9. Bruce ME, McConnell I, Will RG, Ironside JW. Detection of variant Creutzfeldt-Jakob disease infectivity in extraneural tissues. *Lancet*. 2001;358:208–9. [https://doi.org/10.1016/S0140-6736\(01\)05411-3](https://doi.org/10.1016/S0140-6736(01)05411-3)
10. Hilton DA, Fayers E, Edwards P, Ironside JW, Zajicek J. Prion immunoreactivity in appendix before clinical onset of variant Creutzfeldt-Jakob disease. *Lancet*. 1998;352:703–4. [https://doi.org/10.1016/S0140-6736\(98\)24035-9](https://doi.org/10.1016/S0140-6736(98)24035-9)
11. Peden A, McCardle L, Head MW, Love S, Ward HJT, Cousens SN, et al. Variant CJD infection in the spleen of a neurologically asymptomatic UK adult patient with haemophilia. *Haemophilia*. 2010;16:296–304. <https://doi.org/10.1111/j.1365-2516.2009.02181.x>
12. Llewelyn CA, Hewitt PE, Knight RSG, Amar K, Cousens S, Mackenzie J, et al. Possible transmission of variant Creutzfeldt-Jakob disease by blood transfusion. *Lancet*. 2004;363:417–21. [https://doi.org/10.1016/S0140-6736\(04\)15486-X](https://doi.org/10.1016/S0140-6736(04)15486-X)
13. Peden AH, Head MW, Ritchie DL, Bell JE, Ironside JW. Preclinical vCJD after blood transfusion in a PRNP codon 129 heterozygous patient. *Lancet*. 2004;364:527–9. [https://doi.org/10.1016/S0140-6736\(04\)16811-6](https://doi.org/10.1016/S0140-6736(04)16811-6)
14. Bishop MT, Hart P, Aitchison L, Baybutt HN, Plinston C, Thomson V, et al. Predicting susceptibility and incubation time of human-to-human transmission of vCJD. *Lancet Neurol*. 2006;5:393–8. [https://doi.org/10.1016/S1474-4422\(06\)70413-6](https://doi.org/10.1016/S1474-4422(06)70413-6)
15. Douet JY, Zafar S, Perret-Liaudet A, Lacroux C, Lugan S, Aron N, et al. Detection of infectivity in blood of persons with variant and sporadic Creutzfeldt-Jakob disease. *Emerg Infect Dis*. 2014;20:114–7. <https://doi.org/10.3201/eid2001.130353>
16. Edgeworth JA, Farmer M, Sicilia A, Tavares P, Beck J, Campbell I, et al. Detection of prion infection in variant Creutzfeldt-Jakob disease: a blood-based assay. *Lancet*. 2011;377:487–93. [https://doi.org/10.1016/S0140-6736\(10\)62308-2](https://doi.org/10.1016/S0140-6736(10)62308-2)
17. Jackson GS, Burk-Rafel J, Edgeworth JA, Sicilia A, Abdilahi S, Korteweg J, et al. Population screening for variant Creutzfeldt-Jakob disease using a novel blood test: diagnostic accuracy and feasibility study. *JAMA Neurol*. 2014;71:421–8. <https://doi.org/10.1001/jamaneurol.2013.6001>
18. Saborio GP, Permanne B, Soto C. Sensitive detection of pathological prion protein by cyclic amplification of protein misfolding. *Nature*. 2001;411:810–3. <https://doi.org/10.1038/35081095>

19. Soto C, Saborio GP, Anderes L. Cyclic amplification of protein misfolding: application to prion-related disorders and beyond. *Trends Neurosci.* 2002;25:390-4. [https://doi.org/10.1016/S0166-2236\(02\)02195-1](https://doi.org/10.1016/S0166-2236(02)02195-1)
20. Morales R, Duran-Aniotz C, Diaz-Espinoza R, Camacho MV, Soto C. Protein misfolding cyclic amplification of infectious prions. *Nat Protoc.* 2012;7:1397-409. <https://doi.org/10.1038/nprot.2012.067>
21. Concha-Marambio L, Pritzkow S, Moda F, Tagliavini F, Ironside JW, Schulz PE, et al. Detection of prions in blood from patients with variant Creutzfeldt-Jakob disease. *Sci Transl Med.* 2016;8:370ra183. <https://doi.org/10.1126/scitranslmed.aaf6188>
22. Moda F, Gambetti P, Notari S, Concha-Marambio L, Catania M, Park K-W, et al. Prions in the urine of patients with variant Creutzfeldt-Jakob disease. *N Engl J Med.* 2014;371:530-9. <https://doi.org/10.1056/NEJMoa1404401>
23. Lacroux C, Comoy E, Moudjou M, Perret-Liaudet A, Lugan S, Litaise C, et al. Preclinical detection of variant CJD and BSE prions in blood. *PLoS Pathog.* 2014;10:e1004202. <https://doi.org/10.1371/journal.ppat.1004202>
24. Bougard D, Brandel J-P, Bélonrade M, Béringue V, Segarra C, Fleury H, et al. Detection of prions in the plasma of presymptomatic and symptomatic patients with variant Creutzfeldt-Jakob disease. *Sci Transl Med.* 2016;8:370ra182. <https://doi.org/10.1126/scitranslmed.aag1257>
25. McDowell KL, Nag N, Franco Z, Bu M, Piccardo P, Cervenak J, et al. Blood reference materials from macaques infected with variant Creutzfeldt-Jakob disease agent. *Transfusion.* 2015;55:405-12. <https://doi.org/10.1111/trf.12841>
26. Yokoyama T, Takeuchi A, Yamamoto M, Kitamoto T, Ironside JW, Morita M. Heparin enhances the cell-protein misfolding cyclic amplification efficiency of variant Creutzfeldt-Jakob disease. *Neurosci Lett.* 2011;498:119-23. <https://doi.org/10.1016/j.neulet.2011.04.072>
27. Gregori L, McCombie N, Palmer D, Birch P, Sowemimo-Coker SO, Giulivi A, et al. Effectiveness of leucoreduction for removal of infectivity of transmissible spongiform encephalopathies from blood. *Lancet.* 2004;364:529-31. [https://doi.org/10.1016/S0140-6736\(04\)16812-8](https://doi.org/10.1016/S0140-6736(04)16812-8)
28. Holada K, Vostal JG, Theisen PW, MacAuley C, Gregori L, Rohwer RG. Scrapie infectivity in hamster blood is not associated with platelets. *J Virol.* 2002;76:4649-50. <https://doi.org/10.1128/JVI.76.9.4649-4650.2002>
29. Urayama A, Morales R, Niehoff ML, Banks WA, Soto C. Initial fate of prions upon peripheral infection: half-life, distribution, clearance, and tissue uptake. *FASEB J.* 2011;25:2792-803. <https://doi.org/10.1096/fj.11-180729>
30. Urayama A, Concha-Marambio L, Khan U, Bravo-Alegria J, Kharat V, Soto C. Prions efficiently cross the intestinal barrier after oral administration: study of the bioavailability, and cellular and tissue distribution in vivo. *Sci Rep.* 2016;6:32338. <https://doi.org/10.1038/srep32338>
31. Chen B, Morales R, Barria MA, Soto C. Estimating prion concentration in fluids and tissues by quantitative PMCA. *Nat Methods.* 2010;7:519-20. <https://doi.org/10.1038/nmeth.1465>
32. Gill ON, Spencer Y, Richard-Loendt A, Kelly C, Dabaghian R, Boyes L, et al. Prevalent abnormal prion protein in human appendixes after bovine spongiform encephalopathy epizootic: large scale survey. *BMJ.* 2013;347(oct15 5):f5675. <https://doi.org/10.1136/bmj.f5675>
33. Mok T, Jaunmuktane Z, Joiner S, Campbell T, Morgan C, Wakerley B, et al. Variant Creutzfeldt-Jakob disease in a patient with heterozygosity at PRNP codon 129. *N Engl J Med.* 2017;376:292-4. <https://doi.org/10.1056/NEJMc1610003>
34. Knight R. The risk of transmitting prion disease by blood or plasma products. *Transfus Apheresis Sci.* 2010;43:387-91. <https://doi.org/10.1016/j.transci.2010.09.003>
35. Ducrot C, Arnold M, de Koeijer A, Heim D, Calavas D. Review on the epidemiology and dynamics of BSE epidemics. *Vet Res.* 2008;39:15. <https://doi.org/10.1051/vetres:2007053>
36. Collinge J, Whitfield J, McKintosh E, Beck J, Mead S, Thomas DJ, et al. Kuru in the 21st century – an acquired human prion disease with very long incubation periods. *Lancet.* 2006;367:2068-74. [https://doi.org/10.1016/S0140-6736\(06\)68930-7](https://doi.org/10.1016/S0140-6736(06)68930-7)
37. Soto C. Transmissible proteins: expanding the prion heresy. *Cell.* 2012;149:968-77. <https://doi.org/10.1016/j.cell.2012.05.007>
38. Soto C, Pritzkow S. Protein misfolding, aggregation, and conformational strains in neurodegenerative diseases. *Nat Neurosci.* 2018;21:1332-40. <https://doi.org/10.1038/s41593-018-0235-9>
39. Salvadores N, Shahnawaz M, Scarpini E, Tagliavini F, Soto C. Detection of misfolded A β oligomers for sensitive biochemical diagnosis of Alzheimer's disease. *Cell Reports.* 2014;7:261-8. <https://doi.org/10.1016/j.celrep.2014.02.031>
40. Shahnawaz M, Tokuda T, Waragai M, Mendez N, Ishii R, Trenkwalder C, et al. Development of a biochemical diagnosis of Parkinson disease by detection of α -synuclein misfolded aggregates in cerebrospinal fluid. *JAMA Neurol.* 2017;74:163-72. <https://doi.org/10.1001/jamaneuro.2016.4547>
41. Kang UJ, Boehme AK, Fairfoul G, Shahnawaz M, Ma TC, Hutten SJ, et al. Comparative study of cerebrospinal fluid α -synuclein seeding aggregation assays for diagnosis of Parkinson's disease. *Mov Disord.* 2019;34:536-44. <https://doi.org/10.1002/mds.27646>
42. Groveman BR, Orrù CD, Hughson AG, Raymond LD, Zanusso G, Ghetti B, et al. Rapid and ultra-sensitive quantitation of disease-associated α -synuclein seeds in brain and cerebrospinal fluid by α Syn RT-QuIC. *Acta Neuropathol Commun.* 2018;6:7. <https://doi.org/10.1186/s40478-018-0508-2>
43. Fairfoul G, McGuire LJ, Pal S, Ironside JW, Neumann J, Christie S, et al. Alpha-synuclein RT-QuIC in the CSF of patients with alpha-synucleinopathies. *Ann Clin Transl Neurol.* 2016;3:812-8. <https://doi.org/10.1002/acn3.338>
44. Saijo E, Ghetti B, Zanusso G, Oblak A, Furman JL, Diamond MI, et al. Ultrasensitive and selective detection of 3-repeat tau seeding activity in Pick disease brain and cerebrospinal fluid. *Acta Neuropathol.* 2017;133:751-65. <https://doi.org/10.1007/s00401-017-1692-z>

Address for correspondence: Claudio Soto, University of Texas McGovern Medical School, 6431 Fannin St, Houston, TX 77030, USA; email: claudio.soto@uth.tmc.edu

Effect of Acute Illness on Contact Patterns, Malawi, 2017

Judith R. Glynn, Estelle McLean, Jullita Malava, Albert Dube,
Cynthia Katundu, Amelia C. Crampin, Steffen Geis

The way persons interact when ill could profoundly affect transmission of infectious agents. To obtain data on these patterns in Africa, we recorded self-reported named contacts and opportunities for casual contact in rural northern Malawi. We interviewed 384 patients and 257 caregivers about contacts over three 24-hour periods: day of the clinic visit for acute illness, the next day, and 2 weeks later when well. For participants of all ages, the number of adult contacts and the proportion using public transportation was higher on the day of the clinic visit than later when well. Compared with the day after the clinic visit, well participants (2 weeks later) named a mean of 0.4 extra contacts; the increase was larger for indoor or prolonged contacts. When well, participants were more likely to visit other houses and congregate settings. When ill, they had more visitors at home. These findings could help refine models of infection spread.

Knowledge of patterns of contact between persons is central to understanding transmission of infections and for designing control strategies (1–3). Until recently, models estimating the spread of infections assumed random mixing, which is too simplistic, or used data from studies in high-income countries, which may not be relevant for low-income settings (4–7). Although a few studies have been conducted in low-income countries, including 4 published studies from Africa (8–11), none have assessed how contact patterns change after illness. Studies have shown that persons predominantly mix with those of the same age (assortative mixing) (11,12), but the degree of intergenerational mixing and numbers of contacts vary between settings, depending on population characteristics such

as household size and structure, income-generating activities, and population density (9,12).

Almost all studies of contact patterns have involved healthy persons, but infection spread will be greatly influenced by the way persons mix when they are ill (13). Illness probably affects the contacts and movements of the sick persons and their household members. These altered patterns will be a key determinant of infection spread but are largely unknown even in high-income settings (13,14). A study of the effect of influenza-like symptoms in the United Kingdom indicated that changes in contact patterns after illness resulted in a reduced reproduction number (average number of cases generated by 1 case-patient) to <30% of the value it would have had if contact patterns had not changed (13). This effect would dramatically alter spread of infection in the population. To help learn whether similar changes occur in populations in Africa, we studied contact patterns during and after illness in a rural area in Karonga District, northern Malawi.

Methods

The study was conducted within a demographic surveillance area in rural northern Malawi (15). Our previous pilot work showed that keeping a diary and being interviewed about contacts in the previous 24 hours are acceptable to the population but that when each is done independently, both fail to include all contacts. We therefore combined these methods; participants and interviewers could refer to the diary (Appendix 1, <https://wwwnc.cdc.gov/EID/article/26/1/18-1539-App1.pdf>) as a memory aid during the interview, and interviewers were instructed to probe carefully for contacts. Contacts were defined as persons with whom the participant had face-to-face conversations or skin contact, not persons they simply passed and greeted.

Project staff based at the clinic recruited participants from among clinic patients with symptoms suggestive of acute infectious disease (e.g., fever, respiratory symptoms, diarrhea, vomiting). After obtaining

Author affiliations: London School of Hygiene & Tropical Medicine, London, UK (J.R. Glynn, E. McLean, A.C. Crampin, S. Geis); Malawi Epidemiology and Intervention Research Unit, Chilumba, Malawi (E. McLean, J. Malava, A. Dube, C. Katundu, A.C. Crampin, S. Geis)

DOI: <https://doi.org/10.3201/eid2601.181539>

written consent, an interviewer explained to the patient, caregiver, or both how to keep a diary of contacts, starting with the day of the clinic visit.

On the day after the clinic visit, the interviewer visited the home to ask about activities of the previous day and contacts made (number, age, sex, duration, context), who was living in the household at that time and socioeconomic variables, and any movement outside the household in the past 24 hours (including visits to other households; use of taxis/minibuses; and visits to congregate settings such as churches, markets, funerals, school). This visit was also an opportunity to address any difficulties the participants encountered with regard to keeping the diary. The participant was given a new diary to use over the next 24 hours, and another visit and interview were scheduled for the next day. For children <18 years of age, mothers/guardians who attended the clinic with them helped the children keep the diary and were also asked to keep a diary for themselves for the same periods, to assess contacts

Table 1. Characteristics of participants in study of the effect of acute illness on contact patterns, Malawi, 2017

Characteristic	No. (%)
Patient age, y/sex	
0–5/F	87 (23.6)
0–5/M	79 (21.4)
6–17/F	47 (12.7)
6–17/M	37 (10.0)
≥18/F	88 (23.8)
≥18/M	31 (8.4)
Caregiver	
F	207 (83.8)
M	40 (16.2)
Initial symptoms of patients	
Fever	364 (98.6)
Respiratory symptoms	130 (35.2)
Diarrhea/vomiting	74 (20.1)
Patients and caregivers	
Schooling (adults)	
<Primary	129 (35.2)
Primary	198 (54.1)
Secondary (completed)	39 (10.7)
Occupation of adults	
Farmer	259 (70.8)
Skilled nonmanual	51 (13.9)
Other	56 (15.3)
Travel outside village	
Most days	231 (37.5)
≥1×/wk	174 (28.2)
≥ 1×/mo	110 (17.9)
<1×/mo	41 (6.7)
Never	59 (9.6)
Ever travel outside	
District	79 (12.8)
Region	54 (8.8)
Country	6 (1.0)
Animal contact (≥monthly)	
Ducks/chickens	375 (60.9)
Pigs	137 (22.2)
Animal feces	336 (54.5)

while caregiving. We collected data for 2 days of illness because the day of the clinic visit may be atypical and because explaining the diary at the clinic while the patient is ill and wanting to get home cannot take very long, so a refresher session may be necessary.

Two weeks later, each participant was revisited, given a diary, and interviewed the next day to collect information about a 24-hour period while well. If possible, these visits were on the same day of the week as the first home visit and conducted by the same interviewer. If the patient was still sick, the visit was rescheduled.

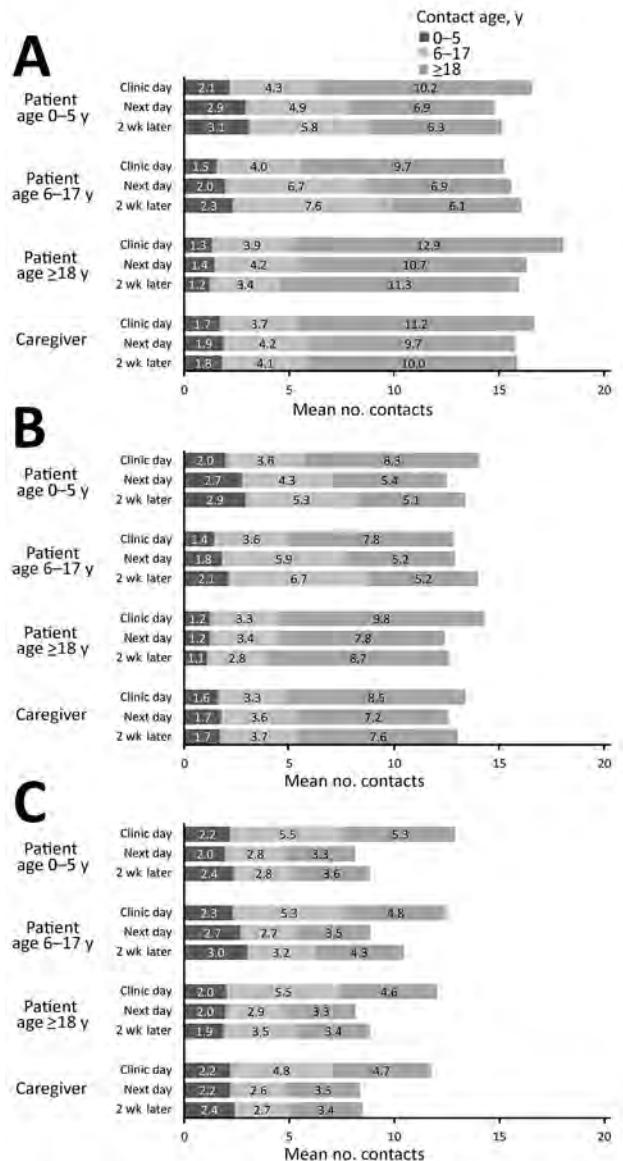


Figure 1. Contact patterns, by age of study participant, age of contact, and visit, in study of the effect of acute illness on contact patterns, Malawi, 2017. Mean number of close contacts per 24-hour period overall (A); restricted to contacts of ≥10 minutes (B); restricted to indoor contacts (C).

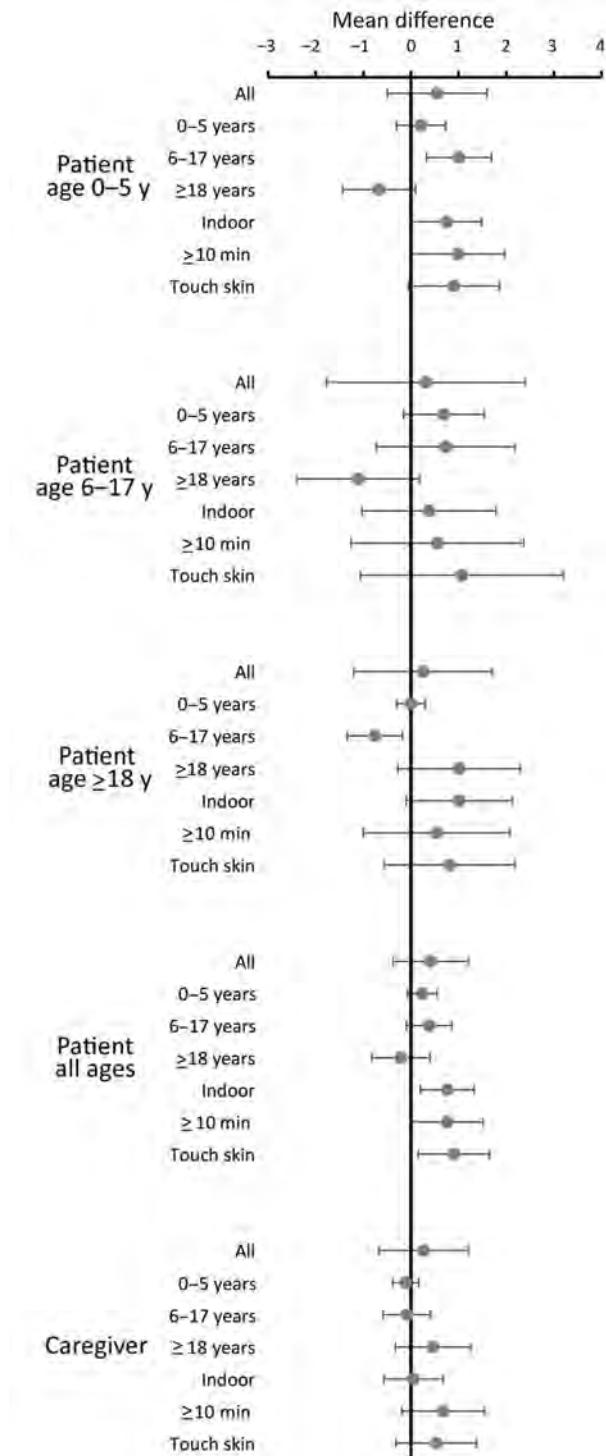


Figure 2. Mean differences in numbers of contacts for study participants when well compared with when ill (the day after the clinic visit), restricted to persons seen on the same day of the week when well and when ill, in study of the effect of acute illness on contact patterns, Malawi, 2017. Characteristics in second column refer to contacts. Mean difference >0 implies more contacts when well, mean difference <0 implies more contacts when ill. Error bars indicate 95% CIs.

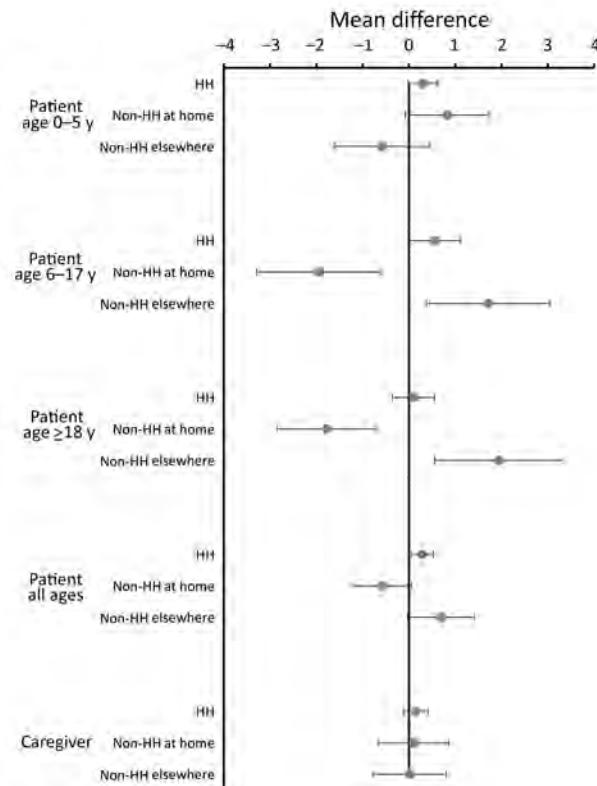


Figure 3. Mean differences in numbers of HH member contacts and non-HH members seen as contacts at the home and elsewhere by study participants when well compared with when ill (the day after the clinic visit), restricted to persons seen on the same day of the week when well and when ill, in study of the effect of acute illness on contact patterns, Malawi, 2017. Mean difference >0 implies more contacts when well; mean difference <0 implies more contacts when ill. Error bars indicate 95% CIs. HH, household.

Sample size calculations for this type of study are not well defined. Previous studies have recorded data for <300 to >1,000 persons. We planned to recruit 400 patients stratified by sex and by age (0-5, 6-17, 18-49, ≥50 years); however, because few adult men and few older adults attended the clinic, we combined the adult age groups. In addition, we recruited caregivers for participants <18 years of age.

Analyses describe contact patterns of participants in the different groups (patient and caregiver, different visits) by using means and differences in mean numbers of contacts. The primary comparison was between when ill, the day after the clinic visit, and when well (2 weeks later) for patients and caregivers, restricted to those visited on the same day of the week for the 2 visits. To assess changes in visits to congregate settings, the mean difference in proportions attending was calculated by scoring a visit as 1 and no

Table 2. Number and proportions of patients and caregivers who visited congregate settings in 24-hour periods in study of the effect of acute illness on contact patterns, Malawi, 2017

Setting, date of visit	Patients, no. (%)			Caregivers
	Age 0–5, y	Age 6–17, y	Age ≥18 y	
All*				
Clinic day	166	84	119	247
Next day	169	85	116	250
2 wk later	165	85	119	248
Church				
Clinic day	1 (0.6)	3 (3.6)	2 (1.7)	11 (4.5)
Next day	11 (6.5)	4 (4.7)	5 (4.3)	23 (9.2)
2 wk later	13 (7.9)	10 (11.8)	11 (9.2)	32 (12.9)
Funeral				
Clinic day	2 (1.2)	0	1 (0.8)	6 (2.4)
Next day	5 (3.0)	0	2 (1.7)	15 (6.0)
2 wk later	4 (2.4)	0	8 (6.7)	14 (5.6)
Market				
Clinic day	45 (27.1)	16 (19.0)	28 (23.5)	86 (34.8)
Next day	14 (8.3)	8 (9.4)	28 (24.1)	68 (27.2)
2 wk later	26 (15.8)	22 (25.9)	52 (43.7)	92 (37.1)
Vehicle				
Clinic day	107 (64.5)	50 (59.5)	65 (54.6)	153 (61.9)
Next day	5 (3.0)	4 (4.7)	15 (12.9)	27 (10.8)
2 wk later	15 (9.1)	4 (4.7)	30 (25.2)	33 (13.3)
School				
Clinic day	1 (0.6)	2 (2.4)	1 (0.8)	3 (1.2)
Next day	3 (1.8)	10 (11.8)	6 (5.2)	3 (1.2)
2 wk later	12 (7.3)	26 (30.6)	7 (5.9)	5 (2.0)
Any other households				
Clinic day	114 (68.7)	47 (56.0)	61 (51.3)	187 (76.1)
Next day	142 (84.0)	68 (80.0)	72 (62.1)	204 (81.6)
2 wk later	155 (93.9)	75 (88.2)	95 (79.8)	225 (90.7)

*Numbers vary between visits because a few persons missed interviews.

visit as 0. For persons with unchanged patterns when ill and well (either visiting or not visiting a congregate setting on both occasions) the difference would be 0; for those visiting when well but not when ill, the difference would be 1; and for those visiting when ill but not when well, the difference would be -1. Assuming a normal distribution of the differences, we calculated exact 95% CIs. The study was approved by the National Health Sciences Research Committee, Malawi (no. 1695) and by the ethics committee of the London School of Hygiene & Tropical Medicine, UK (no. 12023).

Results

At the end of the recruitment period, we had interviewed 384 patients and 257 caregivers. A total of 343 patients and 233 caregivers completed interviews for all 3 visits; 18 patients refused and 41 consented but did not appear for the initial interview in the clinic. The population was rural; 71% (259/366) of adults were farmers and 35% (129/366) of adults had not completed primary school (Table 1). Most participants had regular contact (at least monthly) with animals. Although most participants (84%, 515/616) traveled outside their village at least once a month,

only 13% (79/616) had ever left the district. Almost all patients had fever; 35% (130/369) had respiratory symptoms; and 20% (74/369) had diarrhea, vomiting, or both

Patients had a mean (\pm SD) of 16.8 (\pm 5.6) contacts on the day of the clinic visit, 15.4 (\pm 5.9) the next day, and 15.6 (\pm 5.5) 2 weeks later when well (Figure 1; Appendix 2 Figure 1, <https://wwwnc.cdc.gov/EID/article/26/1/18-1539-App2.pdf>). Mean contact numbers for caregivers were similar to those for patients for the three 24-hour periods: 16.7 (\pm 5.2), 15.7 (\pm 5.6), and 15.9 (\pm 5.8). When analysis was restricted to contacts involving touch, the mean numbers of contacts were 10.6 (\pm 4.6), 9.3 (\pm 4.8), and 10.1 (\pm 5.2) for patients and 9.6 (\pm 4.6), 9.0 (\pm 4.3), and 9.3 (\pm 4.8) for caregivers for each of the 3 periods. When analysis was restricted to contacts of at least 10 minutes' duration, the mean numbers of contacts were 14.3 (\pm 5.0), 13.0 (\pm 5.1), and 13.6 (\pm 5.1) for patients and 14.0 (\pm 4.7), 13.2 (\pm 4.7), and 13.6 (\pm 5.2) for caregivers. Among patients with respiratory symptoms, the mean numbers of contacts were 16.6 (\pm 5.4), 15.4 (\pm 6.3), and 15.9 (\pm 5.8) for the 3 periods; among those with diarrhea/vomiting, mean contact numbers were 15.2 (\pm 4.8), 14.1 (\pm 5.6), and 14.8 (\pm 6.2).

Overall, the contact patterns while ill on the day after the clinic visit were similar to those after recovery in terms of number and age pattern of contacts (Figure 1, panel A; Appendix 2 Figure 2). Contact numbers were similar for men and women, and

because relatively few adult men were interviewed, we combined the results. We found some evidence of assortative mixing (like with like) for age. Attending the clinic led to more adult contacts for all participants and fewer child contacts for children.

When we restricted analysis to contacts of at least 10 minutes' duration, we found 2–3 fewer contacts per person but an overall similar pattern (Figure 1, panel B). Participants had more indoor contacts on the day of the clinic visit, but this number differed little on the other days (Figure 1, panel C).

We found more differences in the type of contact. On the day of the clinic visit, patients met an average of 3.2 contacts they had never met before and caregivers an average of 2.8. On the next day, these numbers were 0.8 and 0.9; 2 weeks later, they were 0.5 and 0.6.

Contact patterns can vary according to day of the week. Further analysis of change of contact patterns compared the day after the clinic visit with 2 weeks later when patients were well for persons who were interviewed on the same day of the week for these 2 periods (228 patients and 154 caregivers). We found no large differences in contact numbers. Overall, patients had a mean (95% CI) of 0.4 (–0.4 to 1.2) extra contacts when well, an increase of 0.8 (0.2–1.3) for indoor contacts, 0.8 (0–1.5) for contacts of ≥ 10 minutes, and 0.9 (0.2–1.7) for contacts involving touch. Children tended to have more contacts with children and fewer with adults when well, and adults tended to have more contacts with adults and fewer with children when well, but all differences were small (< 1 contact/day) (Figure 2).

Household contacts changed little for participants in all age groups (Figure 3; Appendix 2 Figure 3). While well, children 6–17 years of age and adults had an average of 2 fewer nonhousehold contacts at home and 2 more nonhousehold contacts outside the home. We found little difference in household contacts for the youngest children or caregivers.

In addition to the individual contacts, we asked about congregate and other settings. Patients were more likely to visit churches, funerals, markets, school, and to travel by public transportation (taxi/bus) when well than on the day after the clinic visit (Table 2). For caregivers, the differences in these visits were smaller. We also compared the difference in these visits between the day after the clinic visit and when well for those interviewed on the same day of the week (Figure 4). When well, participants of all ages were more likely to visit the market, adults and preschool-age children were more likely to use public transportation, adults were more likely to attend church, school-age children were more likely to go

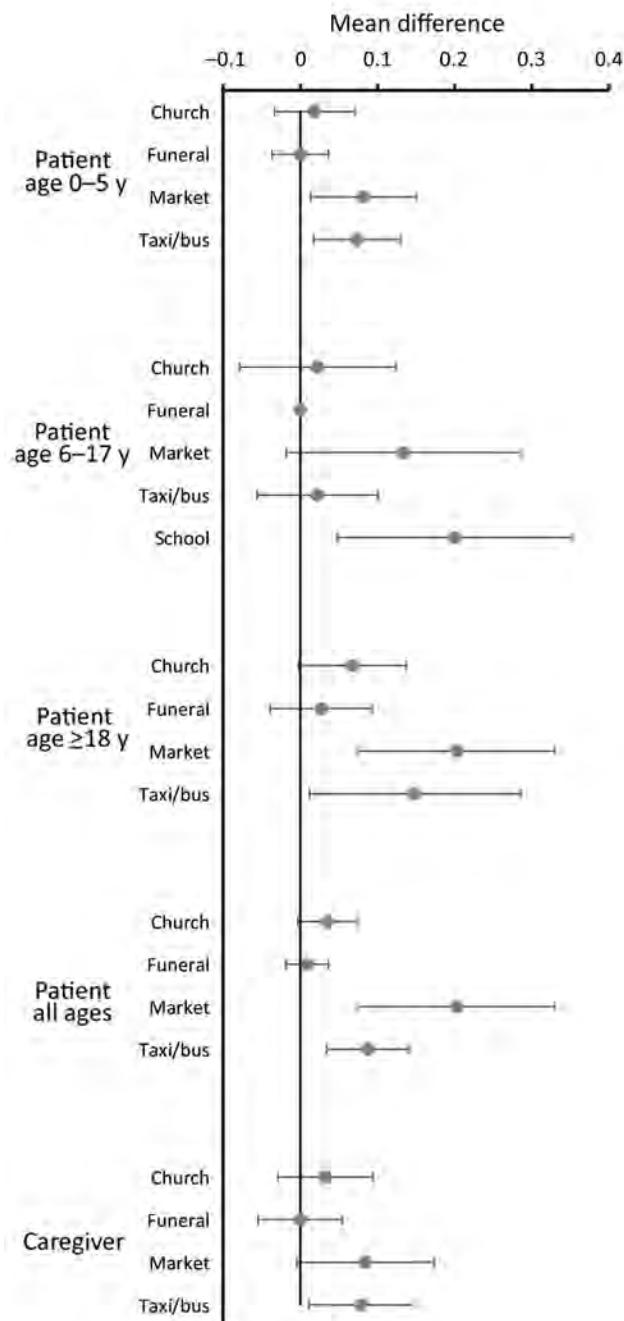


Figure 4. Mean differences in proportions of persons attending congregate settings when well compared with when ill (the day after the clinic visit), restricted to persons seen on the same day of the week when well and when ill, in study of the effect of acute illness on contact patterns, Malawi, 2017. Mean difference > 0 implies more visits when well; mean difference < 0 implies more visits when ill. Error bars indicate 95% CIs.

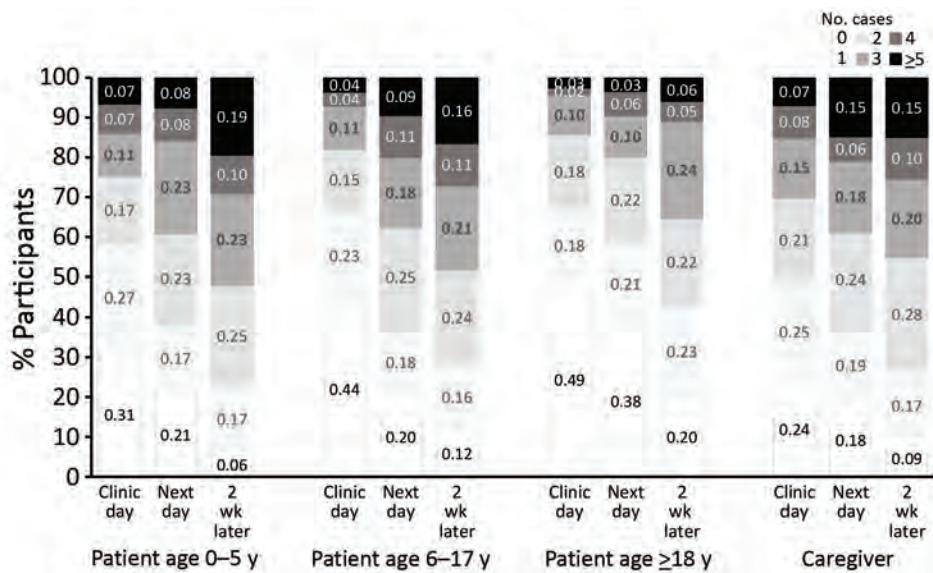


Figure 5. Proportion of all participants visiting 0, 1, 2, 3, 4, or ≥ 5 households in each 24-hour period during study of effects of acute illness on contact patterns, Malawi, 2017.

to school, and participants of all groups were more likely to visit other households and to visit more such households (Figure 5). We found no differences in funeral attendance, but the numbers were small.

Discussion

In this study of the effect of illness on human contact patterns in rural Malawi, we found a slight reduction in named contacts when ill (the day after the clinic visit) than when well (2 weeks later) and a larger reduction in attendance at congregated settings and visits to other households when ill. Also when ill, on the day of the clinic visit the number of contacts was slightly higher than on the other days, and vehicle use was greatly increased. We also showed similar changes in contact patterns of caregivers.

The small change in named contacts between the day after the clinic visit and when well 2 weeks later can partly be explained by many of the contacts being members of the same household and by the reduction in contacts outside the house when ill being offset by visitors to the house (Figure 3). However, given the greater change in the number of households visited and the opportunities for meeting people at congregated settings, the small difference might also result from the difficulties with collecting accurate contact information. We used the combination of a diary as a memory aid and probing by interviewers. The probing often revealed more contacts than initially recorded. It also enabled discussion with the interviewer about who should be included, which should have helped consistency. Even with this help, however, accurately recording contacts is difficult, especially in a

setting of low literacy, and we found some evidence of a tendency to stop at 15 contacts (Appendix 2 Figure 1), which corresponds with the end of the first page of the interviewers' sheets.

Our results from the participants when well are in the range of findings elsewhere in Africa, but definitions have varied, making direct comparison difficult. In Kenya, participants reported a mean of 17.7 contacts per day involving touch (8); in communities in Zambia and South Africa, adult participants reported a mean of 4.9 close contacts (shared conversation longer than a greeting) and 10.4 casual contacts (shared indoor space) per day (9); and in a township in South Africa, participants reported a median of 20 close contacts per day, according to a definition similar to ours (10). Results from studies in Europe that used a definition similar to ours ranged from 8 to 20 contacts per day (12). Some of the variation probably reflects the different settings, and some probably reflects the methods.

As elsewhere, we found evidence of assortative age mixing (8,10,11), which was reduced when persons were ill, as has been found in the United Kingdom (13). The direct comparison of contact patterns for the same persons when well and when ill effectively controls for possible confounding (e.g., by age, sex, or socioeconomic status). It also controls for individual variation in ability to remember contacts. Although it is possible that the higher contact numbers seen on the third visit reflect improved learning and recall, or less distraction by illness, the change in the pattern of types of contact (Figures 2, 3) suggests that these are not the explanation.

The change in households visited and congregate settings attended were more striking than the changes in named contacts. These settings include those that may play a role in infection transmission, such as churches and public transportation, where overcrowding is common, ventilation often poor, and the number of casual contacts can be large (16,17). Healthcare centers are well recognized as places where infection spread is likely, and to get to the healthcare center, most patients used public transportation. On the day after the clinic visit, congregate setting attendance and household visiting were lower than 2 weeks later when well. The lower market attendance on the day after the clinic visit may partly result from having combined a visit to the market with the clinic visit. Of note, visiting other households was very common for persons of all ages, even when ill (Figure 5).

Our results quantify the changes in contact patterns when persons are ill in rural Africa. Although visiting the clinic increased contacts and being ill decreased contacts, changes in named contacts were small (a reduction in outside contacts was offset by visitors at home). For many infections, the changes in casual contact that were seen would probably have more effect on transmission than the smaller changes in named contacts. These findings could be used to refine models of infection spread.

The study was funded by a grant from the UK Public Health Rapid Support Team, which is funded by the UK Department of Health and Social Care.

About the Author

Prof. Glynn is professor of Infectious Disease Epidemiology at the London School of Hygiene & Tropical Medicine and has a particular interest in transmission of infection and mechanisms of disease.

References

- Read JM, Edmunds WJ, Riley S, Lessler J, Cummings DA. Close encounters of the infectious kind: methods to measure social mixing behaviour. *Epidemiol Infect.* 2012;140:2117–30. <https://doi.org/10.1017/S0950268812000842>
- Heesterbeek H, Anderson RM, Andreasen V, Bansal S, De Angelis D, Dye C, et al.; Isaac Newton Institute IDD Collaboration. Modeling infectious disease dynamics in the complex landscape of global health. *Science.* 2015; 347:aaa4339. <https://doi.org/10.1126/science.aaa4339>
- Eames KT, Tilston NL, Brooks-Pollock E, Edmunds WJ. Measured dynamic social contact patterns explain the spread of H1N1v influenza. *PLOS Comput Biol.* 2012;8:e1002425. <https://doi.org/10.1371/journal.pcbi.1002425>
- Edmunds WJ, O'Callaghan CJ, Nokes DJ. Who mixes with whom? A method to determine the contact patterns of adults that may lead to the spread of airborne infections. *Proc Biol Sci.* 1997;264:949–57. <https://doi.org/10.1098/rspb.1997.0131>
- Hens N, Ayele GM, Goeyvaerts N, Aerts M, Mossong J, Edmunds WJ, et al. Estimating the impact of school closure on social mixing behaviour and the transmission of close contact infections in eight European countries. *BMC Infect Dis.* 2009;9:187. <https://doi.org/10.1186/1471-2334-9-187>
- Melegaro A, Jit M, Gay N, Zagheni E, Edmunds WJ. What types of contacts are important for the spread of infections? Using contact survey data to explore European mixing patterns. *Epidemics.* 2011;3:143–51. <https://doi.org/10.1016/j.epidem.2011.04.001>
- Beutels P, Shkedy Z, Aerts M, Van Damme P. Social mixing patterns for transmission models of close contact infections: exploring self-evaluation and diary-based data collection through a web-based interface. *Epidemiol Infect.* 2006;134: 1158–66. <https://doi.org/10.1017/S0950268806006418>
- Kiti MC, Kinyanjui TM, Koech DC, Munywoki PK, Medley GF, Nokes DJ. Quantifying age-related rates of social contact using diaries in a rural coastal population of Kenya. *PLoS One.* 2014;9:e104786. <https://doi.org/10.1371/journal.pone.0104786>
- Dodd PJ, Looker C, Plumb ID, Bond V, Schaap A, Shanaube K, et al. Age- and sex-specific social contact patterns and incidence of *Mycobacterium tuberculosis* Infection. *Am J Epidemiol.* 2016;183:156–66.
- Johnstone-Robertson SP, Mark D, Morrow C, Middelkoop K, Chiswell M, Aquino LD, et al. Social mixing patterns within a South African township community: implications for respiratory disease transmission and control. *Am J Epidemiol.* 2011;174:1246–55. <https://doi.org/10.1093/aje/kwr251>
- le Polain de Waroux O, Cohuet S, Ndazima D, Kucharski AJ, Juan-Giner A, Flasche S, et al. Characteristics of human encounters and social mixing patterns relevant to infectious diseases spread by close contact: a survey in southwest Uganda. *BMC Infect Dis.* 2018;18:172. <https://doi.org/10.1186/s12879-018-3073-1>
- Mossong J, Hens N, Jit M, Beutels P, Auranen K, Mikolajczyk R, et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. *PLoS Med.* 2008;5:e74. <https://doi.org/10.1371/journal.pmed.0050074>
- Van Kerckhove K, Hens N, Edmunds WJ, Eames KT. The impact of illness on social networks: implications for transmission and control of influenza. *Am J Epidemiol.* 2013;178:1655–62. <https://doi.org/10.1093/aje/kwt196>
- Eames KT, Tilston NL, White PJ, Adams E, Edmunds WJ. The impact of illness and the impact of school closure on social contact patterns. *Health Technol Assess.* 2010;14: 267–312. <https://doi.org/10.3310/hta14340-04>
- Crampin AC, Dube A, Mboma S, Price A, Chihana M, Jahn A, et al. Profile: the Karonga Health and Demographic Surveillance System. *Int J Epidemiol.* 2012;41:676–85. <https://doi.org/10.1093/ije/dys088>
- Yates TA, Tanser F, Abubakar I. Plan Beta for tuberculosis: it's time to think seriously about poorly ventilated congregate settings. *Int J Tuberc Lung Dis.* 2016;20:5–10. <https://doi.org/10.5588/ijtld.15.0494>
- Escombe AR, Oeser CC, Gilman RH, Navincopa M, Ticona E, Pan W, et al. Natural ventilation for the prevention of airborne contagion. *PLoS Med.* 2007;4:e68. <https://doi.org/10.1371/journal.pmed.0040068>

Address for correspondence: Judith R. Glynn, London School of Hygiene & Tropical Medicine, Infectious Disease Epidemiology Department, Keppel St, London WC1E 7HT, UK; email: judith.glynn@lshtm.ac.uk

Outbreak of Peste des Petits Ruminants Virus among Critically Endangered Mongolian Saiga and Other Wild Ungulates, Mongolia, 2016–2017

Mathieu Pruvot, Amanda E. Fine, Charlotte Hollinger, Samantha Strindberg, Batchuluun Damdinjav, Bayarbaatar Buuveibaatar, Buyanaa Chimeddorj, Gantulga Bayandonoi, Bodisaikhan Khishgee, Batkhuyag Sandag, Jamiyankhuu Narmandakh, Tserenjav Jargalsaikhan, Batzorig Bataa, Denise McAloose, Munkhduuren Shatar, Ganzorig Basan, Mana Mahapatra, Muni Selvaraj, Satya Parida, Felix Njeumi, Richard Kock, Enkhtuvshin Shiilegdamba

The 2016–2017 introduction of peste des petits ruminants virus (PPRV) into livestock in Mongolia was followed by mass mortality of the critically endangered Mongolian saiga antelope and other rare wild ungulates. To assess the nature and population effects of this outbreak among wild ungulates, we collected clinical, histopathologic, epidemiologic, and ecological evidence. Molecular characterization confirmed that the causative agent was PPRV lineage IV. The spatiotemporal patterns of cases among wildlife were similar to those among livestock affected by the PPRV outbreak, suggesting spillover of virus from livestock at multiple locations and time points and subsequent spread among wild ungulates. Estimates of saiga abundance suggested a population decline of 80%, raising substantial concerns for the species' survival. Consideration of the entire ungulate community (wild and domestic) is essential for elucidating the epidemiology of PPRV in Mongolia, addressing the threats to wild ungulate conservation, and achieving global PPRV eradication.

Author affiliations: Wildlife Conservation Society, Bronx, New York, USA (M. Pruvot, A.E. Fine, C. Hollinger, S. Strindberg, D. McAloose); Wildlife Conservation Society Mongolia Program, Ulaanbaatar, Mongolia (B. Damdinjav, B. Buuveibaatar, E. Shiilegdamba); World Wide Fund for Nature Mongolia Program, Ulaanbaatar (B. Chimeddorj, G. Bayandonoi); Ministry of Food, Agriculture and Light Industry, Ulaanbaatar (B. Khishgee, B. Sandag); Ministry of Nature, Environment and Tourism, Ulaanbaatar (J. Narmandakh); State Central Veterinary Laboratory, Ulaanbaatar (T. Jargalsaikhan, B. Bataa, M. Shatar, G. Basan); The Pirbright Institute, Surrey, UK (M. Mahapatra, M. Selvaraj, S. Parida); Food and Agriculture Organization of the United Nations, Rome, Italy (F. Njeumi); Royal Veterinary College, London, UK (R. Kock)

DOI: <https://doi.org/10.3201/eid2601.181998>

Peste des petits ruminants virus (PPRV; family *Paramyxoviridae*, genus *Morbillovirus*) causes an acute and highly contagious infection in domestic sheep and goats (1) and multiple species of wild ungulates (2). The resultant clinical disease, peste des petits ruminants (PPR), can lead to high morbidity and mortality rates (3) and is recognized as an economically important transboundary disease (4). The substantial effect of PPR on household-level livelihoods, well-being, food security, rural communities, and national economies have made PPR a priority for eradication (5–8). PPR is reported to affect several species of free-ranging or captive wild ruminants (2,9,10) but is rarely expressed clinically in wildlife populations (11), which were therefore thought to play a negligible epidemiologic role (6,10). Nonetheless, losses among several threatened species of wild mountain ungulates (12–14) and susceptibility of many other captive endangered ungulates (15–17) make this virus a major threat to wild ungulate conservation (2,9,18,19).

In the fall of 2016, an outbreak of PPRV among domestic sheep and goats in western Mongolia was confirmed, probably originating from uncontrolled transboundary livestock movements (20,21). In total, 83,889 small ruminants from 1,081 households were reportedly affected by PPR in 14 soums (districts) of 3 aimags (provinces), of which 12,976 small ruminants died (overall case-fatality risk 15.5%) (22). After this initial outbreak, control measures included vaccination of 4,632,200 sheep and 5,800,318 goats in and around the outbreak area in October 2016. Although the vaccination campaign successfully curbed the

epidemic in livestock, on December 27, 2016, deaths among the Mongolian saiga antelope (subspecies *Saiga tatarica mongolica*) from PPRV infection were confirmed; later, deaths from PPRV infection of Siberian ibex (*Capra sibirica*) and goitered gazelle (*Gazella subgutturosa*) were also confirmed (22). In the following months, thousands of critically endangered Mongolian saiga died.

The Mongolian saiga antelope (hereafter saiga) is a nomadic antelope that now occupies <20% of its historic range in 2 provinces of Mongolia (Khovd and Gobi-Altai), representing 36,000 km² of desert steppe bordered by high mountain ranges, lakes, and sand dunes (23). The saiga range partially overlaps that of mountain ungulates, including Siberian ibex, Argali sheep (*Ovis ammon*), and other plains ungulates such as goitered gazelle and Mongolian gazelle (*Procapra gutturosa*). It is also dominated by livestock; >1.5 million sheep and goats in the 8 soums overlapping the saiga range (24) are seasonally grazed over both mountain and desert steppe areas (25).

To describe the PPRV epizootic in the wild ungulate community of Mongolia, we gathered all available

evidence from field missions, histopathology examinations, government records, wildlife population monitoring efforts, and laboratory testing (including molecular characterization of the causative agent). We describe the significance of our findings for the global PPR eradication program and the conservation of wild ungulate species (Figure 1).

Materials and Methods

Emergency Field Mission

On January 20, 2017, shortly after the first confirmation of PPRV infection in saiga, the Crisis Management Center–Animal Health, led by the Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health, and the Mongolia government organized an investigation of PPR among wild and domestic animals (22). Reports of cases before the first official confirmation were collected from multiple sources in the field (Appendix section 1.1, <https://wwwnc.cdc.gov/EID/article/26/1/18-1998-App1.pdf>). In addition, samples were obtained from 27 animals that were found

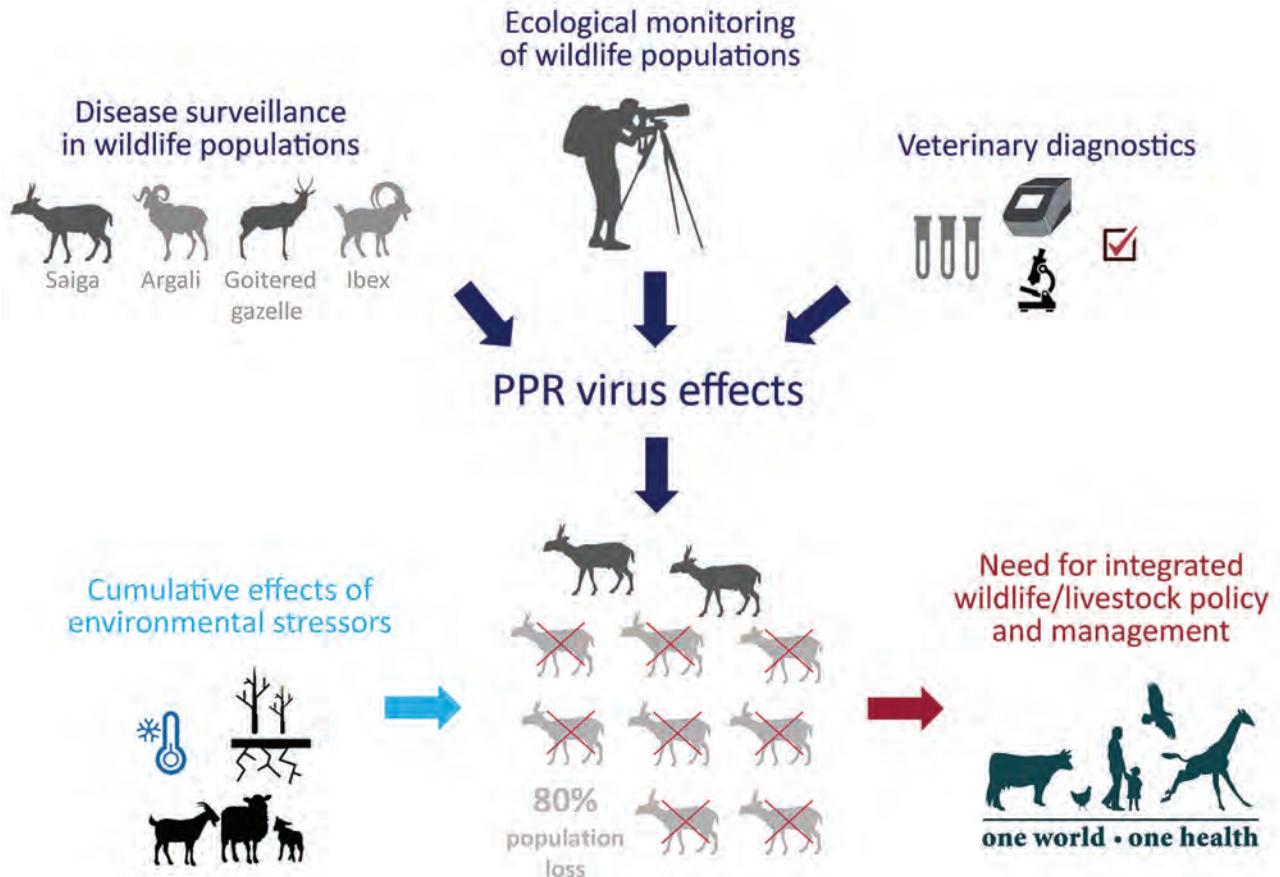


Figure 1. Summary of study of the 2016–2017 outbreak of peste des petits ruminants among wildlife, Mongolia. PPR, peste des petits ruminants.

sick (2 saiga) or dead (2 ibex, 2 goitered gazelle, 21 saiga) during direct field observations. For dead animals, full necropsy examinations were performed when possible (2 ibex, 2 goitered gazelle, 9 saiga); necropsy reports were available for 2 ibex, 1 goitered gazelle, and 4 saiga. Heads were collected from the rest of the animals (12 saiga). Tissues were available for histologic examination from 17 animals (2 ibex, 2 goitered gazelle, 13 saiga) (Appendix section 1.2). We used the lateral flow device Peste-Test (The Pirbright Institute, <https://www.pirbright.ac.uk>) as a rapid penside field test for PPRV (26) on eye swab samples from 20 animals (2 ibex, 1 goitered gazelle, 17 saiga), except for 1 live saiga from which we non-invasively obtained feces and saliva. We sent samples to the State Central Veterinary Laboratory (SCVL) for PPRV confirmation by use of gel-based reverse transcription PCR (RT-PCR) (Appendix section 1.3) (27). For 2 of the saiga samples, the Pirbright Institute sequenced the C-terminus portion of the N-gene (Appendix section 1.4).

Phylogenetic Analysis

We retrieved and used the partial N-gene sequences of PPRV ($n = 56$) available in GenBank for southern, central, and eastern Asia through September 2018 for constructing a neighborhood-joining phylogenetic tree. Sequences included the 2 partial N-gene sequences obtained from saiga from Mongolia in this study (Appendix section 1.4).

Reporting to SCVL

All wildlife samples submitted to SCVL in 2016–2017 were compiled in a dataset, which included georeferences and PPR diagnostic results when available. Most samples were tested by using the RT-PCR procedure mentioned above, and a subset of samples was also tested by using ID Screen PPR Competition and ID Screen PPR Antigen Capture (IDvet, <https://www.id-vet.com>). Cases were considered positive when ≥ 1 of the 3 test results was positive. When georeferences were missing, we used the location description to determine the approximate geographic coordinates and mapped it by using ArcGIS 10.2 (ESRI, <https://www.esri.com>). These coordinates were used to trace the spatiotemporal progression of the PPR outbreak in wildlife, including identifying potentially undiagnosed wildlife illness and deaths that may have been part of the same outbreak.

Government Carcass Disposal

From January 8 through February 28, 2017, as part of the Mongolia government emergency response

(Appendix section 2) in the Khovd and Gobi-Altai Provinces, livestock movements were restricted and saiga carcasses were collected and destroyed. Carcass disposal was conducted at 8 sites where records were kept of the total number of carcasses and sex of the animal (when available). In some soums, at the initiative of the soum-level government, the count and collection of carcasses was maintained until June 30, 2017.

Saiga Population Surveys

In 2010, distance sampling (28) was first applied to the saiga population as a way to improve population abundance estimates (23). Thereafter, it was implemented as part of a routine monitoring program; local saiga rangers conducted distance sampling surveys along 40 transects ranging from 2 to 99 km, for a total of 1,505 km of survey effort. Each survey was conducted by 4 trained teams, who drove vehicles along transects and recorded for each group of saiga seen the radial distance, angle from the transect line, and group size. To better monitor the population-level effect of the outbreak, we repeated the surveys in January, March, and May 2017, and April 2018.

Following systematic data cleaning steps (Appendix section 3.1; datasets, <https://doi.org/10.6084/m9.figshare.7502252.v1>; R code available upon request), we used Distance 7.2 software (29) to fit detection function models to the distance sampling data. Models were fitted separately for each survey and, when sample size was sufficient, were stratified by the 3 regions within the home range (Durgun Steppe, Khuisiin Gobi, and Sharga Gobi). In addition, to estimate population density and abundance, we assessed group size bias (e.g., when smaller groups farther from the transect line tend to be missed) and corrected when necessary (Appendix sections 3.2).

Ethics Considerations

No ethics approval was required for the outbreak response because the investigation was a response to an emergency situation, and no live animal handling was required to obtain the samples (samples obtained from dead animals or from environmental recovery of excreted/secreted material). The driving transect survey technique for estimating the population of Mongolian saiga was reviewed by the wildlife research advisory committee of the Mongolian Academy of Sciences. Members of the Mongolian Academy of Sciences act as the main scientific advisors to the Ministry of Environment in issuing of permits related to wildlife research in Mongolia.

Results

Clinical Manifestations of PPRV Infection in Wild Ungulates

One live clinically ill saiga (confirmed positive for PPRV by Peste-Test) could be approached and displayed the following clinical signs: lethargy with tachypnea and dyspnea, seropurulent ocular discharge with staining of the suborbital area, salivation, diarrhea, and weakness. Of the 20 animals tested with Peste-Test, results were positive for 17 (1 ibex, 1 goitered gazelle, and 15 saiga); of the 17 animals tested by RT-PCR, results were positive for 16. Of the 22 total tested animals, 20 were positive by either Peste-Test or RT-PCR. For the 6 animals PPRV positive by RT-PCR that underwent necropsy (4 saiga, 1 goitered gazelle, 1 ibex), notable gross pathology findings included emaciation (n = 4), red nasal mucosa or discharge (n = 3), erosive to ulcerative lesions of the oral mucosa (n = 3), red or consolidated portions of lungs (n = 6), and red discoloration of the intestinal mucosa or presumptive enteritis (n = 4). Tissues available for histology from PPRV-infected animals (positive test result, histologic evidence, or both) showed acute cellular degeneration and necrosis, with varying degrees of associated acute

inflammation, that affected the oral/pharyngeal mucosa, hepatocytes, cholangiolar epithelium of bile ductules, bronchiolar epithelium, and intestinal crypt epithelium (Table 1; Figure 2). We observed viral inclusion bodies and a few viral syncytia to varying degrees in oral/pharyngeal, liver, lung, and intestinal lesions. In some cases, postmortem artifacts hindered intestinal evaluation. Concurrent diseases in PPR-infected animals were found in 1 saiga with stomatitis typical of parapoxviral infection (contagious ecthyma) and 1 goitered gazelle with bacterial sepsis. Atrophy of adipose tissue and lymphoid depletion were identified in animals with and without evidence of PPRV infection (Appendix section 1.2; individual animal data, <https://doi.org/10.6084/m9.figshare.7502258.v1>).

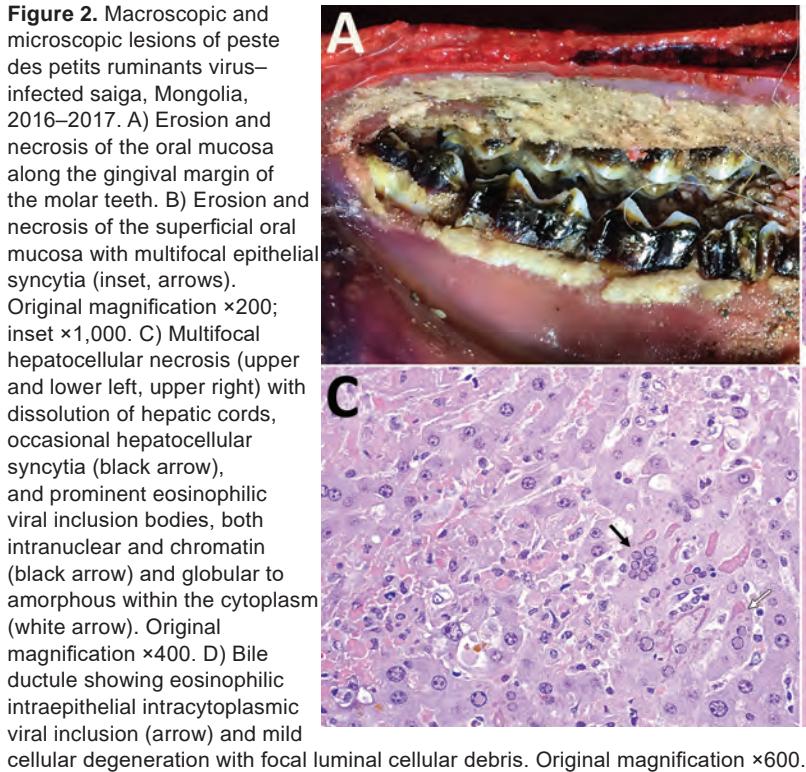
Phylogenetic Analysis

We obtained partial N-gene sequences from 2 PPRV-infected saiga. The phylogenetic analysis, conducted by using 58 partial N-gene sequences (Figure 3), confirmed that the PPRV sequences were of PPRV lineage IV and formed 1 cluster with sequences from livestock in Mongolia in 2016 (20) and from outbreaks in China in 2013–2016 (Figure 3). In addition, these sequences are genetically close to sequences from

Table 1. Major histologic lesions in animals infected with peste des petits ruminants and concurrent diseases, Mongolia, 2016–2017*

Lesion or disease	Animal ID nos.									
	Mongolian saiga						Goitered gazelle		Ibex	
	4	5, 8†	9	10	11	17	13, 15†	18	25	20
PPRV-specific lesions										
Oro/pharyngeal mucosa: erosion, epithelial necrosis, multifocal, acute (stomatitis, necrotizing)	+++ S+	+++ S+	+, S+	–	–	+++ S+ I+ (IN)	–	+, S+	NE	NE
Liver: degeneration and necrosis, hepatocytes, multifocal, random, acute (hepatitis, necrotizing)	NE	NE	++, S+ I+++ (IC and IN)	++	+	++	NE	++, S+ I+ (IN)	++, S+ I+ (IN>IC)	NE
Liver: degeneration and necrosis, biliary epithelium, bile ductules, multifocal, acute	NE	NE	+, I+ (IC>IN)	–	–	–	NE	+	++ I+ (IN>IC)	NE
Liver: cholestasis, canalicular, acute	NE	NE	+	+	–	–	NE	–	–	NE
Liver: hyperplasia, bile ductules, chronic	NE	NE	+	++	–	–	NE	–	–	NE
Lung: degeneration and necrosis, bronchiolar epithelium, multifocal, acute	NE	NE	+, S+ I+(IN and IC)	–	–	–	NE	–	++ S+ I++ (IN and IC)	NE
Intestine: necrosis, crypt epithelium, multifocal, acute	NE	NE	+, I+ (IC>IN)	–/PMA	–	–/PMA	NE	–/PMA	–/PMA	NE
Concurrent diseases	NA	NA	NA	NA	Proliferative stomatitis, parapoxvirus suspected	NA	NA	NA	Bacterial sepsis with bacteremia	NA

*I, presence of viral inclusion bodies; IC, intracytoplasmic inclusion; ID, identification; IN, intranuclear inclusion; NA, not applicable; NE, not examined; PMA, assessment hindered by postmortem autolysis; S, presence of syncytia; +, mildly severe; ++, moderately severe; +++, severe; –, lesion not found. †Tissue samples pooled.



central Asia (i.e., Iran [GenBank accession no. KY550670] and Tajikistan [accession no. DQ840198]).

Mapping of Confirmed and Unconfirmed Cases

From the onset of the outbreak in livestock to December 2018, samples from 30 georeferenced individual animals of 4 species (23 saiga, 5 ibex, 1 argali sheep, and 1 goitered gazelle) were submitted to and confirmed PPRV positive by the SCVL (Figure 4; case mapping data, <https://doi.org/10.6084/m9.figshare.7502264.v1>). Most of the early cases in saiga (until January 2017) were detected in the Durgun Steppe and the Khuisiin Gobi portions of the saiga home range (particularly in Chandmani Soum, Khovd Province); the cases in the Sharga Gobi portion of the saiga range and the rest of the Gobi-Altai Province first appeared in February 2017 (Figure 4). After the last reported saiga case in May 2017, all subsequent confirmed cases were in ibex that died in the Gobi-Altai Province through January 2018.

Suspected but unconfirmed cases were documented through interviews conducted during the Crisis Management Center–Animal Health field mission, review of provincial and central laboratory records of undiagnosed wildlife deaths, and interviews with local herders and rangers patrolling the saiga range (Figure 4). Unconfirmed cases revealed 2 clusters, 1 in the

southwestern part of Khovd Province close to the border with China and 1 between Khar-Uus and Durgun Lake north of the saiga range. The first cluster involved 10 ibex with severe diarrhea that led to death at 3 locations in July and August 2016. At that time, PPR was not suspected because the outbreak in livestock was not confirmed and declared until September 2016 (although retrospective serologic evidence indicates that PPRV was probably circulating in livestock as early as November 2015 [22]). The second cluster was reported by herders, who indicated substantial saiga deaths in December 2016, before the first PPR diagnosis in saiga was confirmed on December 27, 2016. Saiga rangers confirmed the death of at least 27 animals in these locations. This pattern mirrors quite closely the PPR outbreak observed in livestock; initial cases in livestock clustered at the southwestern Mongolia–China border and at a secondary outbreak focus in the Khar-Uus Lake area (22).

Government Carcass Disposal

From January through February 2017, the emergency response team collected and destroyed 4,202 saiga carcasses. Adding the soums for which collection continued until June 2017, the collection efforts totaled 5,425 saiga, 41 goitered gazelles, and 24 ibex. Among soums for which information on animal sex was recorded (Bayan-Uul, Darvi, Khukhmorit and Sharga),

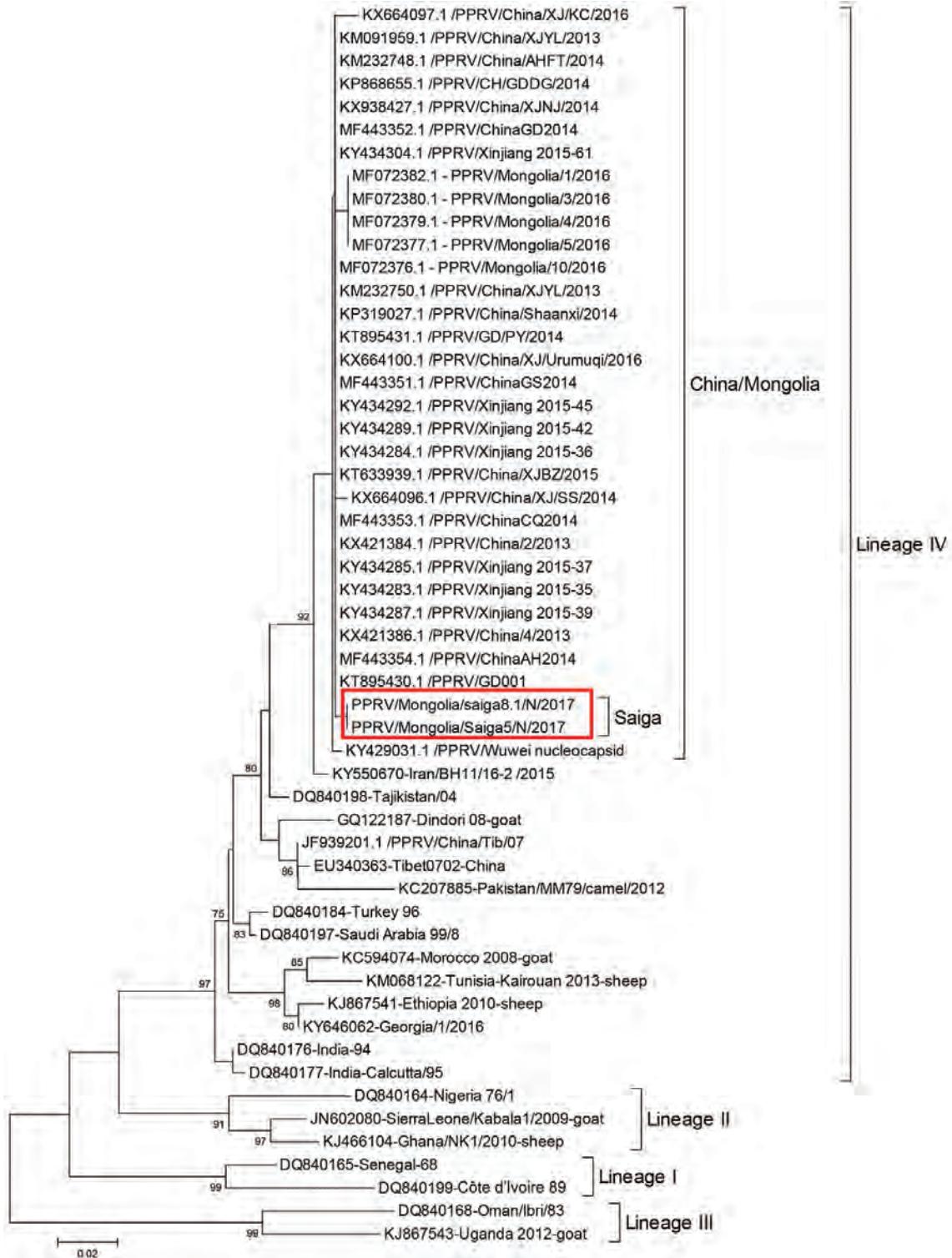


Figure 3. Neighbor-joining tree constructed on the basis of partial N-gene sequences of peste des petits ruminants virus (PPRV), showing relationships among the PPRV isolates. The Kimura 2-parameter model was used to calculate percentages (indicated by numbers beside branches) of replicate trees in which the associated taxa clustered together in 1,000 bootstrap replicates. Red rectangle outlines the 2 PPRV sequences from saiga obtained from this study (BankIt2279588 MOG/saiga5-2017, GenBank accession no. MN648447; BankIt2279588 MOG/saiga8.1-2017, accession no. MN648448). GenBank numbers are indicated. Scale bar indicates nucleotide substitutions per site.

sex ratios ranged from 2 to 6 females for 1 male. The absence of information on exact carcass locations, search routes, and search efforts prevented further assessment of the comprehensiveness of carcass collection and of the spatial distribution of the carcasses.

Saiga Population Surveys

Surveys conducted in January, March, and May 2017 and April 2018 indicated a steep decline in direct saiga observations along transects, from 2,130 saiga in 328 groups in January 2017 to 369 saiga in 46 groups in April 2018, despite similar survey efforts (Table 2). Abundance estimates provided by the best model for each period (Appendix section 3.3) confirmed the

saiga population decline from 25,699 (95% CI 19,249–34,310) in January 2017 to 8,806 (95% CI 6,095–12,721) by May 2017, the last month of reported saiga deaths. However, the saiga population continued declining after May 2017; the last survey in April 2018, almost a year after the outbreak, showed an estimated abundance of 5,142 (95% CI 2,929–9,028), 20% of the January 2017 population size (Figure 5). The average probability of detecting live animals in the surveyed area ranged from 0.38 to 0.59.

Discussion

Epidemiologic, pathology, and ancillary test findings in this PPR outbreak in Mongolia support the

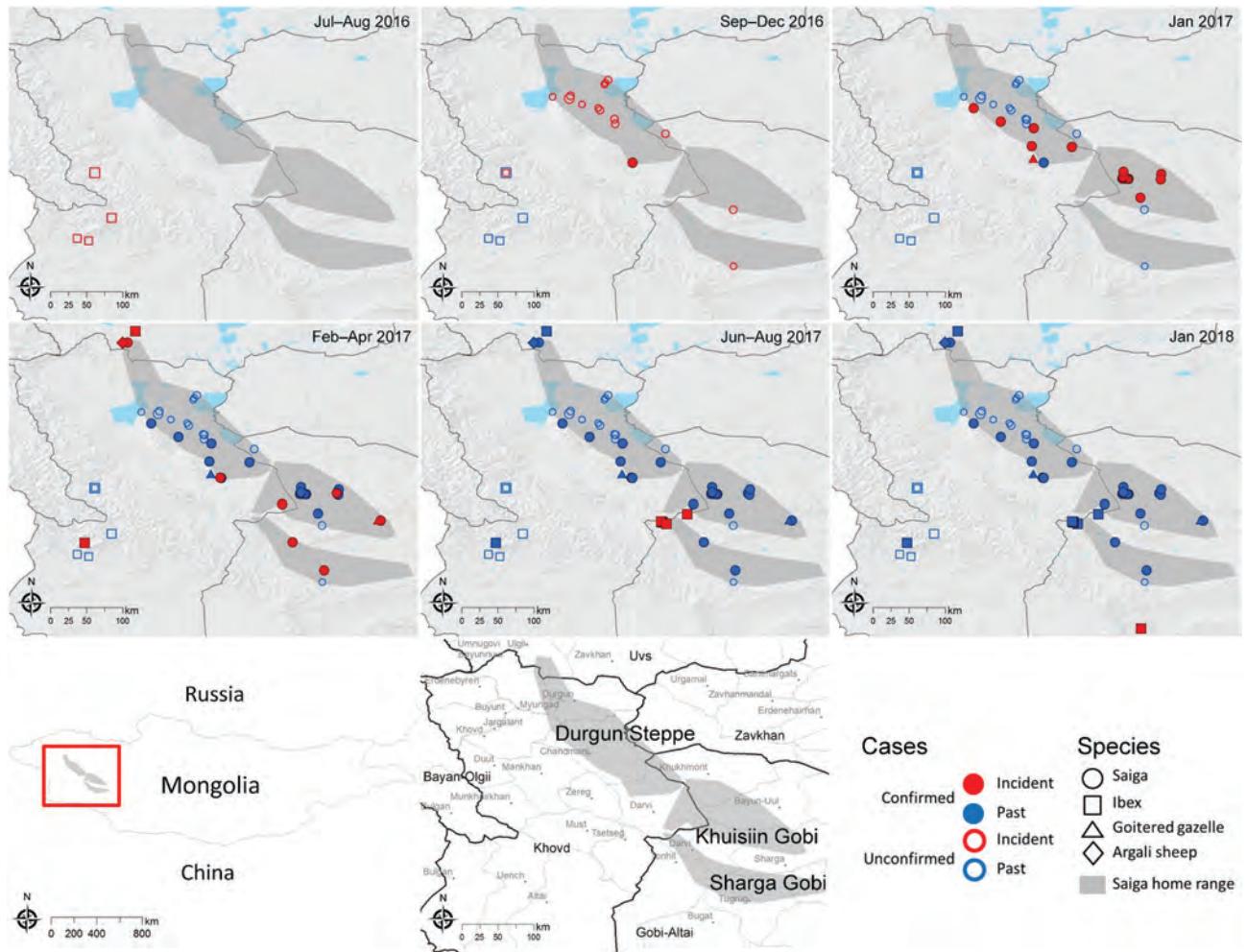


Figure 4. Spatiotemporal distribution of confirmed and unconfirmed cases of peste des petits ruminants (PPR) in several wild ungulate species in Mongolia. Each panel illustrates cases that occurred during the panel-specific time period (incident cases) and cases that occurred during previous periods (past cases). In the 2 periods before the first laboratory confirmation of PPR in saiga in December 2016, 2 clusters of unconfirmed cases (open shapes) were documented and matched the pattern of livestock PPR case distribution. In January 2017, the outbreak spread rapidly through the saiga population and expanded southward and northward from February 2017 through May 2017, when the last confirmed saiga cases were reported. Subsequent PPR cases involved ibex until at least January 2018. Maps at bottom show location of study area in Mongolia and specific location names.

Table 2. Summary statistics from saiga distance sampling surveys conducted in study of outbreak of peste des petits ruminants among critically endangered wild ungulates, Mongolia, January 2017–April 2018*

Date	Days since first PPR confirmation	Total effort, km	No. individuals (groups)	Density of individuals (95% CI)	Abundance (95% CI)	Expected cluster size (95% CI)	Average probability of detecting live saiga
2017 Jan	30	1,505	2,130 (321)	1.18 (0.88–1.58)	25,699 (19,249–34,310)	D: 3.9 (3.2–4.8); K: 4.1 (3.4–4.9); S: 6.3 (4.8–8.4)	D: 0.35 (0.29–0.43); K: 0.37 (0.31–0.43); S: 0.42 (0.35–0.50)
2017 Mar	90	1,505	1,999 (148)	0.73 (0.45–1.20)	15,933 (9,759–26,011)	D: 10.9 (7.5–16.1); K: 13.5 (9.5–19.2); S: 5.4 (4.2–7.0)	D: 0.44 (0.36–0.55); K: 0.69 (0.51–0.94); S: 0.60 (0.50–0.71)
2017 May	150	1,263	742 (157)	0.41 (0.28–0.58)	8,806 (6,095–12,721)	D: 2.6 (2.1–3.3); K: 2.7 (2.2–3.3); S: 2.4 (1.7–3.6)	0.51 (0.45–0.57)
2018 Apr	480	1,505	369 (46)	0.24 (0.14–0.42)	5,142 (2,929–9,028)	D: 11.7 (7.5–18.1); K: 10.9 (6.1–19.6); S: 6.3 (4.4–8.9)	0.51 (0.40–0.65)

*D, Durgun Steppe; K, Khuisiin Gobi; PPR, peste des petits ruminants; S, Sharga Gobi.

diagnosis of PPRV infection causing wild ungulate deaths across both desert steppe and mountain ecosystems. Saiga antelope were confirmed to be susceptible and capable of spreading PPRV infection within their population over a short time, suggesting high viral excretion loads and contact rates over the period of the epidemic. PPR in 3 other species of antelope in a semicaptive private collection in the United Arab Emirates has been previously reported (30), and our report indicates that free-ranging antelope exhibit the same level of susceptibility. The multiple clusters of ibex deaths suggest multiple spillovers from livestock followed by effective intraspecies transmission. The occurrence of apparently sporadic deaths among goitered gazelle and Argali sheep confirms the susceptibility of these species but raises questions about their ability to spread infection.

The pathologic lesions found in this outbreak were largely consistent with typically reported PPR lesions in domestic species (31) (Appendix section 1.2). The prominence of liver lesions and involvement of biliary epithelium were unusual, although they have been reported for other morbillivirus infections, including infection of wild lesser kudu (*Tragelaphus imberbis*) by the closely related rinderpest virus (32). Pathologic findings in the examined animals were not consistent with hemorrhagic septicemia caused by *Pasteurella multocida*, which caused large-scale saiga mortality in Kazakhstan (33,34). Diminished fat reserves and lymphoid depletion in PPR-positive and PPR-negative saiga was a nonspecific finding and could indicate the existence of environmental stressors, possibly explained by midwinter conditions. Further research is required to assess whether poor body condition and potential immunosuppression could contribute to virulent expression of PPRV in these animals. The presence of at least 2 pathogens of live-

stock in the examined saiga also suggested a high permeability of this livestock–wildlife interface to infectious diseases, which may have contributed to the overall mortality rate.

The 2 PPRV N-gene sequences obtained from infected saiga were similar to sequences obtained from livestock during outbreaks in Mongolia in 2016 and China in 2013–2016. This similarity is consistent with a spillover of PPRV from infected domesticated sheep and goats to wild ungulates. In addition, these sequences were genetically close to sequences from central Asia (i.e., Iran [GenBank accession no. KY550670] and Tajikistan [accession no. DQ840198]) but different from sequences from China in 2007 (accession nos. EU340363 and JF939201) (Figure 3), which suggests recent spread of PPRV from central Asia to China and then to livestock and wild ungulates in Mongolia.

PPRV outbreak mapping suggested that wildlife may have been infected earlier (possibly July 2016) than the first confirmed case (December 2016) and that wildlife infections closely followed the timing of the livestock outbreak. The absence of laboratory confirmation of PPRV infection for these initially unconfirmed clusters warrants cautious interpretation, but strong epidemiologic evidence indicates that these suspected cases were part of the same PPRV outbreak. The apparent spatial discontinuity between the 2 outbreak foci supports the hypothesis that the spread of PPR was mainly driven by livestock movement, because the wild mountain ungulates (ibex in the first putative outbreak focus) are relatively resident and unlikely to move long distances across multiple ecotypes. This spatial discontinuity also suggests multiple spillover events from livestock to different wildlife populations, which will require further analysis based on genetic data.

The early onset of PPRV in ibex and the lower and more prolonged incidence of cases in this species (at least until January 2018) are in contrast with the rapid transmission through the saiga population (apparently ceased by June 2017). This contrast in incidence suggests different dynamics of PPRV transmission in the 2 species, influenced by population structure, habitat, and interspecies-intraspecies interactions. Further work, including identification of shared resources between species (e.g., watering points, residual snow patches, and mineral licks), contact rates, and modeling should be conducted to better determine the most likely transmission routes and the respective roles of these wild and domestic ungulates in this multihost system. The probable 5-month delay between the first unconfirmed cases documented and the first confirmation in saiga underscores the value of maintaining operational wildlife health surveillance systems for early detection of wildlife illness and deaths.

The initial mortality estimates, obtained from carcass collection and disposal efforts, were probably underestimated because of imperfect detection (35–38) (Appendix section 3.3). If systematic carcass removal is determined to be cost-effective, adopting standard ecological monitoring methods to ensure reliable and unbiased characterization of mortality patterns is imperative (39). The most compelling estimate of the population-level effect of the PPRV outbreak in saiga was, therefore, derived from the population monitoring efforts by using distance sampling methods (which account for imperfect detection), indicating a saiga population decline of >80%. These estimates depict a serious situation for the Mongolian saiga population and a substantial setback after >10 years of conservation efforts to secure saiga population recovery after a historical low in the early 2000s (40,41). The significance of this event to saiga goes beyond the Mongolian subspecies because other unrelated mass mortality events have recently affected the species and are threatening its global conservation (33). Although saiga have shown great potential for recovery (42), in part because of fertility and frequently giving birth to twins or triplets (43,44), the population estimates a year after the outbreak showed little evidence of recovery. The timing of the outbreak just after rut season (which may have facilitated transmission because of congregation of animals) and during gestation probably delayed recovery through effects on recruitment. In addition, very cold temperatures with exceptionally heavy snowfall (known as dzud) also resulted in saiga deaths during winter 2018 and

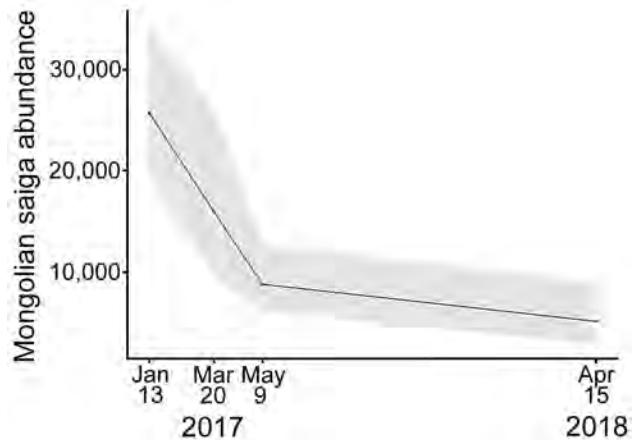


Figure 5. Saiga population in Mongolia during the outbreak of peste des petits ruminants in 2017 and the next year. Shaded area represent 95% CIs around abundance estimates.

probably contributed to the additional population decline from June 2017 through April 2018 (Figure 5). We cannot exclude as potential causes for the sustained population decline the cumulative effects of multiple factors, other concurrent conditions, and undetected PPRV circulation. The lack of similarly detailed data for the other species of ungulates prevented assessment of the full conservation effect of the outbreak, but deaths across the ungulate community suggest broader effects on these ecosystems.

Factors that favored the eradication of rinderpest included an expectation that wildlife did not act as a reservoir of infection for domestic animals (8). This multispecies mass mortality event in Mongolia and recent similar events in eastern Asia and the Middle East (18) challenge the assumption that wildlife play a negligible role in the epidemiology and ecology of PPRV. This observation has substantial implications for the current global eradication program and efforts to outline National Strategic Plans for PPR control and eradication. The explicit integration of wildlife protection into these National Strategic Plans should be considered, and plans should include setting livestock vaccination targets that can effectively prevent spillover of virus from livestock to other susceptible wildlife (45).

The growing number of livestock on rangelands of low productivity, such as in Mongolia and much of central Asia (26,46,47), exerts increasing pressure on sympatric wild ungulates through competition for resources (48,49). Restricted access of wild ungulates to quality forage, water, and minerals may result in poor nutritional status and immune function (50), possibly reducing their resilience to livestock pathogens to which they are increasingly exposed.

Global changes in climate and expected shifts in species distributions and habitat suitability may further reduce resource availability and increase wildlife–livestock interactions. Evidence for possible dislocation of species-habitat-climate relationships leading to increased susceptibility to disease can be found in the mass mortality that occurred because of hemorrhagic septicemia in another subspecies of saiga (*Saiga tartarica tartarica*) in Kazakhstan (34). These combined factors could result in an increasing number of disease spillover events, followed by rapid amplification in populations already under multifactorial stresses. To ensure that objectives of rural development and biodiversity protection are compatible and jointly met, integration of livestock and wildlife management must be improved (Figure 1). Doing so proactively in the face of global climate change and increasing demands of a growing global population is a critical challenge of this century.

Acknowledgments

We thank the saiga ranger network for their commitment to the monitoring of the Mongolian saiga population and their on-the-ground response to the PPR outbreak among saiga; Henny Martineau and Alwyn Jones for parallel examination of pathologic tissues from saiga, reporting findings consistent with those reported in the article; Sarah Olson for her helpful comments reviewing this manuscript; and Tran Minh Phuc for graphic design.

Support was obtained from EU-BBSRC Anihwa grant BB/L013657/1 and BBSRC/DBT FADH grant BB/L004801/1 to conduct the molecular work at the Pirbright Institute, UK. Data synthesis for this manuscript was facilitated by funding from Science for Nature and People Partnership. The response to the PPR outbreak in Mongolian wildlife and this study was made possible through support from the Wildlife Conservation Society, Morris Animal Foundation, Trust for Mutual Understanding, Food and Agriculture Organization of the United Nations, Saiga Conservation Alliance, World Wide Fund for Nature Mongolia Programme Office, and local and national agencies of the government of Mongolia.

About the Author

Dr. Pruvot is a veterinary epidemiologist with the Wildlife Conservation Society, Wildlife Health Program. His primary research interests are disease transmission between wildlife and livestock, the effect of anthropogenic environmental changes on the ecology of emerging diseases, and improvement of wildlife health surveillance.

References

- Balamurugan V, Hemadri D, Gajendragad MR, Singh RK, Rahman H. Diagnosis and control of peste des petits ruminants: a comprehensive review. *VirusDisease*. 2014;25:39–56. <https://doi.org/10.1007/s13337-013-0188-2>
- Munir M. Role of wild small ruminants in the epidemiology of peste des petits ruminants. *Transbound Emerg Dis*. 2014;61:411–24. <https://doi.org/10.1111/tbed.12052>
- Parida S, Couacy-Hymann E, Pope RA, Mahapatra M, El Harrak M, Brownlie J, et al. Pathology of peste des petits ruminants. In: Munir M, editor. *Peste des petits ruminants virus*. Berlin, Heidelberg: Springer Berlin Heidelberg, Berlin, Heidelberg; 2015. p. 51–67.
- Jones BA, Rich KM, Mariner JC, Anderson J, Jeggo M, Thevasagayam S, et al. The economic impact of eradicating peste des petits ruminants: a benefit-cost analysis. *PLoS One*. 2016;11:e0149982. <https://doi.org/10.1371/journal.pone.0149982>
- Anderson J, Baron M, Cameron A, Kock R, Jones B, Pfeiffer D, et al. Rinderpest eradicated; what next? *Vet Rec*. 2011;169:10–1. <https://doi.org/10.1136/vr.d4011>
- Banyard AC, Parida S. Eradicating peste des petits ruminants - the challenges ahead. *British Journal of Virology*. 2016;3(3s):47–52. <https://doi.org/10.17582/journal.bjv/2016.3.3s.47.52>
- Food and Agriculture Organization of the United Nations and the World Organisation for Animal Health. *Peste des petits ruminants global eradication. Contributing to food security, programme poverty alleviation and resilience*. Rome: Food and Agriculture Organization of the United Nations; 2016.
- Mariner JC, Jones BA, Rich KM, Thevasagayam S, Anderson J, Jeggo M, et al. The opportunity to eradicate peste des petits ruminants. *J Immunol*. 2016;196:3499–506. <https://doi.org/10.4049/jimmunol.1502625>
- Aziz-Ul-Rahman, Wensman JJ, Abubakar M, Shabbir MZ, Rossiter P. Peste des petits ruminants in wild ungulates. *Trop Anim Health Prod*. 2018;50:1815–9.
- Parida S, Muniraju M, Mahapatra M, Muthuchelvan D, Buczkowski H, Banyard AC. Peste des petits ruminants. *Vet Microbiol*. 2015;181:90–106. <https://doi.org/10.1016/j.vetmic.2015.08.009>
- Mahapatra M, Sayalel K, Muniraju M, Eblate E, Fyumagwa R, Shilinde L, et al. Spillover of peste des petits ruminants virus from domestic to wild ruminants in the Serengeti ecosystem, Tanzania. *Emerg Infect Dis*. 2015;21:2230–4. <https://doi.org/10.3201/eid2112.150223>
- Bao J, Wang Z, Li L, Wu X, Sang P, Wu G, et al. Detection and genetic characterization of peste des petits ruminants virus in free-living bharals (*Pseudois nayaur*) in Tibet, China. *Res Vet Sci*. 2011;90:238–40. <https://doi.org/10.1016/j.rvsc.2010.05.031>
- Hoffmann B, Wiesner H, Maltzan J, Mustefa R, Eschbaumer M, Arif FA, et al. Fatalities in wild goats in Kurdistan associated with peste des petits ruminants virus. *Transbound Emerg Dis*. 2012;59:173–6. <https://doi.org/10.1111/j.1865-1682.2011.01270.x>
- Marashi M, Masoudi S, Moghadam MK, Modirrousta H, Marashi M, Parvizifar M, et al. Peste des petits ruminants virus in vulnerable wild small ruminants, Iran, 2014–2016. *Emerg Infect Dis*. 2017;23:704–6. <https://doi.org/10.3201/eid2304.161218>
- Elzein EMEA, Housawi FM, Bashareek Y, Gameel AA, Al-Afaleq AI, Anderson E. Severe PPR infection in gazelles kept under semi-free range conditions. *J Vet Med B Infect Dis*

- Vet Public Health. 2004;51:68–71. <https://doi.org/10.1111/j.1439-0450.2004.00731.x>
16. Frölich K, Hamblin C, Jung S, Ostrowski S, Mwanzia J, Streich WJ, et al. Serologic surveillance for selected viral agents in captive and free-ranging populations of Arabian oryx (*Oryx leucoryx*) from Saudi Arabia and the United Arab Emirates. *J Wildl Dis.* 2005;41:67–79. <https://doi.org/10.7589/0090-3558-41.1.67>
 17. Furley CW, Taylor WP, Obi TU. An outbreak of peste des petits ruminants in a zoological collection. *Vet Rec.* 1987;121:443–7. <https://doi.org/10.1136/vr.121.19.443>
 18. Fernandez-Aguilar XF, Fine AE, Pruvot M, Njeumi F, Walzer C, Kock R, et al. PPR virus threatens wildlife conservation. *Science.* 2018;362:165–6.
 19. Leylabadlo HE, Kafil HS, Asgharzadeh M. Peste des petits ruminants (PPR): a serious threat for wild life. *Adv Biosci Clin Med.* 2016;4.
 20. Shatar M, Khanui B, Purevtseren D, Khishgee B, Loitsch A, Unger H, et al. First genetic characterization of peste des petits ruminants virus from Mongolia. *Arch Virol.* 2017;162:3157–60. <https://doi.org/10.1007/s00705-017-3456-4>
 21. Ts U, Uganbayar E, Odbileg R. Sero-surveillance of “Peste des Petits Ruminants” PPR in Mongolia and development of recommendation. *Mong J Agric Sci.* 2017;19:22–6. <https://doi.org/10.5564/mjas.v19i3.731>
 22. Food and Agriculture Organization of the United Nations, Crisis Management Center–Animal Health. Investigation of peste des petits ruminants (PPR) among wild animals and its potential impact on the current PPR situation in livestock [cited 2019 Nov 7]. https://www.researchgate.net/publication/337059734_Mongolia_Investigation_of_Peste_des_Petits_Ruminants_PPR_among_wild_animals_and_its_potential_impact_on_the_current_PPR_situation_in_livestock
 23. Young JK, Murray KM, Strindberg S, Buuveibaatar B, Berger J. Population estimates of endangered Mongolian saiga *Saiga tatarica mongolica*: implications for effective monitoring and population recovery. *Oryx.* 2010;44:285–92. <https://doi.org/10.1017/S0030605309990858>
 24. National Statistical Office of Mongolia. Livestock census. Ulaanbaatar (Mongolia): The Office; 2016.
 25. Lkhagvadorj D, Hauck M, Dulamsuren C, Tsogtbaatar J. Pastoral nomadism in the forest-steppe of the Mongolian Altai under a changing economy and a warming climate. *J Arid Environ.* 2013;88:82–9. <https://doi.org/10.1016/j.jaridenv.2012.07.019>
 26. Baron J, Fishbourne E, Couacy-Hyman E, Abubakar M, Jones BA, Frost L, et al. Development and testing of a field diagnostic assay for peste des petits ruminants virus. *Transbound Emerg Dis.* 2014;61:390–6. <https://doi.org/10.1111/tbed.12266>
 27. Couacy-Hymann E, Roger F, Hurard C, Guillou JP, Libeau G, Diallo A. Rapid and sensitive detection of peste des petits ruminants virus by a polymerase chain reaction assay. *J Virol Methods.* 2002;100:17–25. [https://doi.org/10.1016/S0166-0934\(01\)00386-X](https://doi.org/10.1016/S0166-0934(01)00386-X)
 28. Buckland ST, Anderson DR, Burnham KP, Laake J, Borchers D, Thomas L. Introduction to distance sampling: estimating abundance of biological populations. Oxford (UK): Oxford University Press; 2001. p. 448.
 29. Thomas L, Buckland ST, Rexstad EA, Laake JL, Strindberg S, Hedley SL, et al. Distance software: design and analysis of distance sampling surveys for estimating population size. *J Appl Ecol.* 2010;47:5–14. <https://doi.org/10.1111/j.1365-2664.2009.01737.x>
 30. Kinne J, Kreutzer R, Kreutzer M, Wernery U, Wohlsein P. Peste des petits ruminants in Arabian wildlife. *Epidemiol Infect.* 2010;138:1211–4. <https://doi.org/10.1017/S0950268809991592>
 31. Kul O, Kabakci N, Atmaca HT, Ozkul A. Natural peste des petits ruminants virus infection: novel pathologic findings resembling other morbillivirus infections. *Vet Pathol.* 2007;44:479–86. <https://doi.org/10.1354/vp.44-4-479>
 32. Kock ND, Kock RA, Wambua J, Mwanzia J. Pathological changes in free-ranging African ungulates during a rinderpest epizootic in Kenya, 1993 to 1997. *Vet Rec.* 1999;145:527–8. <https://doi.org/10.1136/vr.145.18.527>
 33. Fereidouni S, Freimanis GL, Orynbayev M, Ribeca P, Flannery J, King DP, et al. Mass die-off of saiga antelopes, Kazakhstan, 2015. *Emerg Infect Dis.* 2019;25:1169–76. <https://doi.org/10.3201/eid2506.180990>
 34. Kock RA, Orynbayev M, Robinson S, Zuther S, Singh NJ, Beauvais W, et al. Saigas on the brink: multidisciplinary analysis of the factors influencing mass mortality events. *Science Advances.* 2018;4:eaa02314.
 35. Kellner KF, Swihart RK. Accounting for imperfect detection in ecology: a quantitative review. *PLoS One.* 2014;9:e111436. <https://doi.org/10.1371/journal.pone.0111436>
 36. Kéry M, Schmid H. Monitoring programs need to take into account imperfect species detectability. *Basic Appl Ecol.* 2004;5:65–73. <https://doi.org/10.1078/1439-1791-00194>
 37. Kéry M, Schmidt B. Imperfect detection and its consequences for monitoring for conservation. *Community Ecol.* 2008;9:207–16. <https://doi.org/10.1556/ComEc.9.2008.2.10>
 38. MacKenzie DI, Kendall WL. How should detection probability be incorporated into estimates of relative abundance? *Ecology.* 2002;83:2387–93. [https://doi.org/10.1890/0012-9658\(2002\)083\[2387:HSDPBI\]2.0.CO;2](https://doi.org/10.1890/0012-9658(2002)083[2387:HSDPBI]2.0.CO;2)
 39. McClintock BT, Nichols JD, Bailey LL, MacKenzie DI, Kendall WL, Franklin AB. Seeking a second opinion: uncertainty in disease ecology. *Ecol Lett.* 2010;13:659–74. <https://doi.org/10.1111/j.1461-0248.2010.01472.x>
 40. Convention on Migratory Species. Memorandum of understanding concerning conservation, restoration and sustainable use of the saiga antelope [cited 2018 Jul 19]. <http://www.cms.int/saiga>
 41. Milner-Gulland EJ, Kholodova MV, Bekenov A, Bukreeva OM, Grachev IA, Amgalan L, et al. Dramatic declines in saiga antelope populations. *Oryx.* 2001;35:340–5. <https://doi.org/10.1046/j.1365-3008.2001.00202.x>
 42. Buuveibaatar B, Young JK, Berger J, Fine AE, Lkhagvasuren B, Zahler P, et al. Factors affecting survival and cause-specific mortality of saiga calves in Mongolia. *J Mammal.* 2013;94:127–36. <https://doi.org/10.1644/11-MAMM-A-077.1>
 43. Bekenov AB, Grachev IA, Milner-Gulland EJ. The ecology and management of the saiga antelope in Kazakhstan. *Mammal Rev.* 1998;28:1–52. <https://doi.org/10.1046/j.1365-2907.1998.281024.x>
 44. Kühl A, Mysterud A, Erdnenov GI, Lushchekina AA, Grachev IA, Bekenov AB, et al. The ‘big spenders’ of the steppe: sex-specific maternal allocation and twinning in the saiga antelope. *Proc Biol Sci.* 2007;274:1293–9. <https://doi.org/10.1098/rspb.2007.0038>
 45. Baron MD, Diop B, Njeumi F, Willett BJ, Bailey D. Future research to underpin successful peste des petits ruminants virus (PPRV) eradication. *J Gen Virol.* 2017;98:2635–44. <https://doi.org/10.1099/jgv.0.000944>
 46. Reading RP, Bedunah DJ, Amgalanbaatar S. Conserving biodiversity on Mongolian rangelands: implications for protected area development and pastoral uses [cited 2019

RESEARCH

- Nov 4]. https://www.fs.fed.us/rm/pubs/rmrs_p039/rmrs_p039_001_017.pdf
47. Wesche K. Plant survival in southern Mongolian desert steppes—ecology of communities, interactions and populations [cited 2019 Nov 7]. <http://www.secheresse.info/spip.php?article83244>
48. Gao W, Angerer JP, Fernandez-Gimenez ME, Reid RS. Is overgrazing a pervasive problem across Mongolia? An examination of livestock forage demand and forage availability from 2000 to 2014 [cited 2019 Nov 7]. https://mountainscholar.org/bitstream/handle/10217/181739/CONF_MOR2-2015-ENG-ALL.pdf
49. Berger J, Buuveibaatar B, Mishra C. Globalization of the cashmere market and the decline of large mammals in central Asia. *Conserv Biol.* 2013;27:679–89. <https://doi.org/10.1111/cobi.12100>
50. Jolles AE, Beechler BR, Dolan BP. Beyond mice and men: environmental change, immunity and infections in wild ungulates. *Parasite Immunol.* 2015;37:255–66. <https://doi.org/10.1111/pim.12153>

Address for correspondence: Mathieu Pruvot, Wildlife Conservation Society, 2300 Southern Blvd, Bronx, NY, 10460, USA; email: mpruvot@wcs.org

The Public Health Image Library (PHIL)



The Public Health Image Library (PHIL), Centers for Disease Control and Prevention, contains thousands of public health-related images, including high-resolution (print quality) photographs, illustrations, and videos.

PHIL collections illustrate current events and articles, supply visual content for health promotion brochures, document the effects of disease, and enhance instructional media.

PHIL images, accessible to PC and Macintosh users, are in the public domain and available without charge.

Visit PHIL at:
<http://phil.cdc.gov/phil>

Elephant Endotheliotropic Herpesvirus Hemorrhagic Disease in Asian Elephant Calves in Logging Camps, Myanmar

Zaw Min Oo, Ye Htut Aung, Tin Tun Aung, Nyo San, Zaw Min Tun, Gary S. Hayward, Arun Zachariah

In recent years, an alarming number of cases of lethal acute hemorrhagic disease have occurred in Asian elephant calves raised in logging camps in Myanmar. To determine whether these deaths were associated with infection by elephant endotheliotropic herpesvirus (EEHV), we conducted diagnostic PCR subtype DNA sequencing analysis on necropsy tissue samples collected from 3 locations. We found that EEHV DNA from 7 PCR loci was present at high levels in all 3 calves and was the same EEHV1A virus type that has been described in North America, Europe, and other parts of Asia. However, when analyzed over 5,610 bp, the strains showed major differences from each other and from all previously characterized EEHV1A strains. We conclude that these 3 elephant calves in Myanmar died from the same herpesvirus disease that has afflicted young Asian elephants in other countries over the past 20 years.

In 1990, a brief description of a case of lethal acute hemorrhagic disease in a young Asian circus elephant in Switzerland that apparently involved a previously unknown herpesvirus was reported by Ossent et al. (1). However, it was not until the study of Richman et al. (2,3), published in 1999, that this disease was shown to be associated with a novel herpesvirus designated elephant endotheliotropic herpesvirus (EEHV) 1 because of detection of nuclear inclusion bodies in damaged vascular endothelial cells in diseased heart and liver tissues. Those 2 studies provided evidence for the presence of high levels of small segments of the DNA genome of EEHV1 found by PCR sequencing

techniques in blood samples and necropsy tissue samples from all major internal organs during active cases of hemorrhagic disease in 5 Asian elephant calves in zoos in North America and Europe, as well as in archival tissue block samples from 6 other cases in older elephants. Only 2 of the afflicted calves with less severe signs survived the acute disease after treatment with the human antiherpesvirus drug famciclovir; and viral DNA load in their blood was documented to decrease below levels of detection over a 4–6-week convalescence and recovery period. One young African elephant calf from a zoo that had a similar lethal hemorrhagic disease case had DNA from a second related virus species (EEHV2).

Since these original reports, numerous studies reviewed by Hayward (4) and Long et al. (5) have shown the presence of either of 2 chimeric subtypes of EEHV1A or EEHV1B in ≤ 50 additional lethal cases and 10 drug-treated confirmed DNA-positive survivors with signs of disease in mostly young Asian elephants in Europe and North America (6–8). A small number of lethal and nonlethal cases of viral DNA-positive hemorrhagic disease cases in young Asian elephants involved 2 additional related but considerably diverged species of *Proboscivirus* (EEHV4 and EEHV5) (9–16). EEHV4 is estimated to have last had common ancestors with EEHV1 35 million years ago and EEHV5 is estimated to have last had common ancestors with EEHV1 20 million years ago.

Furthermore, many examples of occasional shedding of these viruses, especially in trunk-wash and saliva samples have also been documented from adult Asian and African elephants with signs of disease (17–20; V.R. Pearson et al., Fox Chase Cancer Center, pers. comm., 2019 Jan 22). All currently available data (5,13) support the concept that EEHV1A, EEHV1B, EEHV4, and EEHV5 are endemic in Asian elephants (*Elephas maximus*), whereas EEHV2, EEHV3,

Author affiliations: University of Veterinary Sciences, Yezin, Myanmar (Z.M. Oo, Y.H. Aung); Ministry of Environmental Conservation and Forestry, Yangon, Myanmar (Z.M. Oo, T.T. Aung, N. San, Z.M. Tun); Johns Hopkins School of Medicine, Baltimore, Maryland, USA (G.S. Hayward); Kerala Veterinary and Animal Sciences University, Wayanad, India (A. Zachariah)

DOI: <https://doi.org/10.3201/eid2601.190159>

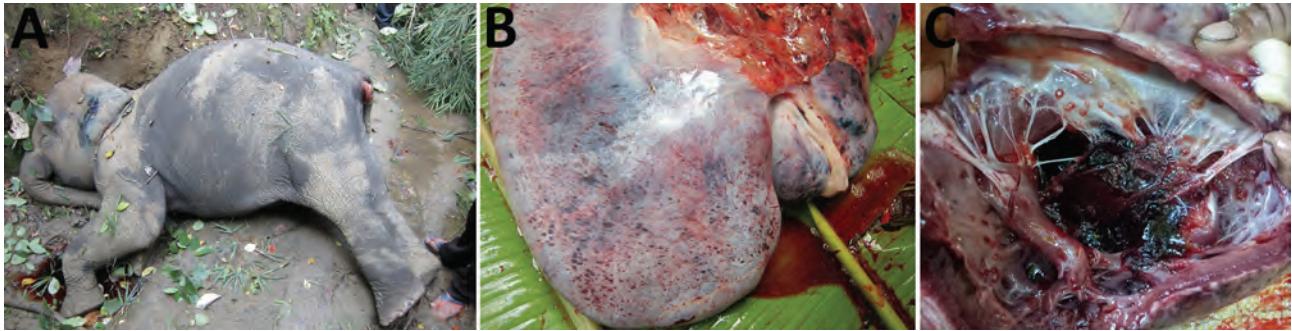


Figure 1. Gross morphologic characteristics of Asian elephant calf no. 3 (MP03) that had endotheliotropic herpesvirus hemorrhagic disease in logging camp, Myanmar. A) Before postmortem necropsy. B) Hemorrhagic lesions on intact surface of the liver. C) Hemorrhagic lesions inside the atrium and valves of the dissected heart.

EEHV6, and EEHV7 are endemic to and largely ubiquitous in African elephants (*Loxodonta africana*). A subset of $\approx 20\%$ of immunologically naive young Asian elephants 1–8 years of age are susceptible to the most severe forms of hemorrhagic disease that are caused predominantly by primary infections with the most pathogenic version of these viruses (EEHV1A).

A major aspect of the situation concerning possible long-term effects on breeding and survival of critically endangered Asian elephants worldwide is whether these viruses and the same hemorrhagic disease occur within countries in Asia and especially in wild populations. Reid et al. (21) reported an EEHV1 DNA-positive case from Cambodia. However, it was not until Zachariah et al. (22) published their results, after setting up a diagnostic PCR DNA laboratory in southern India to examine necropsy tissue collected from young orphan and wild elephants that suddenly died, that the range of the disease and virus became firmly established. These authors initially described 8 lethal cases of hemorrhagic disease associated with EEHV1A and 1 case associated with EEHV1B from India. Later in the same year, Sripiboon et al. (23) also reported 2 cases involving EEHV1A and EEHV4 from Thailand. More recent studies have also confirmed 2 lethal cases of EEHV1 hemorrhagic disease in Laos (24) and as many as 15 additional cases in Thailand (25,26).

We have also expanded the studies in India to 22 lethal cases, including 12 in wild free-ranging calves (27). In this study, we report DNA sequence-confirmed cases of lethal EEHV1A disease in 3 young logging camp elephants in Myanmar, including partial

genetic analysis by PCR sequencing of the strains involved and comparison with all the cases from India and other representative cases worldwide.

Materials and Methods

Case 1 (M1) was in a 20-month-old, captive-born, female *E. maximus* calf that died suddenly on February 11, 2012, in Minbu District, Magway region, Myanmar. Case 2 (M2) was in a 22-month-old, captive-born, male *E. maximus* calf that died suddenly on September 14, 2013, in Nyaung Lay Pin District, Bago (East) region, Myanmar. Case 3 (M3) was in a 16-month-old, captive-born, female *E. maximus* calf that died suddenly on January 8, 2014, in Nay Pyi Taw District, Myanmar.

We observed typical multiorgan hemorrhages, particularly in heart, liver, and peritoneum. We collected tissue samples and placed them in molecular-grade absolute ethanol during postmortem and stored them at -20°C . We used liver samples from each case for extraction of intracellular DNA by using the QIAamp Blood and Tissue Mini Kit (QIAGEN, <https://www.qiagen.com>). Conventional PCR amplifications were performed at the University of Veterinary Sciences (Yezin, Myanmar) during an EEHV workshop. These DNA samples were used as templates to amplify and sequence 7 standard preferred EEHV1 PCR DNA loci (22,27) from all 3 cases, as well as an eighth locus (U51, vGPCR1) from 1 of these cases. We performed Sanger DNA sequencing and DNA sequence editing and constructed phylogenetic trees as described (6,10,22). We used the updated sets of PCR primers reported by Zachariah et al. (27).

Table 1. EEHV PCR gene locus subtype data for cases of hemorrhagic disease in 3 Asian elephant calves, Myanmar*

Case	PCR gene locus							
	E5	vGPCR1	U71-gM	POL-AB	TER	gH-TK	HEL	vOX2
MP01, Myanmar1	B1	ND	A4	A	A1	D	A1	1
MP02, Myanmar2	B1	ND	A4	A	C	D	C2	2
MP03, Myanmar3	C1	E	A5	A	A3	D	A2	3

*EEHV, elephant endotheliotropic herpesvirus; ND, not determined; 1, identical to IP143 from India; 2, novel variant not seen previously; 3, identical to IP93 from India.

Table 2. GenBank DNA file accession numbers for cases of hemorrhagic disease in 3 Asian elephant calves, Myanmar*

Case, virus code	Size, bp	MP01	MP02	MP03
E3(vGPCR5)	962	MF579041	MF579042	MF577043
U38(POL)	485	MF579060	MF579061	MF579062
U48(gH-TK)	850	MF464877	MF464878	MF464879
U51(vGPCR1)	677	ND	ND	MF579097
U60(TERex3) L	741/724/726	MF579075	MF579076	MF579077
U71-gM	651	MF579110	MF579111	MF579112
U77(HEL)	952/921/952	MF579126	MF579127	MF579128
E54(vOX2-1)	854	MF464888	MF464889	MF464890

*ND, not done.

We generated detailed results for genetic differences between EEHV DNA samples at each PCR locus as linear nucleotide-level polymorphisms by using Geneious software (<https://www.geneious.com>) from alignments made in Muscle software

(<https://www.drive5.com/muscle>) or as Bayesian nearest neighbor-based phylogenetic trees at the DNA or protein level. For comparative purposes, we combined results for Myanmar (MP#) with all data for samples from India (IP#) and Sumatra (SP#) evaluated



Figure 2. Nucleotide sequence polymorphism charts for Asian elephant calves that had endotheliotropic herpesvirus hemorrhagic disease in logging camp, Myanmar. Shown are comparisons for hemorrhagic disease cases MP01, MP02, and MP03 across 2 hypervariable EEHV1 PCR loci. A) E5(vGPCR5). B) E54(vOX2-1). Polymorphisms were generated by using Geneious (<https://www.geneious.com>) and MEGA5 (<https://www.megasoftware.net>) Bayesian phylogenetic trees comparing the Myanmar *Proboscivirus* case DNA sequence results with matching data available for all 22 cases from India (IP#) and 2 cases from Sumatra (SP#) plus several representative cases from North America (NAP#) and Europe (EP#) available in GenBank. Both prototype EEHV2(NAP12, Kijana) and EEHV5A(EP24, Vijay) genomes shown in the top lines were used as references in panel B, and EEHV1B(NAP19) was used as the reference for panel A. Assigned subtypes are included with designated code numbers listed on the left-hand side for each genome. Colored short vertical lines indicate single-nucleotide differences from the consensus sequences of all the genomes shown in each panel. Gaps or missing sequence segments appear as blank spaces. EEHV, elephant endotheliotropic herpesvirus.

by our group. We also showed levels of divergence from prototype samples from North America (NAP#) or Europe (EP#) for each known subtype. We used other EEHV reference strains, such as EEHV1B, EEHV2, EEHV5, or EEHV6, as outgroups.

Results

We evaluated 3 cases of fatal hemorrhagic disease in captive-born *E. maximus* calves reared in camps in Myanmar. We provide gross morphology for 1 of the cases (Figure 1). The viral genomes in necropsy tissue DNA samples were designated MP01, MP02, and MP03 (for Myanmar *Proboscivirus* case numbers).

Overall results of gene subtyping PCR analyses (Table 1) and assigned GenBank accession numbers (Table 2) are provided.

At the time this study was conducted, we selected PCR primers provided to participants at a workshop in Myanmar after extensive previous analysis of 10 cases from Europe and 26 cases from North America as preferred standard sets that amplify 8 gene loci distributed across the 180–206-kb genomes of EEHV viruses. These gene loci included 4 well-conserved gene loci, U38(POL), U60(TERex3), U71/gM, and U77(HEL), which enabled identification of specific EEHV viruses present, as well as resolved chimeric EEHV1A subtype versus EEHV1B

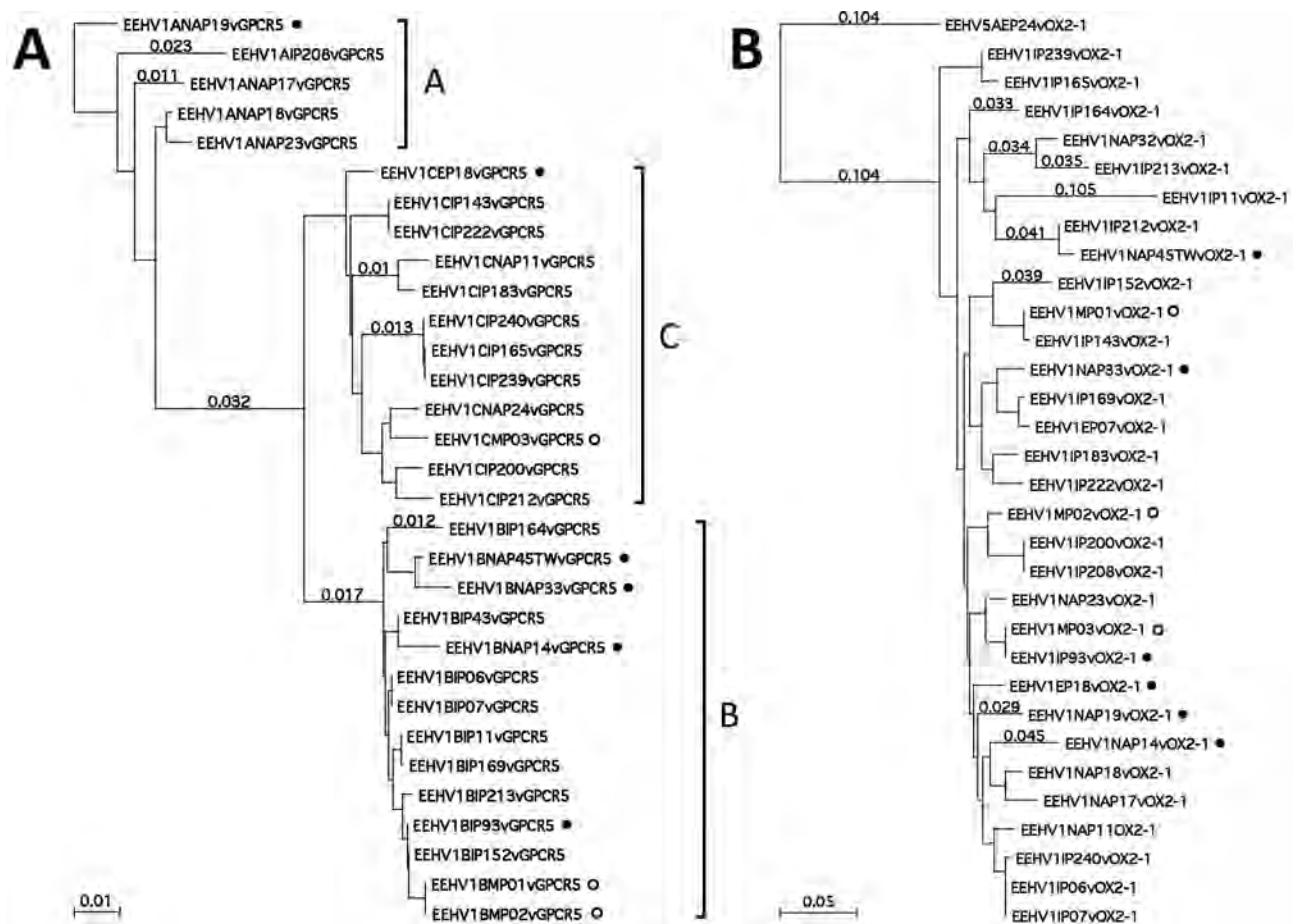


Figure 3. Protein level phylogenetic trees for Asian elephant calves that had endotheliotropic herpesvirus hemorrhagic disease in logging camp, Myanmar. Shown are comparison of examples of Asian EEHV1 at 2 representative hypervariable loci. A) EEHV1 E5(vGPCR5). B) E54(vOX2-1). Bayesian linear phylogenetic trees were generated from translated amino acid data in MEGA5.4.6 (<https://www.megasoftware.net>) by using similar aligned datasets as in Figure 2. Evolutionary history was inferred by using the maximum-likelihood method based on the Jones–Taylor–Thornton matrix-based model with the bootstrap consensus tree. Analysis in panel A involved 31 nt sequences with 303 aa positions in the final dataset with EEHV1B(NAP19) as outgroup. Analysis in panel B involved 32 nt sequences with 244 aa positions in the final dataset and with EEHV5A(EP24) as outgroup. Numbers along branches are bootstrap values. Some representative branch length values are provided. The 3 major subtype clusters are indicated as A, B, or C for E5(vGPCR5) along the right side of panel A, but no similar dramatic subtype clustering was discernable for E54(vOX2-1). All 6 examples in which the genomes have classic EEHV1B type core chimeric domain (CDI, CDII, and CDIII) features elsewhere are indicated by solid circles. Positions of the 3 cases from Myanmar are indicated by open circles. Scale bars indicate amino acid substitutions per site. EEHV, elephant endotheliotropic herpesvirus.

Table 3. Nucleotide differences between EEHV strains from Asian elephant calves in Myanmar and EEHV1A and EEHV1B prototypes at the E5(vGPCR5) locus*

vGPCR5	MP01	MP02	MP03	NAP23	EP18
MP01	–	0	6.9	7.6	5.6
MP02	0	–	6.9	7.6	5.6
MP03	61	61	–	9.3	2.5
NAP23	67	66	83	–	7.9
EP18	50	50	22	71	–

*Values to the right and above the dashes are percentages, and values to the left and below the dashes are number of nucleotides. EEHV, elephant endotheliotropic herpesvirus; EP18, prototype chimeric EEHV1B; NAP23, prototype EEHV1A.

subtype strains (6). In addition, we used 4 other PCR loci that encompass parts of well-characterized hyper-variable genes. These loci were E5(vGPCR5), U48(gH-TK), U51(vGPCR1), and E54(vOX2-1); we used them to address levels and patterns of individual variability for comparison with known, highly divergent, worldwide populations of EEHV1 strains.

We found that all 3 strains from Myanmar were EEHV1A variants that amplified strongly after the first-round conventional PCR. We identified nucleotide-level differences for 2 selected representative hypervariable PCR loci, namely E5(vGPCR5) and E54(vOX2-1) (Figure 2). Equivalent data for the U48(gH-TK) locus have already been reported for cases from India by Zachariah et al. (27). We provide 2 examples of the protein-level phylogenetic trees, again those for the E5(vGPCR5) and E54(vOX2-1) loci (Figure 3). The equivalent protein-level phylogenetic trees for U48(gH-TK) have been included in the report by Zachariah et al. (27). In addition, matching comparative DNA-level phylogenetic trees for all these samples at the 7 PCR loci other than U48(gH-TK) have been reported by Zachariah et al. (27). Finally, we report simple numerical difference comparisons (number and percentage) for the 3 strains from Myanmar with the prototype EEHV1A(NAP23, Kimba) and EEHV1B(EP18, Emelia) strains at the 3 most hypervariable loci, namely E5(vGPCR5), U48(gH-TK), and E54(vOX2-1) (Tables 3–5).

Despite the consistent nucleotide divergence subtyping patterns, the 4 conserved loci evaluated rarely showed large numbers of amino acid polymorphisms. Even within the other 4 hypervariable loci, most nucleotide changes were also synonymous. In addition, unlike conserved loci, there are also numerous nonsynonymous amino acid changes, which provide additional robust supportive evidence for the same

subtyping patterns being recognized in the trees at the DNA and protein levels. This finding is especially valid for the U48(gH) protein, which is the only PCR segment evaluated here that is entirely encompassed within 1 of the EEHV1B chimeric domains (CD-II). The fact that the MP01 and MP02 versions of E5(vGPCR5) protein sequences are identical is a relatively rare exception, but otherwise the divergence of most EEHV1 strains from each other is evident in E5(vGPCR5) and E54(vOX2-1) protein phylogenetic trees (Figure 2), and similar but different gene-specific patterns were also found in U48(gH-TK) and U51(vGPCR1) protein trees.

The 3 cases from Myanmar were caused by distinct strains that showed major differences from each other, as well as from other cases in Asia, North America, and Europe that we evaluated. However, there were unusual similarities among these 3 cases. For example, MP01 and MP02 were in the same subtype groupings at 4 loci, including 2 conserved loci, namely U71-gM and U38(POL), as well as 2 hypervariable loci, namely E5(vGPCR5) and U48(gH)-TK, but showed different subtypes for the other 3 loci, namely U60(TERex3), U77(HEL), and E54(vOX2-1), whereas MP03 was distinguishable from MP01 and MP02 at all loci except U38(POL). Similar to many of the cases from India, MP03 was a subtype E in U51(vGPCR1) and had 28 differences (4.1%) from the subtype A prototype EEHV1A(Kimba). MP01 and MP02 also had different subtypes (B1 for MP01 and C1 for MP02) from MP03 for E5(vGPCR5). Although all 3 cases from Myanmar had the same D subtype group within the U48(gH-TK) locus, MP03 still differed at this locus from the other 2 cases by 8 bp.

Although the captured E54(vOX2-1) gene is unusually well conserved when compared with the original host (African bush elephant [*Loxodonta africana*])

Table 4. Nucleotide differences between EEHV strains from Asian elephant calves in Myanmar and EEHV1A and EEHV1B prototypes at the U48(gH-TK) locus*

gH-TK	MP01	MP02	MP03	NAP23	EP18
MP01	–	0	1.0	8.7	28
MP02	0	–	1.0	8.7	28
MP03	8	8	–	8.5	28
NAP23	73	73	71	–	28
EP18†	227	227	235	233	–

*Values to the right and above the dashes are percentages, and values to the left and below the dashes are number of nucleotides. EEHV, elephant endotheliotropic herpesvirus; EP18, prototype chimeric EEHV1B; NAP23, prototype EEHV1A.

Table 5. Nucleotide differences between EEHV strains from Asian elephant calves in Myanmar and EEHV1A and EEHV1B prototypes at the E54(vOX2-1) locus*

vOX-1	MP01	MP02	MP03	NAP23	EP18
MP01	–	9.7	8.7	10.1	10.2
MP02	82	–	2.8	6.1	5.7
MP03	74	28	–	3.7	5.0
NAP23	86	51	31	–	2.4
EP18	87	48	42	21	–

*Values to the right and above the dashes are percentages, and values to the left and below the dashes are number of nucleotides. EEHV, elephant endotheliotropic herpesvirus; EP18, prototype chimeric EEHV1B; NAP23, prototype EEHV1A.

version at the protein level, it shows the greatest DNA-level divergence between all 3 strains from Myanmar and most other strains, in which MP01 differed from MP02 by 82 bp (9.7%), MP02 from MP03 by 28 bp (3.3%), and MP01 from MP03 by 74 bp (8.7%). Nevertheless, MP01 and MP03 were identical at nt positions 280–480, and MP02 and MP03 differed by only 1 bp over >50% of the locus between nt positions 364–851.

We found that MP01 and MP03 were identical to another case from India (MP01 with IP143 and MP03 with IP93) at the otherwise hypervariable E54(vOX2-1) locus, whereas MP02 had unique features different from all other cases that we have evaluated worldwide. Overall, across all 4 core conserved loci, U38(POL), U71-gM, U60(TERex3), and U77(HEL), the differences from the prototype EEHV1A(Kimba) strain were no more than 15 nt for MP01, 24 nt for MP02, and 22 nt for MP03. In contrast, the 3 most hypervariable loci showed a dramatically different pattern. In E5(vGPCR5), MP01 differed from Kimba by 67 (7.6%), MP02 by 66 (7.7%), and MP03 by 83 (9.3%) nt. In U48(gH-TK), MP01 differed from Kimba by 73 (8.7%), MP02 by 73 (8.7%), and MP03 by 71 (8.5%) nt. Finally, in E54(vOX2-1), MP01 differed from Kimba by 86 (10.1%), MP02 by 66 (6.1%), and MP03 by 31 (3.7%) nt.

Discussion

The DNA sequence and subtyping results for the hemorrhagic disease cases in Myanmar showed that they involved different independent EEHV1A strains that were distinct from all other strains examined in countries in Asia or in zoos in Europe and North America. A similar pattern of variability was also obtained for 21 of the 22 cases in India, but this pattern was not always found. In contrast, in every situation observed in which 2 cases of hemorrhagic disease occurred at the same facility at nearly the same time (i.e., within days or weeks of each other), genomes were always identical at all loci tested. These results include paired cases at 2 facilities in Asia (India and Sumatra), 2 cases in Europe (United Kingdom and Germany), and ≥ 3 cases in the United States (Texas, Florida, and Missouri). However, identical strains have not been reported for cases at different facilities (and different times), even in the same

country. The fact that the 3 cases in Myanmar, although they showed some clear similarities across parts of the genomes and originated from within nearby geographic areas over a relatively short time frame, represent 3 distinct EEHV1A strains is not unexpected. However, more data from additional cases will be required to address whether overall populations of this virus in Myanmar have any common evolutionary features that differentiate them from the numerous and rather broadly diverged examples examined from India or Thailand.

Myanmar is now the sixth country in Asia (after Cambodia, Thailand, India, Laos, and Indonesia) in which apparent EEHV-associated hemorrhagic disease based on gross clinical or pathologic signs involving tissue hemorrhaging has been confirmed by PCR DNA subtype sequencing analysis. A brief preliminary speculation about these and multiple additional potential cases of similar hemorrhagic disease cases in Myanmar logging camps has been reported (28). Our findings will help with programs designed to address the increasing number of cases of lethal acute hemorrhagic disease in Asian elephants and possible long-term effects on breeding and survival of this critically endangered species.

Acknowledgment

We thank Sarah Y. Heaggans for providing valuable contributions for DNA sequence editing and generating phylogenetic trees.

This study was supported by a research grant to G.S.H. from the International Elephant Foundation.

About the Author

Dr. Oo is a veterinarian at the University of Veterinary Sciences, Yezin, Myanmar. His primary research interest is veterinary diseases, specifically diseases in elephants.

References

- Ossent P, Guscetti F, Metzler AE, Lang EM, Rübél A, Hauser B. Acute and fatal herpesvirus infection in a young Asian elephant (*Elephas maximus*). *Vet Pathol.* 1990;27:131–3. <https://doi.org/10.1177/030098589002700212>
- Richman LK, Montali RJ, Garber RL, Kennedy MA, Lehnhardt J, Hildebrandt T, et al. Novel endotheliotropic herpesviruses fatal for Asian and African elephants. *Science.* 1999;283:1171–6. <https://doi.org/10.1126/science.283.5405.1171>

3. Richman LK, Montali RJ, Cambre RC, Schmitt D, Hardy D, Hildbrandt T, et al. Clinical and pathological findings of a newly recognized disease of elephants caused by endotheliotropic herpesviruses. *J Wildl Dis.* 2000;36:1–12. <https://doi.org/10.7589/0090-3558-36.1.1>
4. Hayward GS. Conservation: clarifying the risk from herpesvirus to captive Asian elephants. *Vet Rec.* 2012;170:202–3. <https://doi.org/10.1136/vr.e1212>
5. Long SY, Latimer EM, Hayward GS. Review of elephant endotheliotropic herpesviruses and acute hemorrhagic disease. *ILAR J.* 2016;56:283–96. <https://doi.org/10.1093/ilar/ilv041>
6. Richman LK, Zong J-C, Latimer EM, Lock J, Fleischer RC, Heaggans SY, et al. Elephant endotheliotropic herpesviruses EEHV1A, EEHV1B, and EEHV2 from cases of hemorrhagic disease are highly diverged from other mammalian herpesviruses and may form a new subfamily. *J Virol.* 2014;88:13523–46. <https://doi.org/10.1128/JVI.01673-14>
7. Wilkie GS, Davison AJ, Watson M, Kerr K, Sanderson S, Bouts T, et al. Complete genome sequences of elephant endotheliotropic herpesviruses 1A and 1B determined directly from fatal cases. *J Virol.* 2013;87:6700–12. <https://doi.org/10.1128/JVI.00655-13>
8. Ling PD, Reid JG, Qin X, Muzny DM, Gibbs R, Petrosino J, et al. Complete genome sequence of elephant endotheliotropic herpesvirus 1A. *Genome Announc.* 2013;1:e0010613. <https://doi.org/10.1128/genomeA.00106-13>
9. Garner MM, Helmick K, Ochsenreiter J, Richman LK, Latimer E, Wise AG, et al. Clinico-pathologic features of fatal disease attributed to new variants of endotheliotropic herpesviruses in two Asian elephants (*Elephas maximus*). *Vet Pathol.* 2009;46:97–104. <https://doi.org/10.1354/vp.46-1-97>
10. Latimer E, Zong J-C, Heaggans SY, Richman LK, Hayward GS. Detection and evaluation of novel herpesviruses in routine and pathological samples from Asian and African elephants: identification of two new probosciviruses (EEHV5 and EEHV6) and two new gammaherpesviruses (EGHV3B and EGHV5). *Vet Microbiol.* 2011;147:28–41. <https://doi.org/10.1016/j.vetmic.2010.05.042>
11. Denk D, Stidworthy MF, Redrobe S, Latimer E, Hayward GS, Cracknell J, et al. Fatal elephant endotheliotropic herpesvirus type 5 infection in a captive Asian elephant. *Vet Rec.* 2012;171:380–1. <https://doi.org/10.1136/vr.e6833>
12. Zong J-C, Latimer EM, Long SY, Richman LK, Heaggans SY, Hayward GS. Comparative genome analysis of four elephant endotheliotropic herpesviruses, EEHV3, EEHV4, EEHV5, and EEHV6, from cases of hemorrhagic disease or viremia. *J Virol.* 2014;88:13547–69. <https://doi.org/10.1128/JVI.01675-14>
13. Zong J-C, Heaggans SY, Long SY, Latimer EM, Nofs SA, Bronson E, et al. Detection of quiescent infections with multiple elephant endotheliotropic herpesviruses (EEHVs), including EEHV2, EEHV3, EEHV6 and EEHV7, within lymphoid lung nodules or lung and spleen tissue samples from five asymptomatic adult african elephants. *J Virol.* 2015;90:3028–43. <https://doi.org/10.1128/JVI.02936-15>
14. Wilkie GS, Davison AJ, Kerr K, Stidworthy MF, Redrobe S, Steinbach F, et al. First fatality associated with elephant endotheliotropic herpesvirus 5 in an Asian elephant: pathological findings and complete viral genome sequence. *Sci Rep.* 2014;4:6299. <https://doi.org/10.1038/srep06299>
15. Ling PD, Long SY, Fuery A, Peng RS, Heaggans SY, Qin X, et al. A complete genome sequence of elephant endotheliotropic herpesvirus 4 (EEHV4): the first example of a GC-rich branch proboscivirus. *MSphere.* 2016;1:e00081–15. <https://doi.org/10.1128/mSphere.00081-15>
16. Ling PD, Long SY, Zong JC, Heaggans SY, Qin X, Hayward GS. Comparison of the gene coding contents and other unusual features of the GC-rich and AT-rich branch probosciviruses. *MSphere.* 2016;1:e00091–16. <https://doi.org/10.1128/mSphere.00091-16>
17. Stanton JJ, Zong J-C, Latimer E, Tan J, Herron A, Hayward GS, et al. Detection of pathogenic elephant endotheliotropic herpesvirus in routine trunk washes from healthy adult Asian elephants (*Elephas maximus*) by use of a real-time quantitative polymerase chain reaction assay. *Am J Vet Res.* 2010;71:925–33. <https://doi.org/10.2460/ajvr.71.8.925>
18. Stanton JJ, Zong J-C, Eng C, Howard L, Flanagan J, Stevens M, et al. Kinetics of viral loads and genotypic analysis of elephant endotheliotropic herpesvirus-1 infection in captive Asian elephants (*Elephas maximus*). *J Zoo Wildl Med.* 2013;44:42–54. <https://doi.org/10.1638/1042-7260-44.1.42>
19. Atkins L, Zong J-C, Tan J, Mejia A, Heaggans SY, Nofs SA, et al. EEHV-5, a newly recognized elephant herpesvirus associated with clinical and subclinical infections in captive asian elephants (*Elephas maximus*). *J Zoo Wildl Med.* 2013;44:136–43. <https://doi.org/10.1638/1042-7260-44.1.136>
20. Fuery A, Browning GR, Tan J, Long S, Hayward GS, Cox SK, et al. Clinical infection of captive Asian elephants (*Elephas maximus*) with elephant endotheliotropic herpesvirus 4. *J Zoo Wildl Med.* 2016;47:311–8. <https://doi.org/10.1638/2015-0072.1>
21. Reid CE, Hildebrandt TB, Marx N, Hunt M, Thy N, Reynes JM, et al. Endotheliotropic elephant herpes virus (EEHV) infection. The first PCR-confirmed fatal case in Asia. *Vet Q.* 2006;28:61–4. <https://doi.org/10.1080/01652176.2006.9695209>
22. Zachariah A, Zong J-C, Long SY, Latimer EM, Heaggans SY, Richman LK, et al. Fatal herpesvirus hemorrhagic disease in wild and orphan Asian elephants in southern India. *J Wildl Dis.* 2013;49:381–93. <https://doi.org/10.7589/2012-07-193>
23. Sripiboon S, Tankaew P, Lungka G, Thitaram C. The occurrence of elephant endotheliotropic herpesvirus in captive Asian elephants (*Elephas maximus*): first case of EEHV4 in Asia. *J Zoo Wildl Med.* 2013;44:100–4. <https://doi.org/10.1638/1042-7260-44.1.100>
24. Bouchard B, Xaymountry B, Thongtip N, Lertwatcharasarakul P, Wajjwalku W. First reported case of elephant endotheliotropic herpes virus infection in Laos. *J Zoo Wildl Med.* 2014;45:704–7. <https://doi.org/10.1638/2013-0264R1.1>
25. Lertwatcharasarakul P, Sanyathitserree P, Thongtip N, Charoenphan P, Boonyasart B, Maneewan N, et al. Genetic variant of elephant endotheliotropic herpesvirus detected from captive Asian elephants (*Elephas maximus*) in Thailand from 2007 to 2013. *Wetchnan Sattawaphaet.* 2015;45:73.
26. Sripiboon S, Jackson B, Ditcham W, Holyoake C, Robertson I, Thitaram C, et al. Molecular characterisation and genetic variation of elephant endotheliotropic herpesvirus infection in captive young Asian elephants in Thailand. *Infect Genet Evol.* 2016;44:487–94. <https://doi.org/10.1016/j.meegid.2016.08.004>
27. Zachariah A, Sajesh PK, Santhosh S, Bathrachalam C, Megha M, Pandiyan J, et al. Extended genotypic evaluation and comparison of twenty-two cases of lethal EEHV1 hemorrhagic disease in wild and captive Asian elephants in India. *PLoS One.* 2018;13:e0202438. <https://doi.org/10.1371/journal.pone.0202438>
28. Oo ZM. Health issues of captive Asian elephants in Myanmar. *Gajah.* 2012;36:21–2.

Address for correspondence: Gary S. Hayward, Viral Oncology Program, Department of Oncology, Johns Hopkins School of Medicine, Baltimore, MD 21209, USA; email: ghayward@jhmi.edu

Risk Factors for and Seroprevalence of Tickborne Zoonotic Diseases among Livestock Owners, Kazakhstan

Jennifer R. Head, Yekaterina Bumburidi, Gulfaira Mirzabekova, Kumysbek Rakhimov, Marat Dzhumankulov, Stephanie J. Salyer, Barbara Knust, Dmitriy Berezovskiy, Mariyakul Kulatayeva, Serik Zhetibaev, Trevor Shoemaker, William L. Nicholson, Daphne Moffett

Crimean-Congo hemorrhagic fever (CCHF), Q fever, and Lyme disease are endemic to southern Kazakhstan, but population-based serosurveys are lacking. We assessed risk factors and seroprevalence of these zoonoses and conducted surveys for CCHF-related knowledge, attitudes, and practices in the Zhambyl region of Kazakhstan. Weighted seroprevalence for CCHF among all participants was 1.2%, increasing to 3.4% in villages with a known history of CCHF circulation. Weighted seroprevalence was 2.4% for Lyme disease and 1.3% for Q fever. We found evidence of CCHF virus circulation in areas not known to harbor the virus. We noted that activities that put persons at high risk for zoonotic or tickborne disease also were risk factors for seropositivity. However, recognition of the role of livestock in disease transmission and use of personal protective equipment when performing high-risk activities were low among participants.

Zoonoses account for 61% of human infectious diseases and 75% of emerging pathogens (1). Zoonotic diseases pass from animals to humans through direct contact with animals, inhalation of

infectious aerosols, consumption of contaminated animal products, or a bite from a vector, such as a tick (2). Global incidence of tickborne diseases is increasing and expected to continue rising (3). Given changes in ecologic factors, such as climate and land use, tickborne diseases have emerged in new areas during the past 3 decades, and the incidence of endemic tickborne pathogens has increased (4). Vectorborne infections were responsible for $\approx 28.8\%$ of emerging infectious diseases during 1990–2000 (5).

Crimean-Congo hemorrhagic fever (CCHF), Q fever, and Lyme disease are widespread zoonotic diseases that cause a range of illness and death in humans. CCHF, caused by Crimean-Congo hemorrhagic fever virus (CCHFV), an RNA virus of the family *Nairoviridae*, is highly fatal (6). The virus is maintained through an enzootic cycle involving mammals, ticks, and humans, and is transmitted to humans through contact with viremic livestock or infected ticks. CCHF is endemic to Africa, Asia, eastern and southern Europe, and central Asia (7). Q fever is caused by the bacterium *Coxiella burnetii*, which infects many vertebrates, but ruminant livestock are thought to be its primary reservoir. Transmission to humans most commonly occurs from inhalation of dust contaminated with urine, feces, milk, or birth products from infected animals (8). Q fever has been identified in most countries (8). Severe cases can result in pneumonia or hepatitis in humans, and $\approx 5\%$ of infections become chronic (9). Lyme disease is caused by *Borrelia burgdorferi*, a bacterium transmitted to humans through the bite of *Ixodes* ticks. Untreated Lyme infection can disseminate and spread to the joints, heart, and nervous system. Lyme disease is the most commonly reported arthropodborne disease in North America and is prevalent throughout central Europe,

Author affiliations: Association of Schools and Programs for Public Health, Washington, DC, USA (J.R. Head); Public Health Institute, San Francisco, California, USA (J.R. Head); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (J.R. Head, S.J. Salyer, B. Knust, T. Shoemaker, W.L. Nicholson); Centers for Disease Control and Prevention, Almaty, Kazakhstan (Y. Bumburidi, D. Berezovskiy, D. Moffett); Zhambyl Oblast Public Health Protection Department, Taraz, Kazakhstan (G. Mirzabekova, K. Rakhimov); Zhambyl Oblast Health Department, Taraz (M. Dzhumankulov); Zhambyl Oblast Sanitary Epidemiology Expertise Center, Taraz (M. Kulatayeva, S. Zhetibaev)

DOI: <https://doi.org/10.3201/eid2601.190220>

particularly Germany, Austria, and Slovenia (10,11). Lyme disease is the sixth most commonly reported notifiable infectious disease in the United States (<https://www.cdc.gov/lyme>). In addition, incidence of Lyme disease and the range of tick vectors have been increasing in Europe and Asia (10,11), where Lyme disease is found in western Russia, Mongolia, northeast China, and Japan.

CCHF, Q fever, and Lyme disease are endemic to the southern Kazakhstan region of Zhambyl, but their true burden is largely unknown because few serologic surveys have been conducted in Kazakhstan and central Asia. The Zhambyl region covers >55,000 km² and has a population of ≈1.2 million. The region is characterized by diverse ecology, containing both desert steppes and mountainous pastures, and elevations of 213–4,115 m. The region has 363 villages and 4 cities. Livelihoods are largely pastoral or agricultural, and common occupations involve a high degree of animal contact, placing humans at increased risk for zoonotic infections.

Among the 3 diseases, only CCHF is a reportable disease in Kazakhstan. During 2000–2013, the Zhambyl region had 73 reported human CCHF cases, the second highest case-count among regions in Kazakhstan (12). However, data on human prevalence of CCHF in Kazakhstan are limited to reported clinical cases, even though studies show ≥80% of infections are subclinical (13). Although Q fever was detected in Kazakhstan in the 1950s, the lack of surveillance or serologic studies obscure our understanding of Q fever or Lyme disease incidence in the population (14). Quantifying seroprevalence of these diseases in humans can help identify areas of pathogen circulation and areas where humans could be infected.

For this study, we aimed to determine the seroprevalence of antibodies against CCHFV, *C. burnetii*, and *B. burgdorferi* in humans who interact with livestock in the Zhambyl region. In addition, we sought to assess the population's knowledge of risk factors for disease transmission and how frequently they engage in activities that increase or reduce risk for infection.

Methods

In June 2017, we conducted a knowledge, attitudes, and practices and risk factor survey (KAP/risk factor survey), along with serosurveys for CCHF, Q fever, and Lyme disease, in 30 rural villages in the Zhambyl region. Participants could enroll in the KAP/risk factor survey, the serosurvey, or both. Eligible participants were ≥18 years of age, residents of the village for ≥2 months, and residents of a household containing a sheep or cow of ≥1 year of age.

Sample Size

Sample selection was based on concern about CCHF as a nationally reportable disease. We conducted surveys in households in which sheep and cattle serosurveys simultaneously were conducted; sample size calculations were based on expected seroprevalence of sheep and cattle. We calculated a target sample size of 561 households with sheep and 473 households with cattle. We based the sample size on an α of 0.05, power of 80%, a design effect of 2, and an expected response rate of 90%. We assumed CCHF seroprevalence of 24% in sheep and 19% in cattle, on the basis of a meta-analysis of previous serosurveys (15).

Participant Selection

We stratified the 363 villages in the region by known (CCHF-endemic) or unknown (non-CCHF-endemic) recent circulation of CCHF. We defined recent circulation as 1 confirmed human case reported in hospital-based surveillance from Zhambyl Oblast Health Department (Taraz, Kazakhstan) or 1 CCHF-positive tick confirmed in the previous 5 years and reported in annual tick surveillance data from the Ministry of Agriculture of Kazakhstan. We identified 66 (18.2%) villages that met the definition for having known, recent CCHF circulation.

We selected 15 villages from each stratum; probability of selection was proportional to the number of sheep and cattle in the village. We obtained livestock counts from reports by village veterinarians to the Ministry of Agriculture. Elevation of the 30 villages was 220–2,590 m (mean 781 m, median 488 m). Mean elevation was 513 m for villages with known CCHF circulation and 1,049 m for villages without known circulation.

Local veterinarians provided information on livestock-owning households in each village. To verify, data collectors conducted a census of 5 villages and mapped households containing sheep or cattle. The veterinarian registry was accurate except for 2 instances in which the household recently had sold animals. Survey teams randomly selected 35 households from these registries and 1 adult per household for study participation.

KAP/Risk Factor Survey

We adapted our KAP/risk factor survey from one conducted in Georgia during a 2014 CCHF outbreak (16). We translated the survey into Russian and Kazakh, the 2 most common languages in the region. Survey teams pilot tested the questionnaire in an eligible village not selected for the study.

The survey team administered the questionnaire verbally at each respondent's residence. Survey

questions covered demographics; occupations; history of animal and tick interactions; illness in the previous 4 months or fever and hemorrhaging; and knowledge of CCHF transmission routes, symptoms, and risk factors. The survey did not contain questions specific for Lyme disease or Q fever.

Serosurvey

After answering the questionnaire, respondents were asked to go to their local health clinic to provide a blood sample on the same day. Each village had a health clinic within walking distance of participants. Nurses drew 5 mL of blood from each participant and stored it in a serum separator tube. Blood samples were kept on ice, centrifuged within 6 h, and transported within 24 h to the Zhambyl Regional Laboratory for Especially Dangerous Pathogens in Taraz, where laboratorians aliquoted serum into 4 samples/participant and stored serum at -20°C until analysis.

Laboratorians analyzed samples for evidence of recent CCHF exposure, indicated by presence of IgM, by using VectoCrimean-CCHF-IgM Kits (Vector-Best, <https://vector-best.ru>) and for evidence of past CCHF exposure, indicated by IgG, by using VectoCrimean-CCHF-IgG Kits (Vector-Best). Laboratorians assessed past exposure to *C. burnetii*, indicated by presence of IgG, using ELISA-Anti-Q Kit No. 1 (Pasteur Institute of Epidemiology and Microbiology, <http://www.pasteur-nii.spb.ru>), and exposure to *Borellia* spp., indicated by presence of IgG against *B. afzelii*, *B. garinii*, or *B. burgdorferi*, by using LymeBest-IgG Test Kits (Vector-Best). All testing was performed with commercially available ELISA kits, according to manufacturer instructions (17,18).

Data Analysis

We analyzed data by using R version 3.4.3 (19). We weighted results for each participant by calculating the inverse probability of selection and applying a poststratification adjustment to each stratum to account for nonresponses. We stratified KAP/risk factor answers specific to CCHF according to whether the health department recognized the village as having known, recent history of CCHF. We used χ^2 test in bivariate analysis to compare frequencies between these 2 strata. We used logistic regression models to test associations between risk factors and seropositivity. We defined risk for zoonotic or tickborne disease as participation in ≥ 1 of the following activities: handling ticks with bare hands; working with livestock; working in a healthcare setting; being a veterinarian; or herding, birthing, shearing, slaughtering, or milking animals.

Ethics Review

Each participant provided written, informed consent. No personal identifying information was collected. The Institutional Review Board in Almaty, Kazakhstan, through the Committee for Public Health Protection, approved the study. The protocol was reviewed according to the US Centers for Disease Control and Prevention human subjects review procedures, which determined the agency was not engaged in the study because the Zhambyl Departments of Health and Agriculture owned and collected the data.

Results

KAP/Risk Factor Survey

We selected 969 households; 948 persons completed surveys, a 98% response rate. Reasons for nonresponse included 4 households that were not visited, 2 that were abandoned, and 1 that was not found. In addition, 12 persons did not consent: 4 did not want to participate in the serosurvey, 1 did not have time, 1 distrusted the data team, and 6 gave no reason. Further, 2 persons were excluded from analysis because information on their village of residence was missing and they could not be analyzed according to survey design.

Respondents' median age was 46 (range 19–90) years; 54% were male (Table 1). Most (66.7%) were native to Kazakhstan. The most frequently reported occupations were taking care of the home (23.0%) and farming or herding (20.8%).

Of respondents, 64.4% (95% CI 50.9%–75.8%) reported participating in ≥ 1 activity putting them at elevated risk for zoonotic or tickborne disease during their lives; 55.4% (95% CI 42.8%–67.3%) reported doing so in the previous 4 months (Table 2). Of high-risk activities, butchering or handling raw meat (36.4%) and shearing (26.0%) or herding (25.8%) animals were most common. Of respondents, 139 (22%) who birthed animals in the previous 4 months and 222 (47.4%) who slaughtered an animal in the previous 4 months wore no personal protective equipment (PPE). Few respondents reported tick bites (Table 3), but $>85\%$ said ticks were a major problem (Table 4). Most respondents (93.6%) reported killing ticks with an object; only 0.5% reported killing ticks with bare hands. Most (94.0%) used pesticide to prevent ticks on animals.

Participants from CCHF-endemic villages had a higher knowledge of CCHF, likely because the health department provided education in these villages (Table 5, <https://wwwnc.cdc.gov/EID/article/26/1/19-0220-T5.htm>). Most respondents (95.6%, 95% CI 93.8%–

Table 1. Demographic characteristics of study population in survey of Crimean-Congo hemorrhagic fever, Kazakhstan

Patient characteristics	Median (IQR)	Range
Age, y	46 (36–56)	19–90
Household size	6.1 (4.6–8.4)	2.7–21.6
Land owned, ha	0.18 (0.12–0.25)	0.004–776
Land rented, ha	0.20 (0.14–0.90)	0.024–776
No. animals owned		
Ovine	15.0 (3.0–35.0)	0–1,320
Bovine	2.0 (1.0–5.0)	0–141
Poultry	0 (0–8.0)	0–80
Equine	0 (0–1.0)	0–100
	No. participants	% Participants (95% CI)
Sex		
M	509	56.1 (50.5–61.5)
F	437	43.9 (38.5–49.5)
Country of origin		
Kazakhstan	733	66.7 (44.0–85.9)
Russia	73	10.5 (4.4–23.0)
Turkey	45	4.4 (1.9–9.2)
Kyrgyzstan	3	0.6 (0.2–1.9)
Uzbekistan	3	0.4 (0.1–2.1)
Other	89	15.7 (4.0–45.7)
Occupation		
Farmer, herder, animal tender	163	20.8 (10.1–38.1)
Gardener, fieldworker	50	3.1 (1.3–7.4)
Butcher	1	0.001 (0–0.01)
Healthcare worker	21	2.5 (1.5–4.1)
Veterinarian	15	1.5 (0.6–4.1)
Office, indoor worker	153	14.0 (9.0–21.1)
Family or home caretaker	179	23.0 (18.4–28.5)
Student	10	1.1 (0.4–3.1)
Retired	147	9.8 (6.3–14.9)
Unemployed	105	14.3 (5.8–31.0)
Other	101	9.9 (3.4–25.6)
Education level		
None	12	0.1 (0.03–0.5)
Elementary school	9	0.8 (0.3–2.0)
Middle school	437	44.1 (33.9–54.9)
High school	159	11.5 (7.5–17.2)
Vocational school	71	4.1 (1.7–9.5)
College	251	38.9 (28.3–50.8)
Monthly income, US \$		
≤60	43	4.0 (1.3–11.4)
61–150	373	39.1 (24.6–55.8)
151–300	257	26.8 (20.1–34.6)
301–450	34	0.8 (0.2–3.0)
451–600	9	0.5 (0.1–2.1)
>600	7	0.2 (0.04–1.1)
Unknown, refused to answer	222	28.6 (12.6–52.6)

99.9%) in CCHF-endemic villages had heard of CCHF, compared with only 71.3% (95% CI 61.7%–79.3%) in non-CCHF-endemic villages (Table 5). Information from healthcare workers, pamphlets, and village meetings were common ways participants learned about CCHF. In addition, 95.8% (95% CI 89.8%–98.3%) of respondents in CCHF-endemic villages who knew about CCHF could recognize ≥1 high-risk activity, compared with 75.9% (95% CI 49.1%–91.1%) in non-CCHF-endemic villages. Most recognized tick bites as

Table 2. Participation in activities putting them at high risk for tickborne zoonotic diseases among respondents in survey of Crimean-Congo hemorrhagic fever, Kazakhstan*

Activities	No. respondents	% Respondents (95% CI)
Herding animals		
Ever	297	17.4 (8.4–32.5)
Within the previous 4 mo	190	25.8 (14.3–42.2)
Assisting with animal births		
Ever	226	11.3 (6.8–18.3)
Within the previous 4 mo	140	5.9 (3.5–9.9)
Shearing animals		
Ever	331	26.0 (19.7–33.4)
Within the previous 4 mo	223	17.0 (12.9–22.1)
Milking animals		
Ever	316	23.2 (16.3–31.9)
Within the previous 4 mo	251	18.9 (12.8–27.0)
Slaughtering animals		
Ever	292	25.4 (15.8–38.1)
Within the previous 4 mo	229	20.4 (12.0–32.4)
Butchering or handling raw meat		
Ever	351	36.4 (28.4–45.2)
Within the previous 4 mo	296	30.7 (22.7–40.0)
Eating raw meat		
Ever	8	0.5 (0.1–1.9)
Within the previous 4 mo	0	–
Handling ticks with bare hands		
Ever	61	3.5 (1.1–10.3)
Within the previous 4 mo	27	2.0 (0.4–8.4)
Working in a healthcare setting		
Ever	5	0.3 (0.1–0.9)
Within the previous 4 mo	3	0.2 (0–0.8)
Working in a garden†		
Ever	175	14.5 (7.6–27.4)
Within the previous 4 mo	150	12.4 (6.5–22.6)
Consuming unpasteurized milk or dairy products‡		
Ever	8	1.0 (0.4–2.2)
Within the previous 4 mo	0	–
Participated in ≥1 high-risk activity		
Ever	683	64.4 (50.9–75.8)
Within the previous 4 mo	580	55.4 (42.8–67.3)
Use of personal protective equipment		
Assisting with animal births, n = 139†		
Gloves	73	55.2 (35.8–73.1)
Gown	43	55.1 (30.3–77.6)
Boots	21	30.0 (11.8–58.0)
Glasses	3	12.7 (2.0–51.5)
None	46	20.4 (11.0–34.7)
Shearing animals, n = 222†		
Gloves	172	83.6 (71.7–91.2)
Gown	119	73.9 (55.9–86.4)
Boots	59	20.6 (12.2–32.6)
Glasses	4	2.9 (0.7–11.4)
None	21	5.2 (1.7–15.2)
Milking animals, n = 250†		
Gloves	26	15.5 (5.5–36.8)
Gown	178	81.6 (60.6–92.7)
Boots	12	5.7 (2.2–14.1)
None	71	17.5 (6.4–39.5)
Slaughtering animals, n = 229†		
Gloves	36	23.3 (10.5–44.1)
Gown	91	49.1 (33.6–64.8)
Boots	16	5.2 (2.0–12.9)
Glasses	1	1.6 (0.2–9.4)
None	129	47.4 (29.8–65.7)

*Percentages weighted by calculating the inverse probability of selection and applying a post-stratification adjustment to each stratum to account for nonresponses.

†≥1 response possible.

‡Not considered a high-risk activity.

a mode of transmission, and $\geq 10\%$ in CCHF-endemic villages recognized animal blood as a potential mode of transmission. Despite a lower disease knowledge in non-CCHF-endemic villages, respondents thought CCHF was a major problem (Table 4), but only 52.5% felt prepared to protect themselves from CCHF, compared with 90.5% from CCHF-endemic villages.

Serosurveys

Of 948 persons completing the KAP/risk factor survey, 914 (96.4%) submitted blood samples. Of 34 persons who did not participate in the serosurvey, 10 did not show up for a blood draw, 4 did not have time, 2 feared needles, 1 feared consequences of detection, 1 had recent surgery, and 16 reported no reasons. Serum from 914 samples was tested for evidence of CCHF. In addition, 911 samples were tested for Lyme disease, 910 were tested for Q fever, and 4 did not have adequate sample volume for Lyme disease and Q fever testing.

Of 914 persons tested for CCHFV, 3 were positive for IgM, 12 for IgG, and 2 were positive for both (Table 6, <https://wwwnc.cdc.gov/EID/article/26/1/19-0220-T6.htm>). Among livestock owners in the Zhambyl region, weighted CCHFV seroprevalence was 1.2% (95% CI 0.5%–2.7%). In CCHF-endemic villages,

seroprevalence was 3.4% (95% CI 1.8%–6.43%), compared with 0.9% (95% CI 0.3%–2.7%) in non-CCHF-endemic villages. We found evidence of recent or past CCHFV exposure in persons from 13/30 (43.3%) villages (Figure 1).

Of the 17 persons seropositive for CCHFV, median age was 54 years; 58% were male (Table 6). No persons reported previous CCHF diagnosis or illness with fever and hemorrhaging in the previous 5 years or a tick bite or handling ticks with bare hands in the previous 4 months. Occupations among the 17 seropositive persons were farmer or herder ($n = 2$), healthcare worker ($n = 1$), office or indoor worker ($n = 1$), homemaker ($n = 5$), retired ($n = 3$), unemployed ($n = 4$), and other (guard; $n = 1$).

Of 5 participants with evidence of recent exposure to CCHFV, 4 reported participating in ≥ 1 high-risk activity in the previous 4 months: 3 milked animals, 2 helped birth animals, 1 sheared animals, and 1 slaughtered animals. One participant reported experiencing an illness with joint pain in the previous 4 months. Three were from non-CCHF-endemic villages, which could suggest a wider range of virus circulation than previously thought.

In logistic regression, controlling for age and sex, participation in ≥ 1 high-risk activity had a statistically

Table 3. Interactions with ticks among respondents in survey of Crimean-Congo hemorrhagic fever, Kazakhstan*

Human-tick interactions	No. respondents	% Respondents (95% CI)
Had a tick bite†	17	1.0 (0.3–3.3)
Handled tick with bare hands†	61	3.5 (1.1–10.3)
Method of tick disposal after bare hand removal, $n = 27$		
Threw it out	1	3.2 (0.3–29.3)
Killed with bare hands†	1	0.5 (0–5.9)
Killed with object	16	93.6 (69.2–99.0)
Burned it	10	3.5 (0.6–18.8)
Number of tick bites in previous 4 mo	0	0
Method of human tick bite prevention‡		
None	133	9.3 (3.9–20.8)
Long, layered clothing	694	68.8 (55.2–79.9)
Gloves	588	73.1 (60.5–82.9)
Pesticides in environment	267	13.8 (7.9–22.9)
Insect repellent on self, clothing	155	17.7 (10.0–29.3)
Avoiding woody areas	133	12.2 (4.1–31.0)
Avoiding unnecessary animal contact	111	13.9 (5.0–33.3)
Animal-tick interactions		
Found ticks on livestock	486	29.7 (19.6–42.3)
Primary method used to remove ticks on livestock		
Bare hands†	12	4.3 (1.2–15.0)
Gloved hands	95	29.8 (15.9–48.7)
With an object	291	51.7 (34.0–69.0)
Go to a clinic	15	3.3 (1.2–8.7)
Pour liquid mixture on animal	32	3.0 (1.2–7.1)
Burn the tick	6	0.7 (0.2–2.2)
Leave the tick	31	6.8 (2.6–16.3)
Use tick medication for animals	905	94.0 (76.0–98.8)

*Percentage weighted by calculating the inverse probability of selection and applying a poststratification adjustment to each stratum to account for nonresponses.

†High-risk tick interaction.

‡ ≥ 1 response possible.

Table 4. Comparison of respondent attitudes between CCHF-endemic villages and non-CCHF-endemic villages in survey of Crimean-Congo hemorrhagic fever, Kazakhstan*

Attitudes	CCHF-endemic, n = 442		Non-CCHF-endemic, n = 506		p value
	No. respondents†	% Respondents (95% CI)	No. respondents†	% Respondents (95% CI)	
Among all persons					
Ticks are a problem in the community					0.05
Major problem	410	95.3 (89.9–97.9)	408	86.6 (67.8–95.2)	
Minor problem	4	0.7 (0.2–3.0)	13	2.1 (0.8–5.6)	
Not a problem	3	0.6 (0.1–3.1)	52	5.0 (1.0–21.9)	
Don't know	23	3.4 (1.2–9.0)	33	6.4 (2.7–14.4)	
People in my community frequently get bitten by ticks					0.74
Often	245	49.0 (19.4–79.3)	187	33.5 (12.2–64.7)	
Occasionally	24	7.4 (1.3–32.2)	94	13.3 (5.4–29.4)	
Rarely	149	40.4 (17.7–68.1)	202	50.2 (20.7–79.5)	
Don't know	22	3.2 (1.4–7.2)	23	3.0 (0.7–12.1)	
Among persons who have heard of CCHF					
	n = 420		n = 371		
CCHF is a problem in the community					0.12
Major problem	401	96.2 (90.0–98.6)	326	93.7 (82.7–97.9)	
Minor problem	3	0.7 (0.2–3.1)	9	1.9 (0.5–6.6)	
Not a problem	1	0.1 (0–0.5)	26	2.7 (0.5–13.5)	
Don't know	15	3.0 (1.1–8.4)	10	1.7 (0.5–6.1)	
CCHF is something I should be worried about					0.01
Very worried	371	86.1 (72.5–93.5)	317	93.6 (83.5–97.7)	
Somewhat worried	40	11.5 (4.2–27.8)	19	2.6 (0.9–7.4)	
Not worried	1	0.02 (0–0.2)	25	2.5 (0.4–13.9)	
Don't know	8	2.4 (0.4–12.4)	10	1.2 (0.2–7.1)	
I can protect myself from CCHF					<0.01
Yes	380	90.5 (82.5–95.0)	191	52.5 (33.6–70.6)	
No	4	0.7 (0.2–3.2)	100	22.7 (8.3–48.8)	
Don't know	36	8.9 (4.2–17.9)	80	24.8 (12.6–43.0)	
I would welcome a CCHF survivor into my community	379	89.2 (79.7–94.5)	348	94.2 (87.9–97.4)	0.17

*CCHF, Crimean Congo hemorrhagic fever.

†Percentage weighted by calculating the inverse probability of selection and applying a poststratification adjustment to each stratum to account for nonresponses.

significant association with IgG or IgM seropositivity (adjusted OR [aOR] 5.6, 95% CI 1.1–29.7). Being ≥50 years of age was associated with having a history of infection but was not a risk factor for incident infection. Villages at lower elevations were more likely to have ≥1 person seropositive for CCHFV in logistic regression, but the association was not statistically significant (p = 0.41).

Of 911 participant samples tested for Lyme disease, 27 showed evidence of past exposure by IgG against tickborne borrelioses (Table 6). Weighted seroprevalence in the Zhambyl region was 2.4% (95% CI 1.2%–4.6%). We detected seropositive participants in 16/30 (53.3%) villages (Figure 2); occupations were farmer or herder (n = 5), gardener or fieldworker (n = 1), healthcare worker (n = 1), veterinarian (n = 1), office or indoor worker (n = 9), retired (n = 2), homemaker (n = 3), unemployed (n = 4), and other (geologist; n = 1) (Table 6). We did not identify specific activities statistically associated with seropositivity for Lyme disease, but we identified participants who were seropositive, even in a village at 2,590 m, an elevation at which the disease had not been reported in Kazakhstan.

Of 910 samples tested for Q fever, 11 showed evidence of past exposure by *C. burnetii* IgG. Weighted seroprevalence was 1.3% (95% CI 0.3%–5.0%) with seropositivity in 5/30 (53.3%) villages (Figure 3). Occupations of the 11 seropositive participants were farmer or herder (n = 1), healthcare worker (n = 1), retired (n = 1), homemaker (n = 4), and inside or office worker (teacher, locksmith, or civil servant; n = 4); (Table 6). Controlling for age and sex, history of herding (aOR 2.9, 95% CI 1.5–5.4) and slaughtering animals (aOR 2.7, 95% CI 1.5–4.8) had statistically significant associations with seropositivity. Villages at lower elevations were more likely to have ≥1 person seropositive for Q fever in logistic regression, but the association was not statistically significant (p = 0.49).

Discussion

We conducted a serosurvey to update data on the prevalence of CCHF, Q fever, and Lyme disease in Kazakhstan. Because little is known about the seroprevalence of these diseases in central Asia, this study will increase regional awareness. Cases go undetected because of subclinical infections, non-specific diagnostic methods, or poor surveillance.

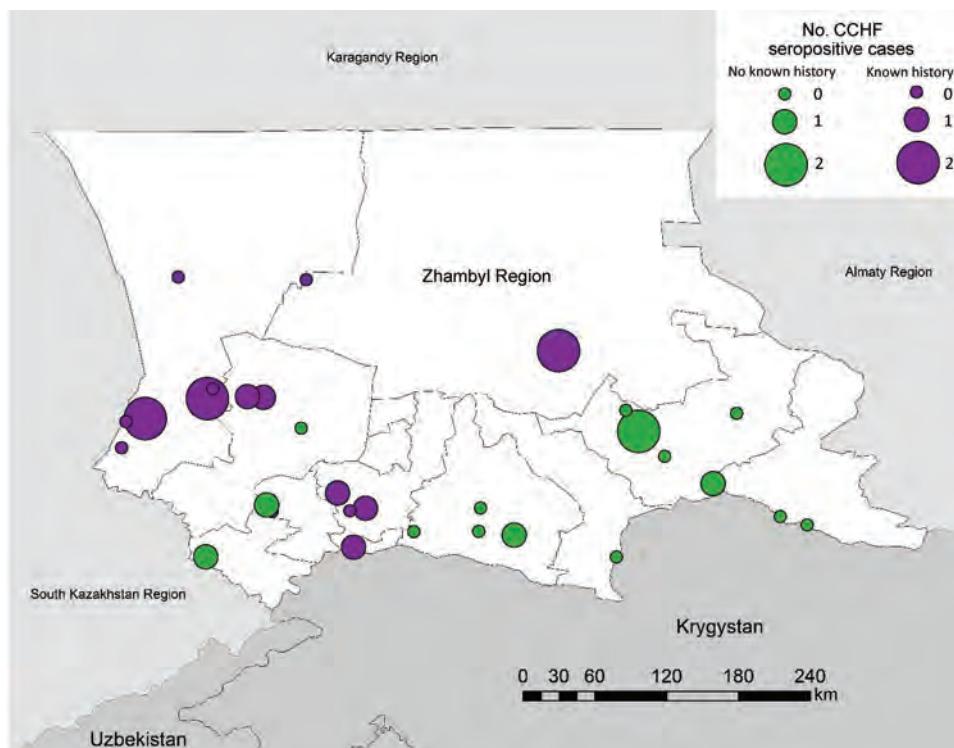


Figure 1. Number of CCHF-seropositive cases in villages included in serologic survey for tickborne diseases, Zhambyl region, Kazakhstan. Circle size denotes the number of IgG antibody-positive serology results indicating past exposure or IgM antibody-positive serology results indicating recent exposure to CCHF. Purple circles indicate that the village had previous known history of CCHF; green circles indicate the village had no known history of CCHF. CCHF, Crimean-Congo hemorrhagic fever.

Our serosurvey identified persons exposed to these pathogens who might have been missed by existing surveillance platforms.

We found a weighted seroprevalence of 1.2% of CCHF in the study region, comparable to findings from studies in Turkey, Iran, and Bulgaria that reported seroprevalences of 2.3%–2.8% (20–22). We found a seroprevalence of 3.4% in villages classified as CCHF-endemic, similar to findings from studies in Bulgaria, China, Georgia, Kosovo, and Turkey that reported seroprevalences of 3%–4% (16,22–28). Most CCHFV serosurveys have been conducted in the Middle East, with a few in Asia, and prevalence estimates range widely, even in the same country.

The comparability of our results to other published surveys is limited because many studies sampled during an outbreak or only sampled high-risk populations. Another CCHFV serosurvey from Kazakhstan found a seroprevalence of 12.7% among patients hospitalized with a fever of unknown origin in the Almaty and Kyzylorda regions (29). Studies of persons exposed to livestock in Iran and Turkey reported CCHFV seroprevalences >12% (30,31). Serosurveys in abattoir workers reported seroprevalences ranging from 0.75% to 16.5% (32,33). Studies in hyperendemic territories reported seroprevalences >10% in the general population (34–41), and a study in Kosovo reported 24% seroprevalence (42).

We found moderate seroprevalence (2.4%) for *B. burgdorferi* compared with findings for other countries in the region. For instance, a serosurvey in Ukraine found seroprevalences of 25%–38% in a healthy population depending on the ecologic zone (43). However, seroprevalence could be caused by other *Borrelia* species in that region and might not be specific to the Lyme disease group of *Borreliae*. In addition, ≤ 3 of 35 persons tested in some villages were seropositive (43).

We also found a lower weighted seroprevalence for Q fever (1.3%) than most reports. Our findings more closely approximate the 3.1% seroprevalence reported in the United States (44). However, as with CCHFV, prevalence of past infection varies widely by location. For instance, reports from Turkey demonstrate $\approx 4\%$ seroprevalence in urban areas but 19% in rural areas (45). As we saw with Lyme disease, some villages in our sample had ≤ 3 of 35 participants who tested seropositive. Previous studies identified higher seroprevalence for Q fever in butchers (46,47), and our study showed 30.6% of participants seropositive for Q fever had butchered animals or handled raw meat.

A limitation of our study is that the Lyme disease assay was designed for broader reactivity and was not analytically specific to a single agent. This assay likely also reacts with relapsing fever *Borreliae*, which

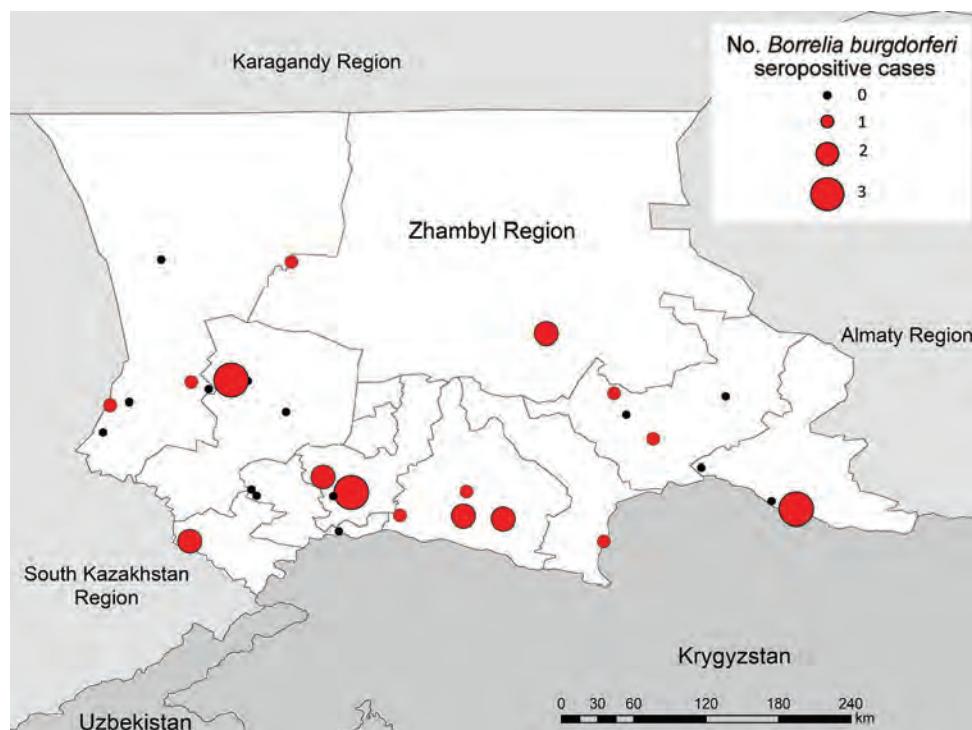


Figure 2. Number of *Borrelia burgdorferi*–seropositive cases in villages included in serologic survey for tickborne diseases, Zhambyl region, Kazakhstan. Circle size denotes the number of IgG antibody–positive serology results indicating past exposure to *B. burgdorferi*.

has unknown distribution in Kazakhstan. Further, validation studies from Vanhomwegen et al. (17) reported an analytic sensitivity of 80% for CCHF IgG Vector-Best kits and 88% for the IgM kits, with a specificity of 100% for both, so the true seroprevalence could be underestimated. The same is true for Lyme disease; a study reported a sensitivity of only 68.8% for the Vector-Best Lyme IgG kit (18).

We were surprised by the few reports of tick bites, considering that $\approx 90\%$ of respondents listed ticks as a major problem and about one third had found ticks on their livestock. A previous survey identified crushing ticks with bare hands as common and a risk factor for CCHF (16). However, most respondents in our study reported crushing ticks with an object, suggesting contact with livestock could be a more common route of exposure among participants. This possibility could be problematic because $<20\%$ of respondents identified infected animals as a potential source of transmission. In addition, nearly half did not wear PPE when slaughtering animals, an exceptionally high-risk activity. The low recognition of the role of livestock in CCHF transmission is seen in other regions (48,49), but targeted educational campaigns have improved knowledge of transmission routes (50).

Our results have been translated into direct public health action. For instance, the serosurvey revealed that CCHFV is circulating in areas previously

unknown to have CCHF activity. Because such areas were not prioritized for educational activities, knowledge of CCHF and modes of transmission was low compared with areas of known transmission. In addition, whereas the KAP/risk factor survey revealed that most respondents understood the risks posed by ticks and many took precautions against tick bites, most did not understand the role animals play in these zoonoses, nor did they wear proper PPE when performing high-risk activities. We helped the health department clarify their pamphlets to state specific high-risk activities and describe which PPE should be worn during each activity. Formative research into the availability and affordability of PPE, as well as the cultural perceptions of PPE when performing activities that may have ritualistic significance, such as slaughtering, is warranted.

A One Health approach that recognizes the interconnectedness of animal, human, and environmental health is needed for effective zoonotic and vectorborne disease control. This study incorporated personnel from the Kazakhstan Ministry of Health, Zhambyl Oblast Public Health Protection Department, and the Ministry of Agriculture. Additional studies in the region will analyze blood and ticks collected from livestock for evidence of past zoonotic infection. Combining the results of the human serosurvey with results of the animal and tick surveys will permit more in-depth investigations into the role of

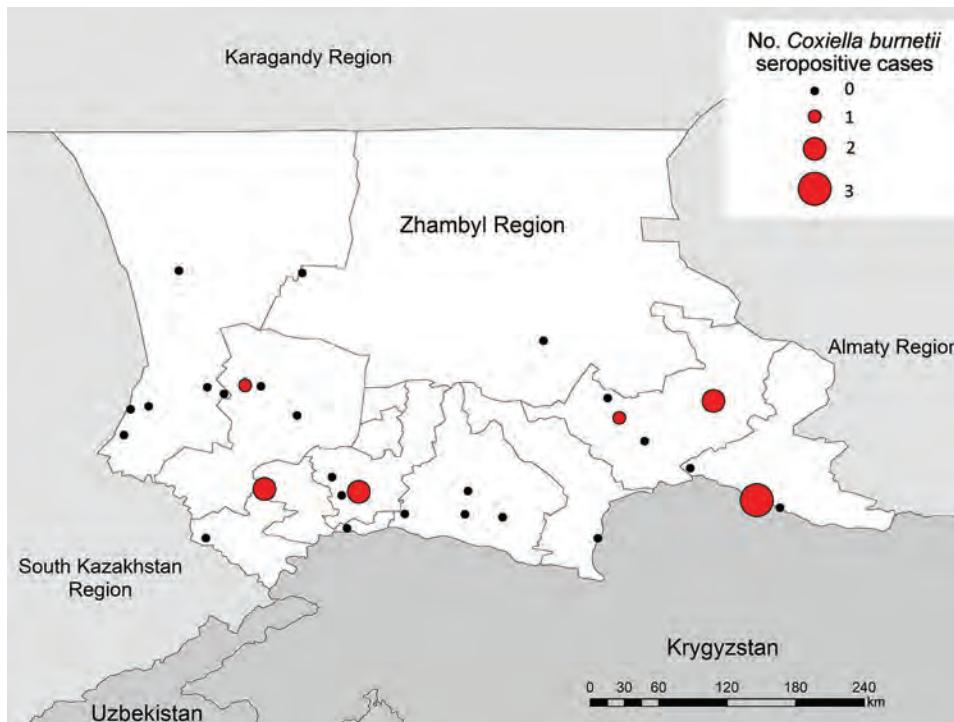


Figure 3. Number of *Coxiella burnetii*-seropositive cases in villages included in serologic survey for tickborne diseases, Zhambyl region, Kazakhstan. Circle size denotes the number of IgG antibody-positive serology results indicating past exposure to *C. burnetii*.

environmental factors, such as climate and elevation, in the transmission of these pathogens.

Acknowledgments

We are grateful to the Akimat of Zhambyl Oblast, the Health Protection Committee of the Ministry of Health and the Committee of Veterinary Control and Surveillance of the Ministry of Agriculture of Kazakhstan, Public Health Protection Department, Health Department, Sanitary Epidemiology Expertise Center, and the Veterinary Inspection unit of Zhambyl Oblast for their help in arranging and performing the investigation. We are grateful for Amber Dimer and Jodi Vanden Eng for technical assistance in the EpiSample application and to Mary Claire Worrell for her assistance generating maps for the application. We thank Ryan Wiegand for assistance in developing the sample selection protocol and Curtis Blanton for discussions regarding trimming of the sample weights.

About the Author

Ms. Head is a doctoral student in epidemiology at the University of California, Berkeley, California, USA, and a previous Global Health Epidemiology Fellow in the Center for Global Health, Centers for Disease Control and Prevention (CDC), Atlanta, Georgia, USA. She has worked with CDC's offices in Kazakhstan on surveillance platforms for influenza, encephalitis, and meningitis. Her research uses statistical and mathematical models to understand the transmission of environmentally mediated or zoonotic pathogens.

References

1. Taylor LH, Latham SM, Woolhouse ME. Risk factors for human disease emergence. *Philos Trans R Soc Lond B Biol Sci.* 2001;356:983–9. <https://doi.org/10.1098/rstb.2001.0888>
2. Christou L. The global burden of bacterial and viral zoonotic infections. *Clin Microbiol Infect.* 2011;17:326–330. <https://doi.org/10.1111/j.1469-0691.2010.03441.x>
3. Paules CI, Marston HD, Bloom ME, Fauci AS. Tickborne diseases – confronting a growing threat. *N Engl J Med.* 2018;379:701–3. <https://doi.org/10.1056/NEJMp1807870>
4. Kilpatrick AM, Randolph SE. Drivers, dynamics, and control of emerging vector-borne zoonotic diseases. *Lancet.* 2012;380:1946–55. [https://doi.org/10.1016/S0140-6736\(12\)61151-9](https://doi.org/10.1016/S0140-6736(12)61151-9)
5. Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. *Nature.* 2008;451:990–3. <https://doi.org/10.1038/nature06536>
6. Whitehouse CA. Crimean-Congo hemorrhagic fever. *Antiviral Res.* 2004;64:145–60. <https://doi.org/10.1016/j.antiviral.2004.08.001>
7. Messina JP, Pigott DM, Golding N, Duda KA, Brownstein JS, Weiss DJ, et al. The global distribution of Crimean-Congo hemorrhagic fever. *Trans R Soc Trop Med Hyg.* 2015; 109:503–13. <https://doi.org/10.1093/trstmh/trv050>
8. Maurin M, Raoult D. Q fever. *Clin Microbiol Rev.* 1999;12:518–53. <https://doi.org/10.1128/CMR.12.4.518>
9. Raoult D, Tissot-Dupont H, Foucault C, Gouvernet J, Fournier PE, Bernit E, et al. Q fever 1985–1998. clinical and epidemiologic features of 1,383 infections. *Medicine (Baltimore).* 2000;79:109–23. <https://doi.org/10.1097/00005792-200003000-00005>
10. Gray J, Kahl O, Lane R, Stanek G. *Lyme borreliosis: biology, epidemiology, and control.* 1st ed. Wallingford, Oxfordshire, United Kingdom: CABI; 2002.

11. Stone BL, Tourand Y, Brissette CA. Brave new worlds: the expanding universe of Lyme disease. *Vector Borne Zoonotic Dis.* 2017;17:619–29. <https://doi.org/10.1089/vbz.2017.2127>
12. Nurmakhanov T, Sansyzbaev Y, Atshabar B, Deryabin P, Kazakov S, Zholsorinov A, et al. Crimean-Congo haemorrhagic fever virus in Kazakhstan (1948–2013). *Int J Infect Dis.* 2015;38:19–23. <https://doi.org/10.1016/j.ijid.2015.07.007>
13. Hoogstraal H. The epidemiology of tick-borne Crimean-Congo hemorrhagic fever in Asia, Europe, and Africa. *J Med Entomol.* 1979;15:307–417. <https://doi.org/10.1093/jmedent/15.4.307>
14. Tsoi DC, Rapoport LP, Samartseva ET. Epidemiological studies of Q fever in the area of Dzhambul in the Kazakh SSR. *J Hyg Epidemiol Microbiol Immunol.* 1980;24:206–11.
15. Spengler JR, Bergeron É, Rollin PE. Seroprevalence studies of Crimean-Congo hemorrhagic fever virus in domestic and wild animals. *PLoS Negl Trop Dis.* 2016;10:e0004210. <https://doi.org/10.1371/journal.pntd.0004210>
16. Greiner AL, Mamuchishvili N, Kakutia N, Stauffer K, Geleishvili M, Chitadze N, et al. Crimean-Congo hemorrhagic fever knowledge, attitudes, practices, risk factors, and seroprevalence in rural Georgian villages with known transmission in 2014. *PLoS One.* 2016;11:e0158049. <https://doi.org/10.1371/journal.pone.0158049>
17. Vanhomwegen J, Alves MJ, Zupanc TA, Bino S, Chinikar S, Karlberg H, et al. Diagnostic assays for Crimean-Congo hemorrhagic fever. *Emerg Infect Dis.* 2012;18:1958–65. <https://doi.org/10.3201/eid1812.120710>
18. Manzeniuk IN, Vorob'eva MS, Nikitiuk NM, Arumova EA, Anan'eva LP, Baranova SG, et al. [Preparations for serodiagnosis of diseases due to causative agents of ixode tick-borne Borreliosis (Lyme disease). Communication 2. Comparative study of recombinant enzyme immunoassay test-systems (rELISA) for serological diagnosis of ixode tick-borne Borreliosis. [in Russian]. *Antibiot Khimioter.* 2004;49(8-9):25–8.
19. R Core Team. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing; 2015.
20. Yagci-Caglayik D, Korukluoglu G, Uyar Y. Seroprevalence and risk factors of Crimean-Congo hemorrhagic fever in selected seven provinces in Turkey. *J Med Virol.* 2014;86:306–14. <https://doi.org/10.1002/jmv.23699>
21. Izadi S, Holakouie-Naieni K, Majdzadeh SR, Chinikar S, Nadim A, Rakhshani F, et al. Seroprevalence of Crimean-Congo hemorrhagic fever in Sistan-va-Baluchestan province of Iran. *Jpn J Infect Dis.* 2006;59:326–8.
22. Christova I, Gladnisha T, Taseva E, Kalvatchev N, Tsergouli K, Papa A. Seroprevalence of Crimean-Congo hemorrhagic fever virus, Bulgaria. *Emerg Infect Dis.* 2013;19:177–9. <https://doi.org/10.3201/eid1901.120299>
23. Gergova I, Kamarinchev B. Seroprevalence of Crimean-Congo hemorrhagic fever in southeastern Bulgaria. *Jpn J Infect Dis.* 2014;67:397–8. <https://doi.org/10.7883/yoken.67.397>
24. Xia H1, Li P, Yang J, Pan L, Zhao J, Wang Z, et al. Epidemiological survey of Crimean-Congo hemorrhagic fever virus in Yunnan, China, 2008. *Int J Infect Dis.* 2011;15:e459–463. <https://doi.org/10.1016/j.ijid.2011.03.013>
25. Sargianou M, Panos G, Tsatsaris A, Gogos C, Papa A. Crimean-Congo hemorrhagic fever: seroprevalence and risk factors among humans in Achaia, western Greece. *Int J Infect Dis.* 2013;17:e1160–1165 <https://doi.org/10.1016/j.ijid.2013.07.015>
26. Sidira P, Maltezou HC, Haidich AB, Papa A. Seroprevalence study of Crimean-Congo haemorrhagic fever in Greece, 2009–2010. *Clin Microbiol Infect.* 2012;18:e16–19. <https://doi.org/10.1111/j.1469-0691.2011.03718.x>
27. Fajs L, Humolli I, Saksida A, Knap N, Jelovšek M, Korva M, et al. Prevalence of Crimean-Congo hemorrhagic fever virus in healthy population, livestock and ticks in Kosovo. *PLoS One.* 2014;9:e110982. <https://doi.org/10.1371/journal.pone.0110982>
28. Gazi H, Özkütük N, Ecemis Ö, Atasoylu G, Köroglu G, Kurutepe S, et al. Seroprevalence of West Nile virus, Crimean-Congo hemorrhagic fever virus, *Francisella tularensis* and *Borrelia burgdorferi* in rural population of Manisa, western Turkey. *J Vector Borne Dis.* 2016;53:112–7.
29. Abdieva K, Turebekov N, Dmitrovsky A, Tukhanova N, Shin A, Yeraliyeva L, et al. Seroprevalence and molecular investigations of infections with Crimean-Congo hemorrhagic fever virus in Kazakhstan. *Int J Infect Dis.* 2019;78:121–7. <https://doi.org/10.1016/j.ijid.2018.10.015>
30. Chinikar S, Ghiasi SM, Naddaf S, Piazak N, Moradi M, Razavi MR, et al. Serological evaluation of Crimean-Congo hemorrhagic fever in humans with high-risk professions living in enzootic regions of Isfahan province of Iran and genetic analysis of circulating strains. *Vector Borne Zoonotic Dis.* 2012;12:733–8. <https://doi.org/10.1089/vbz.2011.0634>
31. Gunes T, Engin A, Poyraz O, Elaldi N, Kaya S, Dokmetas I, et al. Crimean-Congo hemorrhagic fever virus in high-risk population, Turkey. *Emerg Infect Dis.* 2009;15:461–4. <https://doi.org/10.3201/eid1503.080687>
32. Andriamandimby SF, Marianneau P, Rafisandrantsoa JT, Rollin PE, Heraud JM, Tordo N, et al. Crimean-Congo hemorrhagic fever serosurvey in at-risk professionals, Madagascar, 2008 and 2009. *J Clin Virol.* 2011;52:370–2. <https://doi.org/10.1016/j.jcv.2011.08.008>
33. Mostafavi E, Pourhossein B, Esmaeili S, Bagheri Amiri F, Khakifirooz S, Shah-Hosseini N, et al. Seroprevalence and risk factors of Crimean-Congo hemorrhagic fever among butchers and slaughterhouse workers in southeastern Iran. *Int J Infect Dis.* 2017;64:85–9. <https://doi.org/10.1016/j.ijid.2017.09.008>
34. Bayram Y, Parlak M, Özkaçmaz A, Çıkman A, Güdücüoğlu H, Kılıç S, et al. Seroprevalence of Crimean-Congo hemorrhagic fever in Turkey's Van Province. *Jpn J Infect Dis.* 2017;70:65–8. <https://doi.org/10.7883/yoken.JJID.2015.675>
35. Mustafa ML, Ayazi E, Mohareb E, Yingst S, Zayed A, Rossi CA, et al. Crimean-Congo hemorrhagic fever, Afghanistan, 2009. *Emerg Infect Dis.* 2011;17:1940–1. <https://doi.org/10.3201/eid1710.110061>
36. Bukbuk DN, Fukushi S, Tani H, Yoshikawa T, Taniguchi S, Iha K, et al. Development and validation of serological assays for viral hemorrhagic fevers and determination of the prevalence of Rift Valley fever in Borno State, Nigeria. *Trans R Soc Trop Med Hyg.* 2014;108:768–73. <https://doi.org/10.1093/trstmh/tru163>
37. Lwande OW, Irura Z, Tigoi C, Chepkorir E, Orindi B, Musila L, et al. Seroprevalence of Crimean Congo hemorrhagic fever virus in Ijara District, Kenya. *Vector Borne Zoonotic Dis.* 2012;12:727–32. <https://doi.org/10.1089/vbz.2011.0914>
38. Chapman LE, Wilson ML, Hall DB, LeGuenno B, Dykstra EA, Ba K, et al. Risk factors for Crimean-Congo hemorrhagic fever in rural northern Senegal. *J Infect Dis.* 1991;164:686–92. <https://doi.org/10.1093/infdis/164.4.686>
39. Bodur H, Akinci E, Ascioğlu S, Öngürü P, Uyar Y. Subclinical infections with Crimean-Congo hemorrhagic fever virus, Turkey. *Emerg Infect Dis.* 2012;18:640–2. <https://doi.org/10.3201/eid1804.111374>

40. Koksall I, Yilmaz G, Aksoy F, Erensoy S, Aydin H. The seroprevalance of Crimean-Congo haemorrhagic fever in people living in the same environment with Crimean-Congo haemorrhagic fever patients in an endemic region in Turkey. *Epidemiol Infect.* 2014;142:239–45. <https://doi.org/10.1017/S0950268813001155>
41. Cikman A, Aydin M, Gulhan B, Karakecili F, Kesik OA, Ozcicek A, et al. Seroprevalence of Crimean-Congo hemorrhagic fever virus in Erzincan Province, Turkey, relationship with geographic features and risk factors. *Vector Borne Zoonotic Dis.* 2016;16:199–204. <https://doi.org/10.1089/vbz.2015.1879>
42. Humolli I, Dedushaj I, Zupanac TA, Mučaj S. Epidemiological, serological and herd immunity of Crimean-Congo haemorrhagic fever in Kosovo. *Med Arh.* 2010;64:91–3.
43. Biletska H, Podavalenko L, Semenyshyn O, Lozynskyy I, Tarasyuk O. Study of Lyme borreliosis in Ukraine. *Int J Med Microbiol.* 2008;298:154–60. <https://doi.org/10.1016/j.ijmm.2008.04.004>
44. Anderson AD, Kruszon-Moran D, Loftis AD, McQuillan G, Nicholson WL, Priestley RA, et al. Seroprevalence of Q fever in the United States, 2003–2004. *Am J Trop Med Hyg.* 2009;81:691–4. <https://doi.org/10.4269/ajtmh.2009.09-0168>
45. Erturk R, Poyraz Ö, Güneş T. Serosurvey of *Coxiella burnetii* in high risk population in Turkey, endemic to Crimean-Congo haemorrhagic fever virus. *J Vector Borne Dis.* 2017;54:341–7. <https://doi.org/10.4103/0972-9062.225839>
46. Gozalan A, Rolain JM, Ertek M, Angelakis E, Coplu N, Basbulut EA, et al. Seroprevalence of Q fever in a district located in the west Black Sea region of Turkey. *Eur J Clin Microbiol Infect Dis.* 2010;29:465–9. <https://doi.org/10.1007/s10096-010-0885-3>
47. Esmaeili S, Pourhossein B, Gouya MM, Amiri FB, Mostafavi E. Seroepidemiological survey of Q fever and brucellosis in Kurdistan Province, western Iran. *Vector Borne Zoonotic Dis.* 2014;14:41–5. <https://doi.org/10.1089/vbz.2013.1379>
48. Yilmaz R, Ozcetin M, Erkorkmaz U, Ozer S, Ekici F. Public knowledge and attitude toward Crimean Congo hemorrhagic fever in Tokat Turkey. *Iran J Arthropod Borne Dis.* 2009;3:12–7.
49. Gungormus Z, Kiyak E. Evaluation of knowledge about protection against Crimean-Congo hemorrhagic fever. *Southeast Asian J Trop Med Public Health.* 2011;42:737–43.
50. Koculu S, Oncul A, Onal O, Yesilbag Z, Uzun N. Evaluation of knowledge of the healthcare personnel working in Giresun Province regarding Crimean-Congo hemorrhagic fever before and after educational training. *J Vector Borne Dis.* 2015;52:166–70.

Address for correspondence: Jennifer R. Head, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Atlanta, GA 30329-4027, USA; email: jrhead6@gmail.com

EID SPOTLIGHT TOPIC

Ticks transmit a variety of different pathogens, including bacteria, protozoa, and viruses, which can produce serious and even fatal disease in humans and animals. Tens of thousands of cases of tickborne disease are reported each year, including Lyme disease. See the EID Lyme Disease Spotlight. Lyme disease is the most well-known tickborne disease. However, other tickborne illnesses such as Rocky Mountain spotted fever, tularemia, babesiosis, and ehrlichiosis also contribute to severe morbidity and more mortality each year.

Symptoms of tickborne disease are highly variable, but most include sudden onset of fever, headache, malaise, and sometimes rash. If left untreated, some of these diseases can be rapidly fatal.

**EMERGING
INFECTIOUS DISEASES®**



Ticks

<https://wwwnc.cdc.gov/eid/page/tick-spotlight>

High Azole Resistance in *Aspergillus fumigatus* Isolates from Strawberry Fields, China, 2018

Yong Chen,¹ Fengshou Dong,¹ Jingya Zhao, Hong Fan, Chunping Qin, Runan Li, Paul E. Verweij, Yongquan Zheng,² Li Han²

In 2018, we conducted a cross-sectional study to investigate azole resistance in environmental *Aspergillus fumigatus* isolates obtained from different agricultural fields in China. Using 63 soil cores, we cultured for azole-resistant *A. fumigatus* and characterized isolates by their *cyp51A* gene type, short tandem repeat genotype, and mating type. Of 206 *A. fumigatus* isolates, 21 (10.2%) were azole resistant. Nineteen of 21 had mutations in their *cyp51A* gene (5 TR34/L98H, 8 TR34/L98H/S297T/F495I, 6 TR46/Y121F/T289A). Eighteen were cultured from soil samples acquired from strawberry fields, suggesting this soil type is a potential hotspot for azole resistance selection. Twenty resistant isolates were mating type MAT1-1, suggesting asexual sporulation contributed to their evolution. Prochloraz, difenoconazole, and tebuconazole were the most frequently detected fungicides in soil samples with azole-resistant fungus. Our study results suggest that managing the fungicides used in agriculture will help contain the problem of antifungal drug resistance in clinics.

Triazoles are among the main class of drugs used for the treatment of invasive and chronic aspergillosis (1,2). However, the effectiveness of this drug class is being threatened by the emergence and global spread of azole resistance in clinical and environmental *Aspergillus fumigatus* isolates (3,4). Resistance is believed to develop predominantly through 2 distinct routes: long-term clinical azole therapy and the environmental application of azole fungicides, some of which have been shown to have

molecular targets identical to those of medical triazoles and have activity against *A. fumigatus* (4,5). The main resistance mechanism of *A. fumigatus* involves point mutations in *cyp51A* (gene encoding the protein targeted by antifungal azoles) with or without a tandem repeat (TR) insertion in its promoter (6). Two *cyp51A* variants believed to be associated with environmental resistance selection, TR34/L98H and TR46/Y121F/T289A, are highly prevalent worldwide, although the frequency of these resistance alleles varies considerably from country to country (<5%–30%) (4,7–9). Differences in these reported resistance frequencies could be caused by the study design (i.e., the sampling strategy, number of colonies analyzed, and choice of denominator). On the other hand, the reported variances might instead reflect true differences caused by poorly understood phenomena.

One factor that could be contributing to the variation in resistance allele frequencies is differences in regional azole compound use. The use of azole fungicides provides selective pressure for the development of azole resistance among species in soils. Resistance has been reported in environmental *A. fumigatus* isolates in 2 studies conducted in China, and the prevalence of resistance reported in these studies was 1.4% and 2.1% (10,11). However, in these studies, the concentration of fungicides in the environment the isolates came from was not measured. Also, whether environmental hotspots exist for resistance selection is unknown. Sites supporting the growth, reproduction, and genetic variation of *A. fumigatus* and containing residual azole fungicides, which can facilitate the emergence, amplification, and spread of triazole resistance mutations, are considered to be potential

Author affiliations: Chinese People's Liberation Army Center for Disease Control and Prevention, Beijing, China (Y. Chen, J. Zhao, H. Fan, C. Qin, L. Han); Chinese Academy of Agricultural Sciences, Beijing (F. Dong, R. Li, Y. Zheng); Radboud University Medical Center, Nijmegen, the Netherlands (P.E. Verweij)

DOI: <https://doi.org/10.3201/eid2601.190885>

¹These authors contributed equally to this article.

²These senior authors contributed equally to this article.

hotspots for azole resistance (12). Here, we describe a cross-sectional study we conducted to investigate azole resistance in *A. fumigatus* isolates in different agricultural fields, identify hotspots of resistance, and evaluate the relationship between azole resistance and use of azole fungicides.

Methods

Collection of Soil Samples

During July–August 2018, we collected 63 soil cores from agricultural farms or greenhouses located in 8 cities of China (Harbin, Beijing, Weifang, Nanjing, Wuhan, Hangzhou, Yichun, and Loudi; Appendix Figure, <https://wwwnc.cdc.gov/EID/article/26/1/19-0885-App1.pdf>). We acquired soil cores (to a depth of 20 cm) near rice, watermelon, strawberry, tea leaf, mandarin orange, and vegetable (eggplant, pepper, water spinach, shallot, cabbage, and tomato) (Appendix Table 1) crops using a soil sampler.

Isolation and Identification of *A. fumigatus* Isolates

We handled and plated samples according to previously described methods (13–15) with some modifications. In brief, for each soil core, we suspended 2 g of soil from the top (0 cm) and bottom (20 cm) of the column separately into 8 mL of sterile saline with 1% tween and vortexed. We then plated 100 μ L of these suspensions on Sabouraud dextrose agar supplemented with chloramphenicol (50 mg/L; Sigma-Aldrich, <https://www.sigmaaldrich.com>) and incubated at 42°C. We examined plates for *A. fumigatus* growth at 24 h, 48 h, and 72 h. We randomly selected 5 colonies showing *A. fumigatus* morphology for further identification. If the total number of *Aspergillus*-like colonies on the plate was <5 , we subcultured them all. We confirmed colonies were *A. fumigatus* isolates by assessing their capacity to grow at 48°C and by sequencing the β -tubulin gene, as previously described (16).

Detection of Residual Fungicide in Soil Samples

We set aside 10 g of soil from the top (0 cm) and bottom (20 cm) of soil cores for residual fungicide analysis. We detected the 6 main fungicides used in agriculture in China (difenoconazole, tebuconazole, epoxiconazole, prochloraz, imazalil, and tricyclazole) using ultra-high-performance liquid chromatography coupled with tandem mass spectrometry by using an Acquity UPLC BEH Column (2.1 mm \times 50 mm, 1.7- μ m particle size; Waters, <https://www.waters.com>) (Appendix Table 2), as previously described (17). The mobile phase of the

column included chromatographically pure methanol (solution A) and 0.2% formic acid (vol/vol) in Milli-Q water (<http://www.emdmillipore.com>) (solution B), and the flow rate was 0.3 mL/min. We used the following gradient program to detect fungicides with the column: 10% solution A (0 min), 90% solution A (0–1.7 min), 90% solution A (1.7–3.0 min), 10% solution A (3.0–3.1 min), and 10% solution A (3.1–4.0 min).

Screening of Azole Resistance

Because VIP check screening plates (<https://www.vipcheck.nl>) are not commercially available in China, we screened *A. fumigatus* isolates for azole resistance using azole-containing 4-well plates that we prepared. In plate wells, we used RPMI 1640 agar medium (Sigma-Aldrich) supplemented with 4 mg/L itraconazole, 2 mg/L voriconazole, 0.5 mg/L posaconazole, or no fungicide (control well), according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (18). We used 2 azole-resistant isolates (C135 and C02810) from our laboratory (19) and 1 azole-susceptible isolate (ATCC 204305; American Tissue Culture Collection, <https://www.atcc.org>) for quality control purposes. We performed experimental procedures and interpreted results as recommended by EUCAST (18).

Antifungal Drug Susceptibility Testing and *cyp51A* Gene Sequencing

We conducted antifungal drug susceptibility testing for all isolates demonstrating any growth on ≥ 1 azole-containing agar plate. We conducted in vitro drug susceptibility testing with 3 clinical azoles (itraconazole, voriconazole, and posaconazole) and 7 azole fungicides used in agriculture (epoxiconazole, bromucanazole, tebuconazole, difenoconazole, propiconazole, imazalil, and prochloraz) using the EUCAST microbroth dilution E. Def 9.3 method (20). We used the same drug concentration ranges and methods for quality control as done in our previous study (19) and, for confirmed azole-resistant isolates, amplified and sequenced the *cyp51A* gene and its promoter, as described previously (21).

Genotyping of *A. fumigatus* Isolates

For all azole-resistant isolates, we determined cell surface protein (CSP) type and short tandem repeat (STR) type (i.e., type of 9 microsatellite loci [STRAf 2A, 2B, 2C, 3A, 3B, 3C, 4A, 4B, and 4C]) by PCR amplification and sequencing (22,23). We identified the

mating type of all azole-resistant isolates and a randomly selected subset of azole-susceptible isolates using a PCR (with 2 different primer sets) designed to amplify mating type-specific genes (24). We genetically characterized the azole-resistant *A. fumigatus* (ARAF) isolates obtained in this study ($n = 21$) and other studies conducted in China ($n = 30$) by performing a categorical analysis of the previously mentioned 9 microsatellite markers using the UPGMA clustering in BioNumerics 7.5 (<http://www.applied-maths.com>). We also analyzed the STR typing data of all ARAF isolates from this study and 580 representative azole-resistant and azole-susceptible isolates from different countries (19) and presented the information as a minimum spanning tree of categorical data with default settings.

Statistical Analysis

We analyzed data with SPSS 19.0 (IBM Corporation, <https://www.ibm.com>) and used the χ^2 test to evaluate differences in the prevalence of ARAF isolates by sample type. We considered p values <0.05 statistically significant.

Results

Detection of Azole-Resistant *A. fumigatus* Isolates in Soil Samples

From 126 soil sample suspensions cultured for 72 h, we obtained 210 suspected *A. fumigatus* isolates. After further phenotypic and genotypic identification, 206 isolates (140 from topsoil [0 cm] and 66 from deep soil [20 cm]) were identified as *A. fumigatus sensu stricto* (Table 1). After screening for azole resistance on self-prepared 4-well plates, 23 isolates showed the ability to grow on ≥ 1 azole-containing agar. Further confirmatory MIC testing showed that 21 *A. fumigatus* isolates were azole resistant according to EUCAST criteria. The total prevalence of azole resistance among all *A. fumigatus* isolates was 10.2% (21/206).

Overall, 19 ARAF isolates were obtained from 8 topsoil samples acquired near strawberry, vegetable, and rice plants, and 2 ARAF isolates were obtained from 2 deep soil samples acquired near strawberry plants. The prevalence of ARAF isolates was higher in topsoil samples (13.6% [19/140]) than deep soil samples (3.0% [2/66], $\chi^2 = 5.44$; $p = 0.020$). Of 10 soil cores acquired near strawberry plants, ARAF isolates were detected in 6 (60.0%) topsoil samples and 2 (20.0%) deep soil samples. The 8 soil cores positive for ARAF isolates originated from 8 different farms in Nanjing and Hangzhou in eastern China.

Table 1. Prevalence of ARAF isolates in soil samples from different crops, China, 2018*

Crop	Soil depth, cm	No. ARAF-positive soil samples/no. samples (%)	No. ARAF isolates/no. isolates (%)
Watermelon	0	0/10	0/33
	20	0/10	0/13
Rice	0	1/16 (6.3)	1/20 (5.0)
	20	0/16	0/11
Vegetable	0	1/11 (9.1)	2/33 (6.1)
	20	0/11	0/18
Strawberry	0	6/10 (60.0)	16/44 (36.4)
	20	2/10 (20.0)	2/23 (8.7)
Tea leaf	0	0/5	0/6
	20	0/5	0/0
Citrus	0	0/11	0/4
	20	0/11	0/1
Total	0	8/63 (12.7)	19/140 (13.6)
	20	2/63 (3.2)	2/66 (3.0)

*ARAF, azole-resistant *Aspergillus fumigatus*.

Characterization of Azole-Resistant

A. fumigatus Isolates

Of 21 ARAF isolates, 17 were resistant to itraconazole (MIC ≥ 4 mg/L), 15 were resistant to voriconazole (MIC ≥ 4 mg/L), and all were resistant to posaconazole (MIC ≥ 0.5 mg/L) (Appendix Table 3). Sequencing of the *cyp51A* gene and its promoter showed that 19 ARAF isolates harbored 3 commonly identified nucleotide and amino acid change combinations, TR34/L98H ($n = 5$), TR34/L98H/S297T/F495I ($n = 8$), and TR46/Y121F/T289A ($n = 6$); no mutations were identified in the remaining 2 ARAF isolates (E2012-0-2 and E2012-0-4). CSP typing showed that all 5 TR34/L98H ARAF variants corresponded to CSP type t02, 7 of 8 TR34/L98H/S297T/F495I ARAF variants corresponded to CSP type t01 or t11, and all 6 TR46/Y121F/T289A ARAF variants corresponded to CSP type t01 or t04A. The results of mating type identification showed that 20 ARAF isolates were MAT1-1, and only 1 isolate (E2006-0-5) was MAT1-2. Among 21 randomly selected azole-susceptible *A. fumigatus* isolates, 12 were MAT1-1 and 9 MAT1-2.

In Vitro Susceptibility to Azole Fungicides

High MICs of 5 azole fungicides (epoxiconazole, bromuconazole, tebuconazole, difenoconazole, and propiconazole) were required to inhibit the growth of the 19 ARAF isolates with *cyp51A* mutations (Appendix Table 3). For the 2 ARAF isolates that harbored no *cyp51A* mutations (E2012-0-2 and E2012-0-4), reference strain ATCC 204305, and the azole-susceptible *A. fumigatus* isolates in our previous study (19), the MICs of all 7 azole fungicides tested were similar. The MICs of the 2 imidazoles (imazalil and prochloraz) were greater for the TR34/L98H/S297T/F495I and TR46/Y121F/T289A ARAF isolates than they were

for the TR34/L98H ARAF isolates and the ARAF isolates without *cyp51A* mutations. For the TR34/L98H/S297T/F495I and TR46/Y121F/T289A ARAF isolates, the MICs of prochloraz were >32 mg/L.

Detection of Residual Azole Fungicide in Soil Samples

Of the 6 azole fungicides used in agriculture that we tested for, difenoconazole, prochloraz, and tebuconazole were the most frequently detected; epoxiconazole and imazalil were not detected in any soil samples (Table 2). Of the 10 topsoil samples acquired from strawberry-planted fields, difenoconazole (0.0104–0.0385 mg/kg) was detected in 8 and prochloraz (0.0116–0.05 mg/kg) in 7 (Appendix Table 1). We also detected prochloraz in 3 soil samples from 2 sampling sites of vegetable-planted fields. Tebuconazole was detected in 23 of the 32 topsoil and deep soil samples acquired from rice-planted fields. Prochloraz (0.0115–0.05 mg/kg) was detected in 6 of 8 ARAF-positive topsoil samples, difenoconazole (0.0115–0.0385 mg/kg) in 5 of 8, and tebuconazole (0.015–0.0805 mg/kg) in 3 of 8. No azole fungicides were detected in the 2 ARAF-positive deep soil samples. Many azole fungicides, including prochloraz and difenoconazole, had been actively used by the farmers of the fields that we sampled to control for disease during seasons of high temperatures.

Genetic Characterization of Azole-Resistant

A. fumigatus Isolates

ARAF isolates with *cyp51A* mutations had a high diversity of STR types. We observed a close genetic relationship for 5 TR34/L98H/S297T/F495I isolates obtained from strawberry fields of 2 different farms in Hangzhou (Figure 1). The 4 TR46/Y121F/T289A variants isolated from strawberry fields of 3 different farms in Hangzhou were also closely related. The 2

ARAF isolates without *cyp51A* mutations (E2012-0-2 and E2012-0-4) were not genetically related to any other isolate from China, except for a clinical isolate with a G54V amino acid change.

We evaluated the population structure of 601 worldwide *A. fumigatus* isolates on the basis of their STR type (Figure 2). All of the ARAF isolates from Nanjing were part of the major clone complex of ARAF strains disseminated widely throughout the world. All 5 TR34/L98H/S297T/F495I isolates and 4 TR46/Y121F/T289A isolates from Hangzhou clustered within a group mainly consisting of azole-susceptible *A. fumigatus* isolates. These findings suggest that the ARAF isolates from Hangzhou and Nanjing originated from different sources.

Discussion

The rapid dissemination of azole resistance among *A. fumigatus* strains around the world has become an increasing public health problem. An investigation in the Netherlands indicated that an azole resistance mutation (a triple 46-bp repeat in the *cyp51A* promoter) continues to spread in the environment, and compost containing residual azole fungicide was identified as the possible hotspot for this *A. fumigatus* variant (25). As of November 2019, a limited number of studies were available on azole resistance among environmental *A. fumigatus* isolates obtained from agricultural fields in China. In 1 study, the prevalence of azole resistance among 73 *A. fumigatus* isolates collected from soils near crops producing vegetables and fruits (such as strawberries, grapes, carrots, watermelons, pumpkins, shallots, luffas, and eggplants) was investigated in greenhouses in Zhejiang Province (11). In that study, the authors were able to identify 3 (4.1%) resistant isolates: 1 TR34/L98H/S297T/F495I isolate and 1 TR46/Y121F/T289A isolate from soils

Table 2. Fungicides detected in soil samples acquired near different crops, China, 2018

Crop	Soil depth, cm	No. soil samples	No. (%) samples containing fungicide*					
			Difenoconazole	Prochloraz	Tebuconazole	Epoxiconazole	Imazalil	Tricyclazole
Watermelon	0	10	0	0	1 (10.0)	0	0	0
	20	10	0	0	0	0	0	0
Rice	0	16	0	1 (6.3)	12 (75.0)	0	0	4 (25.0)
	20	16	0	1 (6.3)	11 (68.8)	0	0	0
Vegetable	0	11	1 (9.1)	3 (27.3)	1 (9.1)	0	0	0
	20	11	0	0	0	0	0	0
Strawberry	0	10	8 (80.0)	7 (70.0)	3 (30.0)	0	0	0
	20	10	1 (10.0)	1 (10.0)	1 (10.0)	0	0	0
Tea leaf	0	5	0	0	0	0	0	0
	20	5	0	0	0	0	0	0
Citrus	0	11	0	0	0	0	0	0
	20	11	0	0	0	0	0	0
Total	0	63	9 (14.3)	11 (17.5)	17 (27.0)	0	0	4 (6.3)
	20	63	1 (1.6)	2 (3.2)	12 (19.0)	0	0	0

*Detection limit for all 6 fungicides was 0.01 mg/kg.

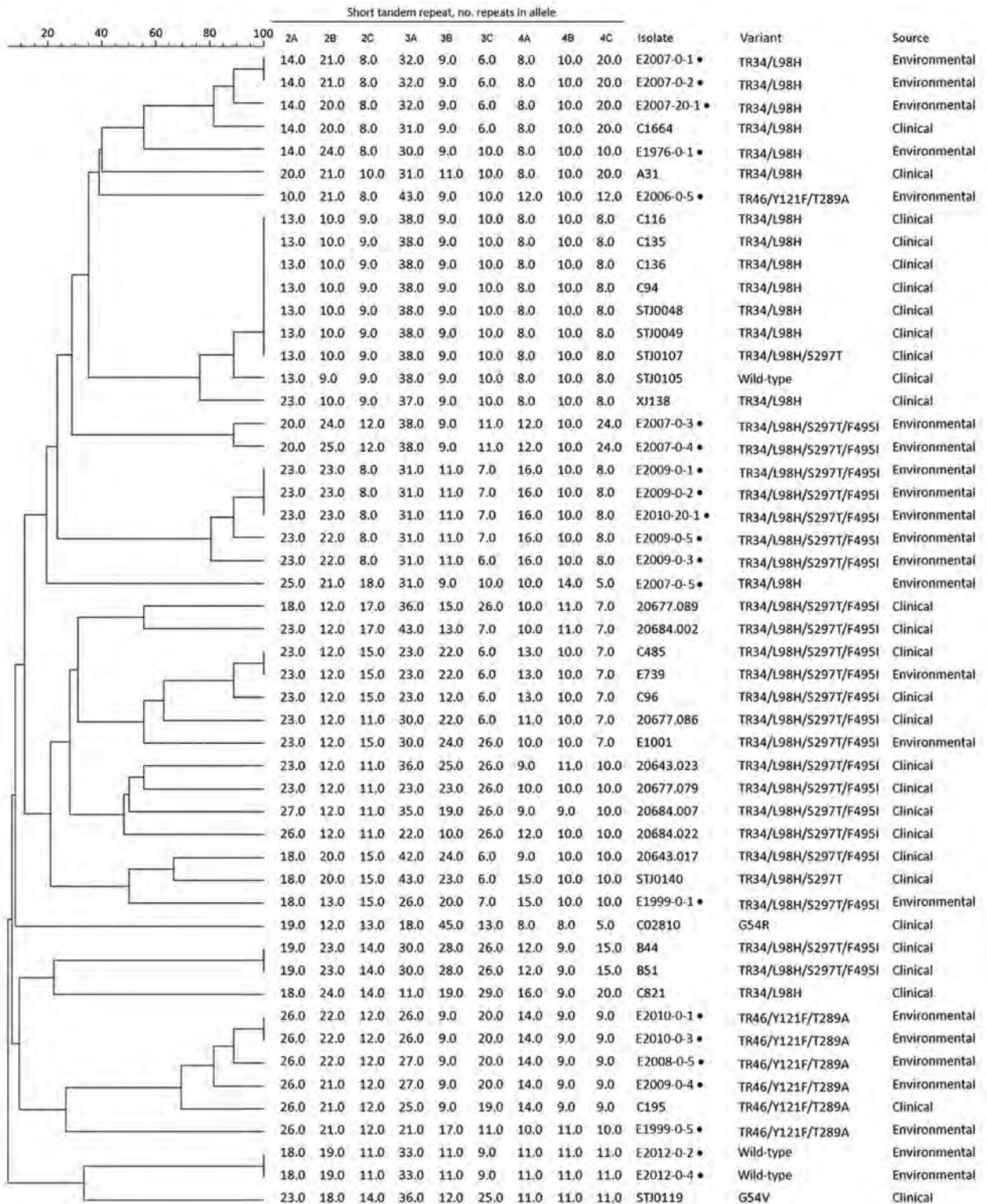


Figure 1. Genotypes of 21 azole-resistant *Aspergillus fumigatus* isolates obtained from farm soils in China, 2018 (black dots), and other azole-resistant *A. fumigatus* isolates from China. This dendrogram was constructed on the basis of a categorical analysis of 9 microsatellite markers (short tandem repeats 2A–4C) by using the UPGMA. Scale bar indicates percentage identity.

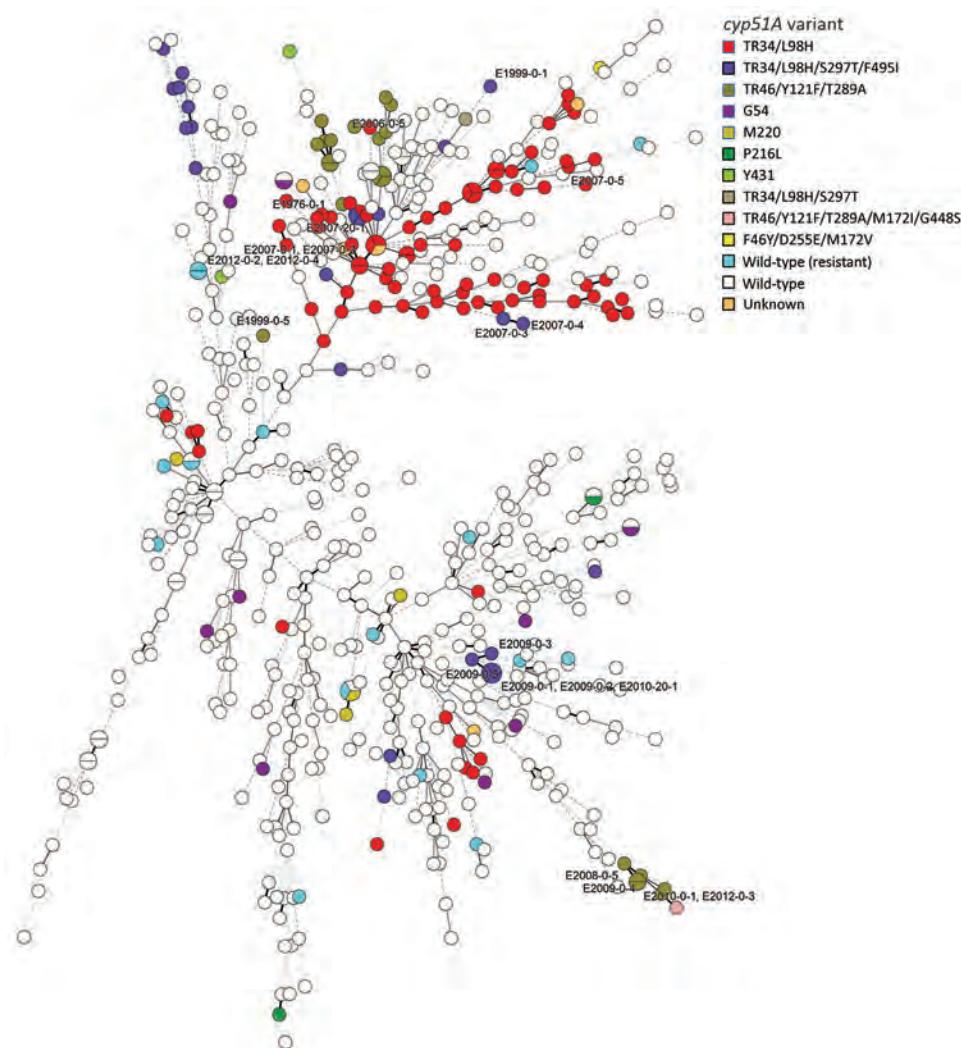


Figure 2. Minimum spanning tree of 21 environmental azole-resistant *Aspergillus fumigatus* (ARAF) isolates, China, 2018 (labeled), and 580 other ARAF and azole-susceptible *A. fumigatus* isolates. The tree was constructed on the basis of short tandem repeat type for all 9 microsatellite markers. Each circle represents 1 unique short tandem repeat genotype but might include multiple *cyp51A* variants. All ARAF isolates from Nanjing are located in the upper right clade of the tree, which represents a major clone complex of ARAF strains disseminated worldwide. All 5 TR34/L98H/S297T/F495I and 4 TR46/Y121F/T289A isolates from Hangzhou are located in the lower right clade of the tree, consisting mainly of azole-susceptible *A. fumigatus* isolates.

near strawberry plants and 1 TR46/Y121F/T289A isolate from soil near a luffa plant. However, the azole fungicide levels in the samples were not investigated, and no TR34/L98H isolate was cultured.

Our study suggests that, in China, ARAF with different *cyp51A* mutations is abundant in strawberry field soils and might be a potential hotspot for the emergence of *A. fumigatus* azole resistance. In a study conducted in the United Kingdom, azole-resistant *A. fumigatus* isolates were identified in several products, including tea and peppers, some of which originated from China (26). In this study, we found 2 ARAF isolates in soil sampled near pepper plants. The findings of these 2 studies suggest a high possibility for the transmission of ARAF isolates through international trade, which could pose a great challenge for containing the problem of azole resistance.

We characterized azole resistance of *A. fumigatus* isolates collected at 2 different soil depths, at the

surface and 20 cm below the surface. Our results showed that the prevalence of ARAF isolates was much higher in topsoil samples than deep soil samples, a finding potentially attributable to different selective pressures at different soil depths. Compared with the azole fungicide detection rates in 20-cm deep soil samples, the detection rates, particularly for 3 fungicides (difenoconazole, tebuconazole, and prochloraz), in topsoil samples were substantially higher. In the 8 topsoil samples harboring the 19 ARAF isolates, we detected ≥ 1 azole fungicide, prochloraz being the most prevalent.

In vitro susceptibility testing showed that the MIC of prochloraz was much higher for *A. fumigatus* TR34/L98H/S297T/F495I isolates than TR34/L98H isolates. This finding is consistent with our previous study (19), which suggested that F495I is needed for high imidazole MICs for TR34/L98H/S297T/F495I isolates.

The primary reason for azole fungicide application is not to prevent *A. fumigatus* growth but to prevent green mold, the most destructive postharvest disease of citrus plants caused by *Penicillium digitatum*. Imidazole is the primary fungicide used to control for this disease in China. Surveillance data have shown that imidazole-resistant *P. digitatum* has been isolated from the provinces of Zhejiang, Hubei, and Jiangxi, and the prevalences in these provinces are >30% (27,28). Alignments of *cyp51* protein sequences have shown that F495I in *cyp51A* of *A. fumigatus* corresponds to F506I in *cyp51B* of *P. digitatum*, suggesting that these 2 pathogens harbor similar resistance mechanisms. Therefore, agricultural use of imidazole fungicides might also contribute to the emergence of azole resistance in *A. fumigatus*.

China produces a substantial number of agricultural products and uses a wide array of fungicides for crop protection (29). The total amount of fungicides used in agriculture in China was ≈80 million kg/year during 2013–2016, and azole fungicides accounted for more than one third of these fungicides. Triazoles (e.g., tebuconazole) and imidazoles (e.g., prochloraz) are 2 of the most commonly used azole fungicide drug classes. The national registry from the Chinese Ministry of Agriculture showed that, within the azole fungicide class, the usage of tebuconazole and prochloraz almost doubled during 2012–2016. Unlike in countries in Europe, where imidazoles are used less often than triazoles, in China, the frequency of use of imidazoles and triazoles are comparable.

The Chinese Ministry of Agriculture previously released a series of policies on pesticide use (the Zero Growth of Pesticide Usage program) to reduce overuse and inappropriate use of pesticides in agriculture, and the goal of this program was achieved in 2016. Reducing the amount of fungicide used on some crops is likely to happen in China in the near future, which will provide us the opportunity to evaluate the effect of agricultural fungicide use on clinical resistance.

The genetic analysis of ARAF isolates from this study and previous studies provided us information about the emergence of azole resistance in *A. fumigatus* in China. First, nearly all ARAF isolates were MAT1–1, except 1 isolate, E2006-0-5, which was a TR46/Y121F/T289A variant, suggesting that these ARAF isolates mainly evolved and disseminated through asexual sporulation. A possible role for sexual reproduction in the emergence of azole resistance was reported in the study of isolates from compost samples containing residual azole fungicide (25). Compost heaps are warm, dark environments

low in oxygen and high in carbon dioxide that promote sexual reproduction and thus genetic recombination; hence, sexual reproduction might also facilitate the emergence of azole resistance. However, mating type has rarely been reported in most studies, so a conclusion on the role of sexual and asexual reproduction in azole resistance cannot be drawn. Second, the ARAF variants that we isolated (which harbored different *cyp51A* mutations) were genetically unrelated to each other, suggesting that these isolates might have evolved from different sources. Third, compared with the major ARAF clone complex of strains disseminated worldwide, the genotypes of the ARAF isolates from Hangzhou were closely related to azole-susceptible *A. fumigatus* isolates (Figure 2); this finding suggests that the isolates from Hangzhou might be new strains evolving under the selective pressure of the azole fungicides used in that environment.

In conclusion, we identified strawberry planting sites as potential hotspots for the development of azole resistance in *A. fumigatus* in China. The 3 most common *cyp51A* variants, namely TR34/L98H, TR34/L98H/S297T/F495I, and TR46/Y121F/T289A, which accounted for nearly 90% of all the ARAF isolates in China, might be regarded as the 3 fitness peaks in the fitness landscape of *A. fumigatus* (30). ARAF isolates with different *cyp51A* mutations can coexist in the same soil sample. Both triazole and imidazole fungicides might provide the selective pressure for the development of azole resistance in *A. fumigatus*. The management of fungicide use in agricultural fields, especially those serving as potential resistance hotspots, such as strawberry fields, is needed to curb the emergence of antifungal drug resistance in clinics.

Acknowledgments

We thank the National Agricultural Technology Extension Service Center, Ministry of Agriculture, China, for providing the data on use of azole fungicides in agriculture in China.

This study was supported by a grant from the Beijing Nova Program (Z181100006218107), National Key Program for Infectious Diseases of China (2018ZX10712001-009, 2018ZX10733-402), National Natural Science Foundation of China (31872004, 81772163), and Beijing Natural Science Foundation (7172157).

About the Author

Dr. Chen is a researcher from the Chinese PLA Center for Disease Control and Prevention, Beijing, China. His primary research interest is the epidemiology of antimicrobial resistance.

References

- Patterson TF, Thompson GR III, Denning DW, Fishman JA, Hadley S, Herbrecht R, et al. Practice guidelines for the diagnosis and management of aspergillosis: 2016 update by the Infectious Diseases Society of America. *Clin Infect Dis*. 2016;63:e1–60. <https://doi.org/10.1093/cid/ciw326>
- Denning DW, Cadranel J, Beigelman-Aubry C, Ader F, Chakrabarti A, Blot S, et al.; European Society for Clinical Microbiology and Infectious Diseases and European Respiratory Society. Chronic pulmonary aspergillosis: rationale and clinical guidelines for diagnosis and management. *Eur Respir J*. 2016;47:45–68. <https://doi.org/10.1183/13993003.00583-2015>
- Garcia-Rubio R, Cuenca-Estrella M, Mellado E. Triazole resistance in *Aspergillus* species: an emerging problem. *Drugs*. 2017;77:599–613. <https://doi.org/10.1007/s40265-017-0714-4>
- Verweij PE, Chowdhary A, Melchers WJ, Meis JF. Azole resistance in *Aspergillus fumigatus*: can we retain the clinical use of mold-active antifungal azoles? *Clin Infect Dis*. 2016;62:362–8. <https://doi.org/10.1093/cid/civ885>
- Snelders E, Camps SM, Karawajczyk A, Schaftenaar G, Kema GH, van der Lee HA, et al. Triazole fungicides can induce cross-resistance to medical triazoles in *Aspergillus fumigatus*. *PLoS One*. 2012;7:e31801. <https://doi.org/10.1371/journal.pone.0031801>
- Berger S, El Chazli Y, Babu AF, Coste AT. Azole resistance in *Aspergillus fumigatus*: a consequence of antifungal use in agriculture? *Front Microbiol*. 2017;8:1024. <https://doi.org/10.3389/fmicb.2017.01024>
- Vermeulen E, Maertens J, De Bel A, Nulens E, Boelens J, Surmont I, et al. Nationwide surveillance of azole resistance in *Aspergillus* diseases. *Antimicrob Agents Chemother*. 2015;59:4569–76. <https://doi.org/10.1128/AAC.00233-15>
- van der Linden JW, Arendrup MC, Warris A, Lagrou K, Pelloux H, Hauser PM, et al. Prospective multicenter international surveillance of azole resistance in *Aspergillus fumigatus*. *Emerg Infect Dis*. 2015;21:1041–4. <https://doi.org/10.3201/eid2106.140717>
- Wiederhold NP, Gil VG, Gutierrez F, Lindner JR, Albataineh MT, McCarthy DJ, et al. First detection of TR34 L98H and TR46 Y121F T289A cyp51 mutations in *Aspergillus fumigatus* isolates in the United States. *J Clin Microbiol*. 2016;54:168–71. <https://doi.org/10.1128/JCM.02478-15>
- Chen Y, Lu Z, Zhao J, Zou Z, Gong Y, Qu F, et al. Epidemiology and molecular characterizations of azole resistance in clinical and environmental *Aspergillus fumigatus* isolates from China. *Antimicrob Agents Chemother*. 2016;60:5878–84. <https://doi.org/10.1128/AAC.01005-16>
- Ren J, Jin X, Zhang Q, Zheng Y, Lin D, Yu Y. Fungicides induced triazole-resistance in *Aspergillus fumigatus* associated with mutations of TR₃₄/Y121F/T289A and its appearance in agricultural fields. *J Hazard Mater*. 2017;326:54–60. <https://doi.org/10.1016/j.jhazmat.2016.12.013>
- Schoustra SE, Debets AJM, Rijs AJMM, Zhang J, Snelders E, Leendertse PC, et al. Environmental hotspots for azole resistance selection of *Aspergillus fumigatus*, the Netherlands. *Emerg Infect Dis*. 2019;25:1347–53. <https://doi.org/10.3201/eid2507.181625>
- Mortensen KL, Mellado E, Lass-Flörl C, Rodriguez-Tudela JL, Johansen HK, Arendrup MC. Environmental study of azole-resistant *Aspergillus fumigatus* and other aspergilli in Austria, Denmark, and Spain. *Antimicrob Agents Chemother*. 2010;54:4545–9. <https://doi.org/10.1128/AAC.00692-10>
- Snelders E, Huis In 't Veld RA, Rijs AJ, Kema GH, Melchers WJ, Verweij PE. Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl Environ Microbiol*. 2009;75:4053–7. <https://doi.org/10.1128/AEM.00231-09>
- Chowdhary A, Sharma C, van den Boom M, Yntema JB, Hagen F, Verweij PE, et al. Multi-azole-resistant *Aspergillus fumigatus* in the environment in Tanzania. *J Antimicrob Chemother*. 2014;69:2979–83. <https://doi.org/10.1093/jac/dku259>
- Balajee SA, Gribskov JL, Hanley E, Nickle D, Marr KA. *Aspergillus lentulus* sp. nov., a new sibling species of *A. fumigatus*. *Eukaryot Cell*. 2005;4:625–32. <https://doi.org/10.1128/EC.4.3.625-632.2005>
- Li R, Chen Z, Tao Y, Pan X, Jiang D, Dong F, et al. Determination of valifenalate in grape, vegetables, and soil using ultrahigh performance liquid chromatography tandem mass spectrometry and exploration of its degradation behavior in grape field. *Food Anal Methods*. 2019;12:742–51. <https://doi.org/10.1007/s12161-018-1399-3>
- Guinea J, Verweij PE, Meletiadis J, Mouton JW, Barchiesi F, Arendrup MC, et al.; Subcommittee on Antifungal Susceptibility Testing of the ESCMID European Committee for Antimicrobial Susceptibility Testing. How to: EUCAST recommendations on the screening procedure E.Def 10.1 for the detection of azole resistance in *Aspergillus fumigatus* isolates using four-well azole-containing agar plates. *Clin Microbiol Infect*. 2019;25:681–7. <https://doi.org/10.1016/j.cmi.2018.09.008>
- Chen Y, Li Z, Han X, Tian S, Zhao J, Chen F, et al. Elevated MIC values of imidazole drugs against *Aspergillus fumigatus* isolates with TR₃₄/L98H/S297T/F495I mutation. *Antimicrob Agents Chemother*. 2018;62:e01549-17. <https://doi.org/10.1128/AAC.01549-17>
- Arendrup MC, Guinea J, Cuenca-Estrella M, Meletiadis J, Mouton JW, Lagrou K, et al. EUCAST definitive document E. Def 9.3: method for the determination of broth dilution minimum inhibitory concentrations of antifungal agents for conidia forming moulds. 2015 Dec [cited 2019 Jun 20]. https://www.aspergillus.org.uk/sites/default/files/pictures/Lab_protocols/EUCAST_E_Def_9_3_Mould_testing_definitive_0.pdf
- Bader O, Weig M, Reichard U, Lugert R, Kuhns M, Christner M, et al.; MykoLabNet-D Partners. *cyp51A*-based mechanisms of *Aspergillus fumigatus* azole drug resistance present in clinical samples from Germany. *Antimicrob Agents Chemother*. 2013;57:3513–7. <https://doi.org/10.1128/AAC.00167-13>
- Garcia-Rubio R, Escribano P, Gomez A, Guinea J, Mellado E. Comparison of two highly discriminatory typing methods to analyze *Aspergillus fumigatus* azole resistance. *Front Microbiol*. 2018;9:1626. <https://doi.org/10.3389/fmicb.2018.01626>
- Hagiwara D, Takahashi H, Watanabe A, Takahashi-Nakaguchi A, Kawamoto S, Kamei K, et al. Whole-genome comparison of *Aspergillus fumigatus* strains serially isolated from patients with aspergillosis. *J Clin Microbiol*. 2014;52:4202–9. <https://doi.org/10.1128/JCM.01105-14>
- Sugui JA, Losada L, Wang W, Varga J, Ngamskulrungron P, Abu-Asab M, et al. Identification and characterization of an *Aspergillus fumigatus* “supermater” pair. *MBio*. 2011;2:e00234-11. <https://doi.org/10.1128/mBio.00234-11>
- Zhang J, Snelders E, Zwaan BJ, Schoustra SE, Meis JF, van Dijk K, et al. A novel environmental azole resistance mutation in *Aspergillus fumigatus* and a possible role of sexual reproduction in its emergence. *MBio*. 2017;8:e00791-17. <https://doi.org/10.1128/mBio.00791-17>

26. Dutch Ministry of Health. Azole resistance selection in *Aspergillus fumigatus* final report: 2015-2017. 2017 Jul [cited 2019 Jun 20]. <https://www.rijksoverheid.nl/binaries/rijksoverheid/documenten/rapporten/2017/12/07/rapport-azole-resistance-selection-in-aspergillus-fumigatus-final-report/Rapport+Azole+resistance+selection+in+aspergillus+fumigatus+final+report.pdf>
27. Sun X, Wang J, Feng D, Ma Z, Li H. *PdCYP51B*, a new putative sterol 14 α -demethylase gene of *Penicillium digitatum* involved in resistance to imazalil and other fungicides inhibiting ergosterol synthesis. *Appl Microbiol Biotechnol*. 2011;91:1107–19. <https://doi.org/10.1007/s00253-011-3355-7>
28. Wang J, Yu J, Liu J, Yuan Y, Li N, He M, et al. Novel mutations in *cyp51B* from *Penicillium digitatum* involved in prochloraz resistance. *J Microbiol*. 2014;52:762–70. <https://doi.org/10.1007/s12275-014-4112-2>
29. Zhang WJ, Jiang FB, Ou JF. Global pesticide consumption and pollution: with China as a focus. *Proc Int Acad Ecol Environ Sci*. 2011;1:125–44.
30. de Visser JA, Krug J. Empirical fitness landscapes and the predictability of evolution. *Nat Rev Genet*. 2014;15:480–90. <https://doi.org/10.1038/nrg3744>

Address for correspondence: Li Han, Chinese PLA Center for Disease Control and Prevention, no. 20 Dongda St, 100071, Beijing, China; email: hanlicdc@163.com; Yongquan Zheng, Chinese Academy of Agricultural Sciences, State Key Laboratory for Biology of Plant Diseases and Insect Pests, Institute of Plant Protection, No. 2 Yuanmingyuan West Rd, Haidian District, Beijing, 100193, China; email: zhengyongquan@ippcaas.cn

etymologia

Picobirnavirus [pi-ko-burr'nə-vi"rəs]

Yashpal S. Malik, Souvik Ghosh

Picobirnavirus, the recently recognized sole genus in the family *Picobirnaviridae*, is a small (*Pico*, Spanish for small), bisegmented (*bi*, Latin for two), double-stranded RNA virus. Picobirnaviruses were initially considered to be birna-like viruses, and the name was derived from birnavirus (bisegmented RNA), but the virions are much smaller (diameter 35 nm vs. 65 nm).

Picobirnaviruses are reported in gastroenteric and respiratory infections. These infections were first described in humans and black-footed pigmy rice rats in 1988. Thereafter, these infections have been reported in feces and intestinal contents from a wide variety of mammals with or without diarrhea, and in birds and reptiles worldwide.

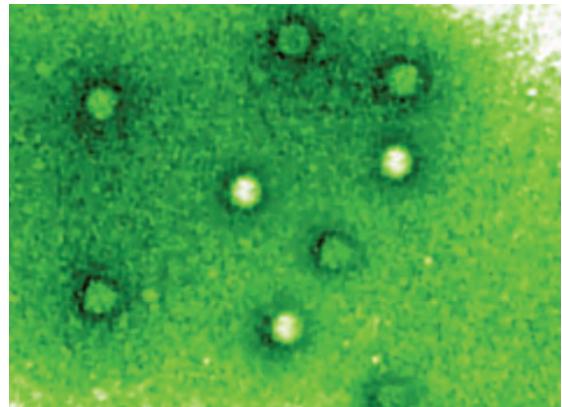


Figure. Picobirnavirus by negative stain electron microscopy, from Wikipedia, <https://en.wikipedia.org/wiki/File:Picobirnavirus.jpg>

Sources

- Delmas B, Attoui H, Ghosh S, Malik YS, Mundt E, Vakharia VN; ICTV Report Consortium. ICTV virus taxonomy profile: Picobirnaviridae. *J Gen Virol*. 2019;100:133–4. <https://doi.org/10.1099/jgv.0.001186>
- Malik YS, Kumar N, Sharma K, Dhama K, Shabbir MZ, Ganesh B, et al. Epidemiology, phylogeny, and evolution of emerging enteric Picobirnaviruses of animal origin and their relationship to human strains. *BioMed Res Int*. 2014;2014:780752. <https://doi.org/10.1155/2014/780752>
- Pereira HG, Flewett TH, Candeias JA, Barth OM. A virus with a bisegmented double-stranded RNA genome in rat (*Oryzomys nigripes*) intestines. *J Gen Virol*. 1988;69:2749–54. <https://doi.org/10.1099/0022-1317-69-11-2749>
- Smits SL, van Leeuwen M, Schapendonk CM, Schürch AC, Bodewes R, Haagmans BL, et al. Picobirnaviruses in the human respiratory tract. *Emerg Infect Dis*. 2012;18:1539–40. <https://doi.org/10.3201/eid1809.120507>

Author affiliations: Indian Veterinary Research Institute, Izatnagar, India (Y.S. Malik); Ross University School of Veterinary Medicine, Basseterre, St. Kitts, West Indies (S. Ghosh)

Address for correspondence: Yashpal S. Malik, Division of Biological Standardization, Indian Veterinary Research Institute, Izatnagar 243122, India; email: malikyps@gmail.com; yashpal.makil@icar.gov.in

Tick-Borne Encephalitis Virus, United Kingdom

Maya Holding, Stuart D. Dowall, Jolyon M. Medlock, Daniel P. Carter, Steven T. Pullan, James Lewis, Richard Vipond, Mara S. Rocchi, Matthew Baylis, Roger Hewson

During February 2018–January 2019, we conducted large-scale surveillance for the presence and prevalence of tick-borne encephalitis virus (TBEV) and louping ill virus (LIV) in sentinel animals and ticks in the United Kingdom. Serum was collected from 1,309 deer culled across England and Scotland. Overall, 4% of samples were ELISA-positive for the TBEV serocomplex. A focus in the Thetford Forest area had the highest proportion (47.7%) of seropositive samples. Ticks collected from culled deer within seropositive regions were tested for viral RNA; 5 of 2,041 ticks tested positive by LIV/TBEV real-time reverse transcription PCR, all from within the Thetford Forest area. From 1 tick, we identified a full-length genomic sequence of TBEV. Thus, using deer as sentinels revealed a potential TBEV focus in the United Kingdom. This detection of TBEV genomic sequence in UK ticks has important public health implications, especially for undiagnosed encephalitis.

The only tickborne flavivirus in the United Kingdom documented to cause disease in vertebrates is louping ill virus (LIV), a virus transmitted by the deer/sheep tick, *Ixodes ricinus* (1). This tick species is

the most abundant and widely distributed tick species in the United Kingdom and a known vector of Lyme borreliosis. LIV is most commonly detected in sheep, cattle, and red grouse and has been reported in Scotland, Wales, and England (primarily Cumbria, Devon, and North Yorkshire) (1). Humans are incidental hosts for LIV, and infection has been reported infrequently; ≈45 clinical cases have been linked to encephalitis during the past 85 years (1,2). However, the short window of acute infection leads to uncertainty about whether suspected cases resulted from LIV infection or some other cause, although serologic analysis to analyze recent exposure through induction of IgM-specific responses, in combination with clinical symptoms, could inform a presumptive diagnosis. Human cases are mostly linked to occupational exposure, particularly in abattoir or farm workers and occasionally in laboratory staff (2). Although the UK Animal and Plant Health Agency holds a database of confirmed diagnoses of LIV in livestock (3,4), the distribution and regional prevalence of LIV has not been fully defined. Records of distribution and regional prevalence are based on voluntary submissions by farmers and veterinarians from symptomatic livestock (1), from which private submissions are not integrated. Serologic analysis has been complicated; some animals received vaccination before its withdrawal.

Tick-borne encephalitis virus (TBEV) is a closely related flavivirus that, although known to be less virulent than LIV for sheep (5), causes a neurologic disease (tick-borne encephalitis [TBE]) after transmission to humans by infected ticks, producing clinical disease in an estimated one third of TBEV infections (6). TBE typically has a biphasic course starting with a prodromal phase with influenza-like symptoms, followed by a symptom-free interval before neurologic disease occurs; neurologic disease ranges from mild meningitis to severe encephalitis with or without myelitis and spinal paralysis (7). Three classic subtypes of TBEV are recognized: European (TBEV-Eu), Siberian, and Far Eastern. Two additional TBEV subtypes have recently

Author affiliations: National Institute for Health Research Health Protection Research Unit in Emerging and Zoonotic Infections, Liverpool, UK (M. Holding, S.D. Dowall, J.M. Medlock, D.P. Carter, R. Vipond, M.S. Rocchi, M. Baylis, R. Hewson); Virology and Pathogenesis Group, National Infection Service, Public Health England, Porton Down, UK (M. Holding, S.D. Dowall, R. Vipond, R. Hewson); Medical Entomology and Zoonoses Ecology, Emergency Response Department, Public Health England, Porton Down (M. Holding, J.M. Medlock); Genomics, National Infection Service, Public Health England, Porton Down (D.P. Carter, S.T. Pullan); Geographic Information Systems, Emergency Response Department, Public Health England, Porton Down (J. Lewis); Virus Surveillance Unit, Moredun Research Institute, Edinburgh, Scotland, UK (M.S. Rocchi); Institute of Infection and Global Health, University of Liverpool, Liverpool (M. Baylis)

DOI: <https://doi.org/10.3201/eid2601.191085>

been proposed: Baikalian subtype and the Himalayan subtype (8). TBEV-Eu is the prevailing subtype in Western Europe where it is primarily transmitted by *I. ricinus* ticks and is maintained within forest and meadow biotypes in endemic foci. In the United Kingdom, TBE is considered an imported disease; opportunities for the virus to become established principally are limited because the UK climate was not thought to support the specific conditions required for enzootic cycles to be established for TBEV to become endemic (9). However, changes in climate have affected the emergence, distribution, and abundance of *I. ricinus* in the United Kingdom (10); thus, the risk for tickborne disease has increased (11). A recent study provided evidence that co-infestation of tick larvae and nymphs occurs in small mammals in UK woodland (12). The increasing range of TBEV in Western Europe was underscored recently when the Netherlands reported its first human case in 2016 (13). Moreover, retrospective serologic screening of deer serum samples and molecular analysis of questing ticks found evidence of TBEV circulation in the Netherlands as far back as 2010 and 2015 (13,14). Given the increasing possibility that TBEV could be circulating in the United Kingdom, Public Health England developed a surveillance program focusing on wild animals and ticks.

In TBEV-endemic areas in continental Europe, the prevalence of TBEV in questing ticks is low, rarely exceeding 1% even in regions where the incidence of human infections is high (15). Therefore, instead of screening ticks directly, we used sentinel animals first to identify serologic evidence of TBEV to highlight sites for focused tick testing by specific TBEV detection using real-time reverse transcription PCR (rRT-PCR). Deer are proven as reliable sentinels for identifying areas where TBEV is present (13,15) because they have a limited home range, are available in large numbers, and are broadly dispersed within the surveillance areas. They also show long-lasting antibody responses after natural exposure to flaviviruses (15,16).

For our study, collectors retrieved blood samples from deer culled in England and Scotland during February 2018–January 2019; when available, they also collected tick samples. We tested the blood samples for TBEV or LIV antibodies and the ticks for the presence of viral RNA by rRT-PCR.

Methods

Sample Collection

We recruited persons involved in routine management of deer from across the United Kingdom to

collect serum and tick samples from any species of deer. This program was promoted through organizations involved in deer management. These deerstalkers submitted 1,323 serum samples (and tick samples where present) from deer culled in England and Scotland during February 2018–January 2019. The University of Liverpool Ethics Committee (ref: VREC596) granted ethics approval for this study on February 1, 2018.

Blood samples were collected in serum-separation vacutainers from the chest cavity during gralloching, and blood-fed ticks were collected from any location on the deer carcass. Samples were centrifuged at 1,500 relative centrifugal force for 10 min and aliquoted. Serum and tick samples were stored at -80°C until further processing.

ELISA Testing

We tested serum samples for antibodies to TBEV using the commercial Immunozyg FSME IgG All Species ELISA (Progen, <https://www.progen.com>) according to the manufacturer's instructions. We read plates at an optical density ratio of 450 nm. We considered samples with a reading of ≥ 127 Vienna units/mL to be seropositive.

Hemagglutination Inhibition Testing

We tested serum samples for antibodies to LIV using a hemagglutination inhibition (HAI) test (17,18). We considered samples with a titer ≥ 20 seropositive. A small number of samples did not have sufficient serum for HAI testing.

Tick Identification and RNA Extraction

We morphologically identified all ticks collected from culled deer within a 15-km radius of any TBEV ELISA-seropositive deer (19) to life stage and species level. We individually homogenized the ticks in 300 μL RLT buffer (QIAGEN, <https://www.qiagen.com>) in MK28-R Precellys homogenizing tubes using a Precellys 24 homogenizer (Bertin, <https://www.bertin-instruments.com>) at 5,500 rpm for 5 sec, followed by a 30-sec break; we repeated this process 4 times. We then added 300 μL of isopropanol and passed the tick homogenate through a QIAshredder (QIAGEN). We extracted total RNA using the BioSprint 96 One-For-All Vet Kit (QIAGEN) and eluted it into 100 μL AVE buffer according to the manufacturer's instructions.

rRT-PCR

We tested individual tick samples for LIV/TBEV RNA using a sensitive LIV/TBEV assay (20). We

amplified RNA in 20 μ L rRT-PCR mix containing 0.8 μ L Invitrogen (<https://www.thermofisher.com>) Superscript III/Platinum Taq Mix, 10 μ L Invitrogen 2X reaction mix, 1.6 μ L 50 mmol/L $MgSO_4$, 1 μ L of 1 μ mol/L forward primer, 1 μ L of 18 μ mol/L reverse primer, 0.2 μ L of 25 μ mol/L probe, 5 μ L template, and 0.4 μ L molecular-grade water.

We also tested all RNA-positive samples using a secondary assay designed to detect only LIV (21). We amplified RNA in 20 μ L rRT-PCR mix containing 0.8 μ L Invitrogen Superscript III/Platinum Taq Mix, 10 μ L Invitrogen 2X reaction mix, 0.8 μ L of 10 μ mol/L forward primer, 1.8 μ L of 10 μ mol/L reverse primer, 1.0 μ L of 5 μ mol/L probe, 5 μ L template, and 0.6 μ L molecular-grade water.

Sequencing and Phylogenetic Analysis

We prepared the tick sample that showed a high level of TBEV RNA for metagenomic RNA sequencing (22) and assembled the sequencing data using SPAdes version 3.1.1 (23). We inferred the evolutionary history by using the maximum-likelihood method based on the Tamura 3-parameter model (24). We used the tree with the highest log likelihood. We automatically obtained initial trees for the heuristic search by applying neighbor-joining and BioNJ (25) algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The analysis involved 10 full-length genomic TBEV nucleotide-

sequences and was performed using Molecular Evolutionary Genetics Analysis version 7.0 software (26).

Results

Deerstalkers submitted a total of 1,323 serum samples, of which 14 samples were excluded from analysis because of insufficient location or deer species information. Serum samples were obtained from 5 deer species and a hybrid of 2 species; 61% of samples submitted were from male deer. The most frequently sampled species were roe deer (*Capreolus capreolus*) (51%), followed by fallow deer (*Dama dama*) (19%). Samples were submitted from across Scotland and England, but distribution and density of samples varied by county (Figure 1, panel C). A limited number of samples were submitted from across the Midlands and parts of Northern England; no samples were submitted from Wales.

Of serum samples from across the United Kingdom, 4% were positive by ELISA, and 5% by HAI. Cohen's κ indicated substantial agreement (0.61) between the methods, indicating ELISA results agreed closely with HAI test results (Table 1). ELISA yielded positive results in all deer species for which it was used. These were 27/663 roe, 10/246 fallow, 9/242 red deer (*Cervus elaphus*), 6/108 muntjac (*Muntiacus reevesi*), 1/48 sika (*Cervus nippon*), and 0/2 red/sika hybrids. HAI determined the following positives: 28/662 roe, 15/245 fallow, 18/242 red, 7/106 muntjac, 0/45 sika, and 1/2 red/sika hybrid.

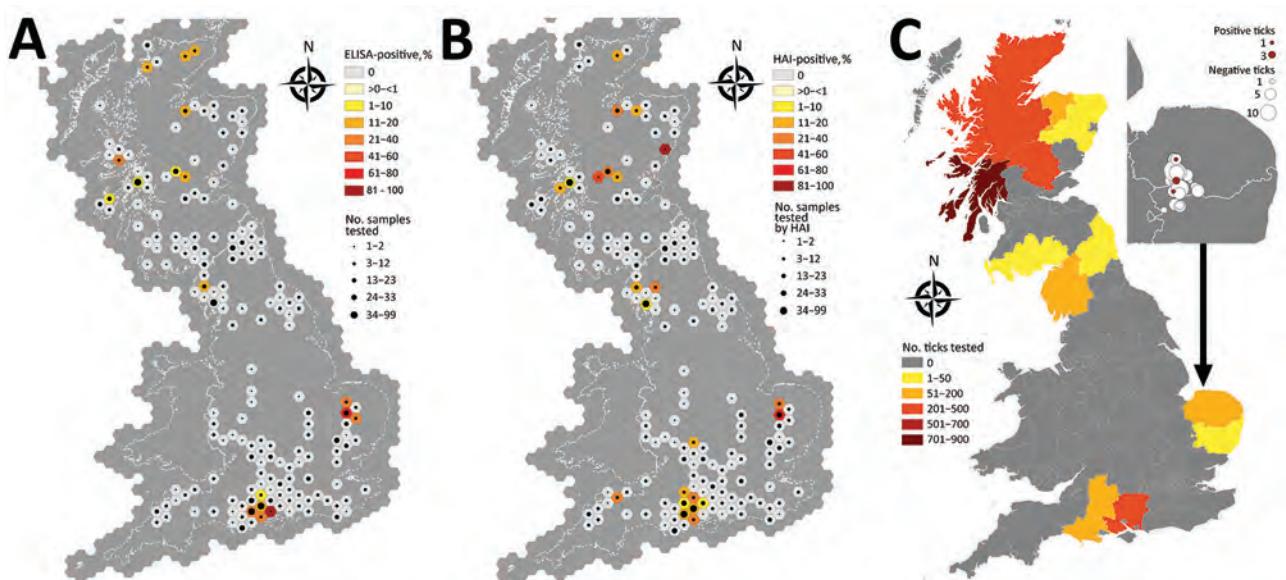


Figure 1. Results for deer serum samples and ticks tested for tick-borne encephalitis virus, United Kingdom. A, B) Number of samples tested and seroprevalence of samples positive by ELISA (A) and HAI (B). C) Number of ticks tested by county; inset shows magnification of testing area with ticks positive by real-time reverse transcription PCR. HAI, hemagglutination inhibition. Source: Ordnance Survey data, © Crown copyright and database right 2019; and National Statistics data, © Crown copyright and database right 2019.

ELISA- and HAI-positive samples were geographically distributed to specific areas (Figure 1, panels A, B); seroprevalence was high in southwestern Norfolk and northwestern Suffolk (Thetford Forest) in the east of England. Norfolk had the highest seroprevalence detected by ELISA (51.4%), followed by Hampshire (14.3%), Suffolk (10.7%), and Scottish Highlands (8.6%) (Table 2).

Of all ticks submitted from deer carcasses, 2,041 collected from 339 deer were from within 15 km of an ELISA-positive result. All ticks were identified as *I. ricinus*; 1,450 were adult females, 585 adult males, and 6 nymphs. Tick availability for testing by area of seropositive foci varied (Figure 1, panel C); most ticks tested were collected from Argyll and Bute, and an average of 6 ticks were tested per deer. Five (4 adult males, 1 adult female) of the 2,041 ticks tested positive by the LIV/TBEV rRT-PCR (20) and were all within the Norfolk/Suffolk focus (Figure 1, panel C). No LIV RNA was detected in these 5 ticks when they were tested by rRT-PCR designed to detect only LIV (21). The 192 ticks tested from within the Norfolk/Suffolk focus resulted in a prevalence of 2.6% in this area.

One tick (male) showed high levels of TBEV RNA (cycle threshold 15.4). Sequencing revealed a full-length TBEV genome designated TBEV-UK (GenBank accession no. MN128700). Phylogenetic analysis illustrates this as a TBEV-Eu subtype; it is most closely related to the Norwegian Mandal strain of TBEV isolated from ticks in 2009 (Figure 2), sharing a 99% sequence identity.

Discussion

The detection of TBEV in the United Kingdom is important because TBEV can infect humans, causing febrile illness and neurologic complications including

Table 1. Variation between ELISA for tick-borne encephalitis virus and HAI for louping ill virus, United Kingdom*

ELISA result	HAI result		Not tested	Total
	Positive	Negative†		
Positive	38	14	1	53
Negative‡	31	1,219	6	1,255
Total	69	1,233	7	1,309

*HAI, hemagglutination inhibition.
 †HAI negative, borderline, unknown.
 ‡ELISA negative/borderline.

encephalitis. This evidence is contrary to earlier predictions based on climate change (9) that did not forecast a spread of TBEV to the United Kingdom. However, in addition to climate change, the spread of the tick vector, TBEV, and associated viruses into new regions can be influenced by a variety of other factors, such as transportation of animals and alterations in land management (27).

Serologic evidence suggests a high prevalence (47.7%) of exposure of deer to flaviviruses, such as TBEV and LIV, in the Norfolk/Suffolk (Thetford Forest) focal area. This seroprevalence is within the upper levels detected in TBEV risk areas of Europe, where seroprevalence studies in deer rarely exceed 50% (15,16,28,29). In addition, the detected prevalence of flavivirus RNA in ticks collected from deer of 2.6% within the Thetford Forest area falls within the range of findings from other studies in mainland Europe that tested blood-fed ticks (15). The deer were culled within a large forest habitat, which aligns closer with ecology required for TBEV, rather than LIV, maintenance (7). Based on these findings, and the evidence that all rRT-PCR-positive results were for TBEV and not LIV, we propose that TBEV is established and is being maintained through enzootic cycles within the Thetford Forest area, rather than resulting from multiple importation events, which is in line with

Table 2. ELISA- and HAI-positive results for tick-borne encephalitis virus from counties from which serum samples were submitted, United Kingdom*

County and country	ELISA		HAI	
	No. positive/no. tested	% Positive (95% CI)†	No. positive/no. tested	% Positive (95% CI)†
Norfolk, England	18/35	51.43 (35.57–67.01)	16/35	45.71 (30.46–61.82)
Hampshire, England	15/105	14.29 (8.74–22.35)	14/104	13.46 (8.07–21.46)
Suffolk, England	3/28	10.71 (2.90–28.01)	2/28	7.14 (0.90–23.73)
Highland, Scotland	7/81	8.64 (3.99–17.04)	8/81	9.88 (4.86–18.53)
Perth and Kinross, Scotland	2/33	6.06 (0.68–20.60)	10/33	30.30 (17.25–47.46)
Dorset, England	2/72	2.78 (0.19–10.15)	0/70	0.00 (0.00–6.23)
Cumbria, England	2/95	2.11 (0.12–7.81)	4/95	4.21 (1.31–10.67)
Argyll and Bute, Scotland	3/158	1.90 (0.40–5.69)	5/158	3.16 (1.16–7.39)
Wiltshire, England	1/56	1.79 (0.00–10.34)	5/55	9.09 (3.53–19.99)
Stirling, Scotland	0/2	0.00 (0.00–70.98)	1/2	50.00 (9.45–90.55)
Somerset, England	0/13	0.00 (0.00–26.59)	1/13	7.69 (0.00–35.42)
Moray, Scotland	0/19	0.00 (0.00–19.79)	1/19	5.26 (0.00–26.48)
Gloucestershire, England	0/24	0.00 (0.00–16.31)	1/24	4.17 (0.00–21.87)
Aberdeenshire, Scotland	0/32	0.00 (0.00–12.73)	1/32	3.13 (0.00–17.11)

*HAI, hemagglutination inhibition.

†95% CIs computed by Agresti Coull method.

findings in many endemic focal areas of TBEV (30). The hypothesis that TBEV infection might be maintained in Thetford Forest is supported by previous work in the United Kingdom that provided evidence of co-feeding between ticks from different life stages on small mammals in a southern English woodland, which is a crucial factor for the maintenance of TBEV (9,12). In addition, our positive serology data support the concept that the virus is circulating nonviremically in local wildlife and by cycling among the co-feeding nymphs, larvae, and adult ticks, through nonviremic transmission. Good evidence shows that TBEV is maintained in other parts of Europe through nonviremic transmission (31).

In other study areas, we detected serologic evidence of flavivirus exposure but not viral RNA in ticks. The close homology between LIV and TBEV presents challenges when serologic methods are used alone because the tests cannot distinguish between

them. Thus, based on such data, confirming which virus is responsible for the seroreactivity in the areas where LIV has previously been reported is not possible. Previous reports of LIV prevalence are limited; just one study showed up to 15.3% of ticks positive for LIV (32). However, other researchers have not confirmed these data, and our results indicate a much lower prevalence. Nevertheless, we did not find any published clinical reports of LIV in Hampshire livestock despite our detected seroprevalence of 14.3% by ELISA (1,3,4). Although additional tick and small mammal ecology studies are needed to build on serologic data, evidence shows the maintenance of TBEV in the identified focal endemic area.

The genomic sequence of TBEV-UK shows close identity to a TBEV-Eu virus isolated in 2009 from questing ticks collected in Norway. This similarity suggests that TBEV-UK might have been brought to the United Kingdom on migratory birds, such as blackbirds (*Turdus merula*) and redwings (*Turdus iliacus*) (33), which are known to transport ticks over wide distances (34–37). The United Kingdom experiences a large influx of migratory birds each autumn from several TBEV-endemic countries in northern Europe, including Norway. During this migration, birds first arrive on the east coast of the United Kingdom, and it is feasible that TBEV-UK could have originated from a tick imported by an autumn migratory bird. We are collecting tick samples from migratory birds to assess the proportion of tick-infested birds arriving in the United Kingdom and testing these imported ticks for TBEV (among other potential pathogens). In addition, because of lower viral RNA levels, we are looking into primer-amplification sequencing approaches to further decipher the virus responsible for the rRT-PCR-positive samples detected from Thetford Forest.

For zoonotic infections, detection of a pathogen in the animal reservoir/host, vector, or both often precedes the emergence of human infection (38). Such was the case in the Netherlands, where deer serum samples, collected 6 years before the first cases in humans (13), demonstrated serologic evidence of TBEV infection (14). Similarly, in Spain, Crimean-Congo hemorrhagic fever virus was first reported in ticks in 2010 (39), before autochthonous infections in humans was identified in 2017 (40). Within the focal TBEV-endemic areas we identified in this study, seroepidemiologic studies should be undertaken, particularly in risk groups that include patients presenting to general practitioners and hospitals with central nervous system symptoms.

Although UK-TBEV has not been linked to human disease, it nevertheless shows close homology

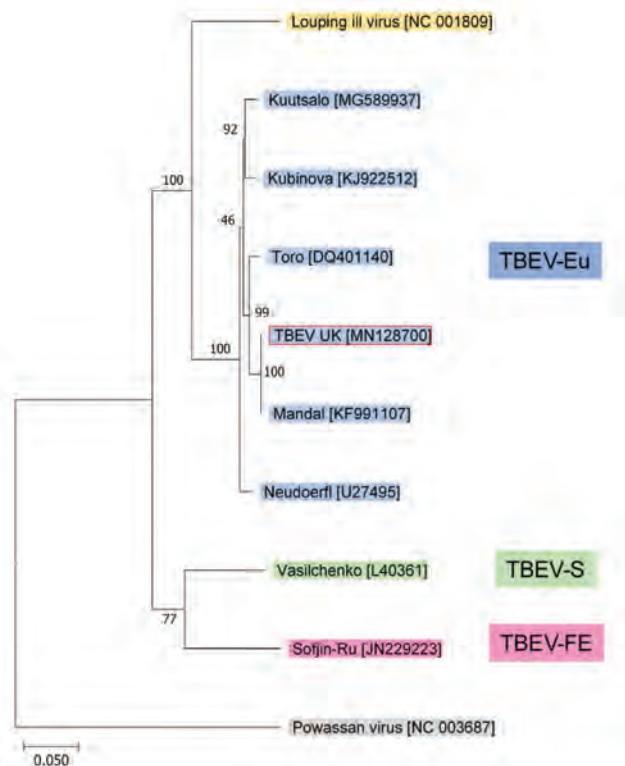


Figure 2. Phylogenetic relationship between TBEV-UK from a tick in the United Kingdom and contemporary strains of TBEV. The tree was constructed with a maximum-likelihood analysis using full-length complete TBEV genomes and is rooted with the tickborne Powassan virus. GenBank accession numbers of each sequence are provided in brackets. TBEV, tick-borne encephalitis virus; TBEV-Eu, TBEV-European; TBEV-FE, TBEV-Far Eastern; TBEV-S, TBEV-Siberian; TBEV-UK, TBEV-United Kingdom.

to pathogenic isolates of TBEV and should be considered to be a potential public health risk. Thus, clinicians in the United Kingdom should consider the European Union case definition of TBE (41) and include TBE in the differential diagnosis of patients with symptoms of meningoencephalitis, especially if they have been exposed to a tick bite, even if they have not traveled recently to a known TBE-endemic country. The European Union case definition specifies clinical criteria as any person presenting with inflammation of the central nervous system. In addition to meeting clinical criteria, laboratory case confirmation requires ≥ 1 of the following 5 criteria to be satisfied: 1) detection of TBEV nucleic acid, 2) viral isolation from clinical specimens, 3) TBEV-specific IgM and IgG in blood, 4) TBEV-specific IgM in cerebrospinal fluid, and 5) seroconversion or 4-fold increase of TBEV-specific antibodies in paired serum samples (41).

Although no autochthonous cases of clinical human disease have been diagnosed in the United Kingdom, up to 60% of encephalitis cases reach no diagnosis (42). Therefore, our results indicate that TBEV should be considered as a potential cause in encephalitis patients, and the wide distribution of the natural vector in the United Kingdom indicates a need for close monitoring and a potential for geographic spread and expanding risk areas.

Acknowledgments

We are grateful to the Forestry Commission, British Deer Society, Deer Initiative, and Barry Dowsett for their support and promotion of this study. We thank all of the participating deerstalkers for their support in collecting samples for this study; Dylan Turnbull and Ellie Laming for performing HAI testing; Gillian Slack, John Chamberlain, Emma Kennedy, and Victoria Graham for their help processing samples; Penny Roberts for producing study packs; Hein Sprong for his advice in setting up the study; and Nick Johnson for providing control serum for assay testing.

This research was funded by the Virology & Pathogenesis Group's Arbovirus Programme, Public Health England, and by the National Institute for Health Research Health Protection Research Unit in Emerging and Zoonotic Infections at the University of Liverpool in partnership with Public Health England, in collaboration with Liverpool School of Tropical Medicine. M.H. is funded by the National Institute of Health Research Health Protection Research Unit in Emerging and Zoonotic Infections. M.S.R. is funded by the Scottish Government.

About the Author

Ms. Holding is a PhD candidate at Public Health England. Her primary research interests are the ecology and epidemiology of vectorborne pathogens.

References

1. Jeffries CL, Mansfield KL, Phipps LP, Wakeley PR, Mearns R, Schock A, et al. Louping ill virus: an endemic tick-borne disease of Great Britain. *J Gen Virol*. 2014;95:1005–14. <http://dx.doi.org/10.1099/vir.0.062356-0>
2. Davidson MM, Williams H, Macleod JAJ. Louping ill in man: a forgotten disease. *J Infect*. 1991;23:241–9. [http://dx.doi.org/10.1016/0163-4453\(91\)92756-U](http://dx.doi.org/10.1016/0163-4453(91)92756-U)
3. Animal & Plant Health Agency; Scotland's Rural College. GB sheep disease surveillance dashboard [cited 2019 May 20]. https://public.tableau.com/profile/siu.apha#!/vizhome/SheepDashboard_/Overview
4. Animal & Plant Health Agency; Scotland's Rural College. GB cattle disease surveillance dashboard [cited 2019 May 20]. <https://public.tableau.com/profile/siu.apha#!/vizhome/CattleDashboard/Overview>
5. Gritsun TS, Nuttall PA, Gould EA. Tick-borne flaviviruses. *Adv Virus Res*. 2003;61:317–71. [http://dx.doi.org/10.1016/S0065-3527\(03\)61008-0](http://dx.doi.org/10.1016/S0065-3527(03)61008-0)
6. European Centre for Disease Prevention and Control. Epidemiological situation of tick-borne encephalitis in the European Union and European Free Trade Association countries. ECDC Technical Report. 2012 [cited 2019 Jun 6]. <https://www.ecdc.europa.eu/sites/portal/files/media/en/publications/Publications/TBE-in-EU-EFTA.pdf>
7. Lindquist L, Vapalahti O. Tick-borne encephalitis. *Lancet*. 2008;371:1861–71. [http://dx.doi.org/10.1016/S0140-6736\(08\)60800-4](http://dx.doi.org/10.1016/S0140-6736(08)60800-4)
8. Dai X, Shang G, Lu S, Yang J, Xu J. A new subtype of eastern tick-borne encephalitis virus discovered in Qinghai-Tibet Plateau, China. *Emerg Microbes Infect*. 2018;7:74. <http://dx.doi.org/10.1038/s41426-018-0081-6>
9. Randolph SE, Rogers DJ. Fragile transmission cycles of tick-borne encephalitis virus may be disrupted by predicted climate change. *Proc Biol Sci*. 2000;267:1741–4. <http://dx.doi.org/10.1098/rspb.2000.1204>
10. Medlock JM, Hansford KM, Vaux AGC, Cull B, Gillingham E, Leach S. Assessment of the public health threats posed by vector-borne disease in the United Kingdom (UK). *Int J Environ Res Public Health*. 2018;15:2145. <http://dx.doi.org/10.3390/ijerph15102145>
11. Medlock JM, Leach SA. Effect of climate change on vector-borne disease risk in the UK. *Lancet Infect Dis*. 2015;15:721–30. [http://dx.doi.org/10.1016/S1473-3099\(15\)70091-5](http://dx.doi.org/10.1016/S1473-3099(15)70091-5)
12. Cull B, Vaux AGC, Ottowell LJ, Gillingham EL, Medlock JM. Tick infestation of small mammals in an English woodland. *J Vector Ecol*. 2017;42:74–83. <http://dx.doi.org/10.1111/jvec.12241>
13. Jahfari S, de Vries A, Rijks JM, Van Gucht S, Vennema H, Sprong H, et al. Tick-borne encephalitis virus in ticks and roe deer, the Netherlands. *Emerg Infect Dis*. 2017;23:1028–30. <http://dx.doi.org/10.3201/eid2306.161247>
14. de Graaf JA, Reimerink JH, Voorn GP, Bij de Vaate EA, de Vries A, Rockx B, et al. First human case of tick-borne encephalitis virus infection acquired in the Netherlands, July 2016. *Euro Surveill*. 2016;21:30318. <http://dx.doi.org/10.2807/1560-7917.ES.2016.21.33.30318>
15. Imhoff M, Hagedorn P, Schulze Y, Hellenbrand W, Pfeffer M, Niedrig M. Review: Sentinels of tick-borne

- encephalitis risk. *Ticks Tick Borne Dis.* 2015;6:592–600. <http://dx.doi.org/10.1016/j.ttbdis.2015.05.001>
16. Kiffner C, Vor T, Hagedorn P, Niedrig M, Rühle F. Determinants of tick-borne encephalitis virus antibody presence in roe deer (*Capreolus capreolus*) sera. *Med Vet Entomol.* 2012;26:18–25. <http://dx.doi.org/10.1111/j.1365-2915.2011.00961.x>
 17. Ytrehus B, Vainio K, Dudman SG, Gilray J, Willoughby K. Tick-borne encephalitis virus and louping-ill virus may co-circulate in Southern Norway. *Vector Borne Zoonotic Dis.* 2013;13:762–8. <http://dx.doi.org/10.1089/vbz.2012.1023>
 18. Clarke DH, Casals J. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. *Am J Trop Med Hyg.* 1958;7:561–73. <http://dx.doi.org/10.4269/ajtmh.1958.7.561>
 19. Hillyard PD. *Ticks of north-west Europe.* Shrewsbury (UK): Field Studies Council; 1996.
 20. Schwaiger M, Cassinotti P. Development of a quantitative real-time RT-PCR assay with internal control for the laboratory detection of tick borne encephalitis virus (TBEV) RNA. *J Clin Virol.* 2003;27:136–45. [http://dx.doi.org/10.1016/S1386-6532\(02\)00168-3](http://dx.doi.org/10.1016/S1386-6532(02)00168-3)
 21. Marriott L, Willoughby K, Chianini F, Dagleish MP, Scholes S, Robinson AC, et al. Detection of Louping ill virus in clinical specimens from mammals and birds using TaqMan RT-PCR. *J Virol Methods.* 2006;137:21–8. <http://dx.doi.org/10.1016/j.jviromet.2006.05.025>
 22. Kafetzopoulou LE, Efthymiadis K, Lewandowski K, Crook A, Carter D, Osborne J, et al. Assessment of metagenomic Nanopore and Illumina sequencing for recovering whole genome sequences of chikungunya and dengue viruses directly from clinical samples. *Euro Surveill.* 2018;23:1800228. <http://dx.doi.org/10.2807/1560-7917.ES.2018.23.50.1800228>
 23. Nurk S, Bankevich A, Antipov D, Gurevich A, Korobeynikov A, Lapidus A, et al. Assembling genomes and mini-metagenomes from highly chimeric reads. In: Deng M, Jiang R, Sun F, Zhang X, editors. *Research in computational molecular biology.* RECOMB 2013. Lecture notes in computer science, vol 7821. Berlin: Springer; 2013. p. 158–70.
 24. Tamura K. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C-content biases. *Mol Biol Evol.* 1992;9:678–87.
 25. Gascuel O. BIONJ: an improved version of the NJ algorithm based on a simple model of sequence data. *Mol Biol Evol.* 1997;14:685–95. <http://dx.doi.org/10.1093/oxfordjournals.molbev.a025808>
 26. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 2016;33:1870–4. <http://dx.doi.org/10.1093/molbev/msw054>
 27. Gould EA, Higgs S, Buckley A, Gritsun TS. Potential arbovirus emergence and implications for the United Kingdom. *Emerg Infect Dis.* 2006;12:549–55. <http://dx.doi.org/10.3201/eid1204.051010>
 28. Duscher GG, Wetscher M, Baumgartner R, Walder G. Roe deer sera used for TBE surveillance in Austria. *Ticks Tick Borne Dis.* 2015;6:489–93. <http://dx.doi.org/10.1016/j.ttbdis.2015.03.018>
 29. Tavernier P, Sys SU, De Clercq K, De Leeuw I, Caij AB, De Baere M, et al. Serologic screening for 13 infectious agents in roe deer (*Capreolus capreolus*) in Flanders. *Infect Ecol Epidemiol.* 2015;5:29862. <http://dx.doi.org/10.3402/iee.v5.29862>
 30. Burton A. Tick-borne encephalitis emerges in Norway. *Lancet Infect Dis.* 2004;4:189. [http://dx.doi.org/10.1016/S1473-3099\(04\)00985-5](http://dx.doi.org/10.1016/S1473-3099(04)00985-5)
 31. Labuda M, Jones LD, Williams T, Danielova V, Nuttall PA. Efficient transmission of tick-borne encephalitis virus between co-feeding ticks. *J Med Entomol.* 1993;30:295–9. <http://dx.doi.org/10.1093/jmedent/30.1.295>
 32. Watts EJ, Palmer SCF, Bowman AS, Irvine RJ, Smith A, Travis MJ. The effect of host movement on viral transmission dynamics in a vector-borne disease system. *Parasitology.* 2009;136:1221–34. <http://dx.doi.org/10.1017/S0031182009990424>
 33. Sparagano O, George D, Giangaspero A, Špitalská E. Arthropods and associated arthropod-borne diseases transmitted by migrating birds. The case of ticks and tick-borne pathogens. *Vet Parasitol.* 2015;213:61–6. <http://dx.doi.org/10.1016/j.vetpar.2015.08.028>
 34. Waldenström J, Lundkvist A, Falk KI, Garpmo U, Bergström S, Lindegren G, et al. Migrating birds and tickborne encephalitis virus. *Emerg Infect Dis.* 2007;13:1215–8. <http://dx.doi.org/10.3201/eid1308.061416>
 35. Geller J, Nazarova L, Katargina O, Leivits A, Järvekülg L, Golovljova I. Tick-borne pathogens in ticks feeding on migratory passerines in Western part of Estonia. *Vector Borne Zoonotic Dis.* 2013;13:443–8. <http://dx.doi.org/10.1089/vbz.2012.1054>
 36. Hasle G. Transport of ixodid ticks and tick-borne pathogens by migratory birds. *Front Cell Infect Microbiol.* 2013;3:48. <http://dx.doi.org/10.3389/fcimb.2013.00048>
 37. Klaus C, Gethmann J, Hoffmann B, Ziegler U, Heller M, Beer M. Tick infestation in birds and prevalence of pathogens in ticks collected from different places in Germany. *Parasitol Res.* 2016;115:2729–40. <http://dx.doi.org/10.1007/s00436-016-5022-5>
 38. Randolph SE, Sumilo D. Tick-borne encephalitis in Europe: dynamics of changing risk. In: Takken W, Knols BGJ, editors. *Emerging pests and vector-borne diseases in Europe.* Wageningen (the Netherlands): Wageningen Academic Publishers; 2007. p. 187–206.
 39. Estrada-Peña A, Palomar AM, Santibáñez P, Sánchez N, Habela MA, Portillo A, et al. Crimean-Congo hemorrhagic fever virus in ticks, southwestern Europe, 2010. *Emerg Infect Dis.* 2012;18:179–80. <http://dx.doi.org/10.3201/eid1801.111040>
 40. Negrodo A, de la Calle-Prieto F, Palencia-Herrejón E, Mora-Rillo M, Astray-Mochales J, Sánchez-Seco MP, et al.; Crimean Congo Hemorrhagic Fever@Madrid Working Group. Autochthonous Crimean-Congo Hemorrhagic Fever in Spain. *N Engl J Med.* 2017;377:154–61. <http://dx.doi.org/10.1056/NEJMoa1615162>
 41. European Commission. Commission implementing decision of 8 August 2012 amending Decision 2002/253/EC laying down case definitions for reporting communicable diseases to the Community network under Decision No 2119/98/EC of the European Parliament and of the Council. *Official Journal of the European Union.* 2012;55(L 262/1):1–57.
 42. Kennedy PGE, Quan P-L, Lipkin WI. Viral encephalitis of unknown cause: current perspective and recent advances. *Viruses.* 2017;9:138. <http://dx.doi.org/10.3390/v9060138>

Address for correspondence: Maya Holding, Public Health England, Virology and Pathogenesis, Manor Farm Road, Porton Down, Salisbury, Wiltshire SP4 0JG, UK; email: maya.holding@phe.gov.uk

Phenotypic and Genotypic Correlates of Penicillin Susceptibility in Nontoxigenic *Corynebacterium diphtheriae*, British Columbia, Canada, 2015–2018

Jason Zou, Samuel D. Chorlton, Marc G. Romney, Michael Payne, Tanya Lawson, Anna Wong, Sylvie Champagne, Gordon Ritchie, Christopher F. Lowe

In 2015, the Clinical and Laboratory Standards Institute (CLSI) updated its breakpoints for penicillin susceptibility in *Corynebacterium* species from ≤ 1 mg/L to ≤ 0.12 mg/L. We assessed the effect of this change on *C. diphtheriae* susceptibility reported at an inner city, tertiary care center in Vancouver, British Columbia, Canada, during 2015–2018 and performed whole-genome sequencing to investigate phenotypic and genotypic resistance to penicillin. We identified 44/45 isolates that were intermediately susceptible to penicillin by the 2015 breakpoint, despite meeting previous CLSI criteria for susceptibility. Sequencing did not reveal β -lactam resistance genes. Multilocus sequence typing revealed a notable predominance of sequence type 76. Overall, we saw no evidence of penicillin nonsusceptibility at the phenotypic or genotypic level in *C. diphtheriae* isolates from our institution. The 2015 CLSI breakpoint change could cause misclassification of penicillin susceptibility in *C. diphtheriae* isolates, potentially leading to suboptimal antimicrobial treatment selection.

Since the introduction of the diphtheria toxoid vaccine, cases of diphtheria caused by toxigenic strains of *Corynebacterium diphtheriae* have decreased (1). More recently, however, *C. diphtheriae* appears to have reemerged, with outbreaks of diphtheria

occurring globally and with increasing frequency. In the 1990s, states in the former Soviet Union experienced several epidemics (2). Since 2010, outbreaks have been described almost yearly and span the globe, including South America (3,4), Southeast Asia (5), South Africa (6,7), and Europe (8,9). Data from national surveillance programs, such as one in Latvia, have shown that diphtheria incidence can increase despite adequate vaccination programs (10). Furthermore, serologic studies performed in Europe show that waning or inadequate immunity to diphtheria is becoming more common, indicating populations increasingly are susceptible to diphtheria re-emergence (11,12).

Those living in impoverished, urban settings, even in developed countries, appear to be especially susceptible to *C. diphtheriae* infection. Nontoxigenic strains have been shown to have epidemic potential, causing infections in persons afflicted by homelessness, alcohol abuse, and injection drug use (9,13–15). Nontoxigenic strains of *C. diphtheriae*, against which the toxoid vaccine does not provide immunity, are being reported with greater frequency as a source of severe disease, both in the form of cutaneous diphtheria and more invasive infections, such as bacteremia and endocarditis (14–21). In addition, nontoxigenic strains have the potential to become toxigenic through exposure to corynebacteriophages carrying the toxin gene, particularly through contact with toxin-producing strains carried by travelers returning from diphtheria-endemic countries (22). Use of available, effective, and well-tolerated antimicrobial drugs

Author affiliations: St. Paul's Hospital, Vancouver, British Columbia, Canada (J. Zou, M.G. Romney, M. Payne, T. Lawson, A. Wong, S. Champagne, G. Ritchie, C.F. Lowe); University of British Columbia, Vancouver (S.D. Chorlton, M.G. Romney, M. Payne, S. Champagne, G. Ritchie, C.F. Lowe)

DOI: <https://doi.org/10.3201/eid2601.191241>

targeted to *C. diphtheriae* infection can counter the growing threat.

Currently, penicillin and erythromycin are considered first-line antimicrobial drugs for diphtheria treatment (23). Since 2010, a limited number of case reports from Canada, the United States, and the United Kingdom have described *C. diphtheriae* isolates resistant to penicillin and other conventional antimicrobial drugs (24–26). In 2015, the Clinical and Laboratory Standards Institute (CLSI) lowered the penicillin-susceptible breakpoint for *C. diphtheriae* from a MIC of ≤ 1 mg/L to ≤ 0.12 mg/L, citing expert opinions and discordance with breakpoints determined by the European Committee on Antimicrobial Susceptibility Testing as reasons for the change (27,28). With the change, many *C. diphtheriae* isolates previously considered penicillin-susceptible are now classified as penicillin-intermediate (i.e., intermediately susceptible to penicillin). Consequently, clinicians might opt for alternative antimicrobial drug regimens, such as clindamycin, vancomycin, or erythromycin, the alternative first-line agent. However, these drugs are limited by gastrointestinal side effects, increased risk for *C. difficile* infection, and unnecessary broad-spectrum antimicrobial exposure.

No published reports have demonstrated whether the reclassification of some *C. diphtheriae* isolates to penicillin-intermediate truly reflects an increasing prevalence of penicillin resistance at the phenotypic and genotypic levels. Limited evidence of penicillin-resistant *C. diphtheriae* infections have been reported in cases in which failure of initial penicillin therapy necessitated a change to broad-spectrum antimicrobial drugs before the patients' clinical signs and symptoms improved (24,26). The absence of large-scale susceptibility testing leaves a scarcity of data. One available study reviewed susceptibility testing performed on ≈ 200 *C. diphtheriae* isolates collected from various provincial reference laboratories across Canada during 2006–2015 and found 100% of isolates tested were susceptible to penicillin, as defined by a MIC of ≤ 1 mg/L (29).

C. diphtheriae is a reemerging pathogen of public health concern and penicillin breakpoint changes could have implications for clinical treatment. We assessed the evolving trends in *C. diphtheriae* antimicrobial nonsusceptibility at the phenotypic and genotypic levels by performing susceptibility testing and whole-genome sequencing (WGS) analysis on isolates collected in Vancouver, British Columbia, Canada, during 2015–2018.

Materials and Methods

Collection of *C. diphtheriae* isolates

We isolated *C. diphtheriae* from blood, throat, and wound cultures collected from inpatients and outpatients at St. Paul's Hospital, an inner city, tertiary care center in Vancouver, during March 2015–September 2018. We included all unique *C. diphtheriae* isolates. For patients with multiple *C. diphtheriae* isolates during the study period, we only included the first isolate. One patient in the study had 2 isolates recovered the same day from blood and wound cultures; we only obtained MICs from the blood isolate, but we performed WGS on the wound isolate, assuming the 2 isolates would represent the same strain. We confirmed *C. diphtheriae* isolates by using methods described previously (14). The National Microbiology Laboratory of the Public Health Agency of Canada confirmed all isolates were nontoxigenic by using a modified Elek test and PCR. We obtained ethics approval for this study from the University of British Columbia–Providence Health Care Research Ethics Board.

Antimicrobial Susceptibility Testing and Interpretation

We performed antimicrobial susceptibility testing for penicillin, erythromycin, clindamycin, and vancomycin by using Etest (bioMérieux, <https://www.biomerieux.com>). We interpreted results by using breakpoints from the second edition of the CLSI M45 guidelines (30) published in 2010 and from the third edition (27) published in 2015.

WGS, Multilocus Sequence Type, and Antimicrobial Resistance Marker Analysis

We stored *C. diphtheriae* isolates in trypticase soy broth with 13.8% glycerol at -70°C . For WGS, we thawed and subcultured the isolates onto 5% sheep blood agar plates. We incubated single isolates in Mueller-Hinton broth at 37°C for 48 h. After incubation, we resuspended the cultures in phosphate buffered saline with 1% sodium dodecyl sulfate and 0.25 mg/mL proteinase K and incubated them overnight at 55°C before heating to 95°C for 15 min and bead lysing on the TissueLyser LT (QIAGEN, <https://www.qiagen.com>) at a setting of 50 for 2 min. We performed DNA extraction on the MagNA Pure Compact (Roche Diagnostics, <https://www.roche.com>) and eluted in 50 μL elution buffer.

We used the KAPA HyperPlus Kit with KAPA dual-indexed adapters (Roche Sequencing, <https://sequencing.roche.com>) for WGS. We assessed DNA library quality by using Agilent High Sensitivity DNA

Chips on the Bioanalyzer 2000 (Agilent Technologies, <https://www.agilent.com>). After normalization of the samples, we sequenced DNA on the MiSeq (Illumina, <https://www.illumina.com>) platform using MiSeq Reagent 2x300 V3 Kit (Illumina). We submitted processing codes to GitHub (https://github.com/schrlton/cdip_sequencing). We preprocessed reads to remove low-quality and contaminated sequences, and then performed de novo assembly. We identified multilocus sequence types (STs) through analysis of assembled scaffolds by using the Center for Genomic Epidemiology database version 2.0.0 and multilocus sequence type (MLST) tool 2.0.1 (<https://www.genomicepidemiology.org>). We identified antimicrobial nonsusceptibility by using the Resistance Gene Identifier in the CARD database version 3.0.2 (31,32; <http://card.mcmaster.ca>) to locate individual markers of nonsusceptibility.

Results

Isolate Characteristics

We identified 60 nontoxicogenic *C. diphtheriae* isolates during the study period, 1 from a blood culture, 1 from a throat culture, and 58 from wound cultures. We identified 4 isolates in 2015, 12 in 2016, 26 in 2017, and 18 in 2018. We conducted WGS on 56/60 (93.3%) isolates and obtained MICs from susceptibility testing for 45/60 (75%) isolates.

Antimicrobial Susceptibility Testing

We obtained MICs by susceptibility testing for penicillin, erythromycin, clindamycin, and vancomycin (Figure). The MIC required for 50% growth inhibition for penicillin was 0.25 mg/L and for 90% growth inhibition was 0.38 mg/L. Using the 2010 edition of the CLSI breakpoints, all isolates were penicillin-susceptible. Using the 2015 edition of CLSI breakpoints, all isolates, except 1 with a MIC of 0.125 mg/L, were nonsusceptible (Figure, panel A). The distribution of MICs for penicillin did not change greatly over the study period. One isolate was resistant to erythromycin and clindamycin with a MIC of >256 mg/L for both agents (Figure, panel B). All isolates tested were susceptible to vancomycin (Figure, panel B). Interpretations for erythromycin, clindamycin, and vancomycin testing were unchanged between the 2010 and 2015 CLSI M45 breakpoints.

WGS Analysis, MLST, and Genotypic Correlates of Resistance

MLST typing revealed ST76 was the predominant strain in our study, in 52/56 isolates. We also noted 1

each of ST5, ST32, ST319, and 1 novel ST most similar to ST441/442/444.

We sequenced 56 isolates to investigate resistance markers, yielding a median of 0.4 million (interquartile range [IQR] 0.3–1.05 million) paired-end reads per sample and an estimated median coverage of

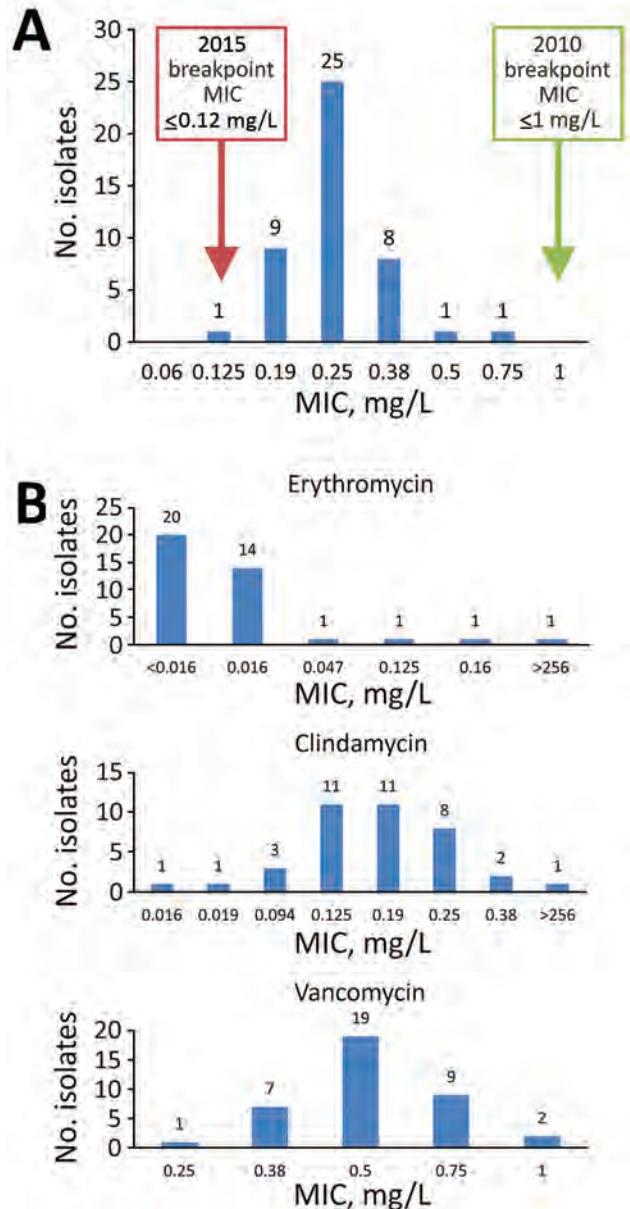


Figure. Distribution of MICs from antimicrobial susceptibility testing on *Corynebacterium diphtheriae* isolates collected at St. Paul’s Hospital, Vancouver, British Columbia, Canada during March 2015–September 2018. A) MICs from penicillin susceptibility testing. Green box indicates penicillin-susceptible breakpoints from the 2010 Clinical Laboratory Standards Institute (CLSI) M45 guidelines (30); red box indicates penicillin-susceptible breakpoints from the 2015 CLSI M45 guidelines (27). B) MICs for erythromycin, clindamycin, and vancomycin susceptibility testing.

100' (IQR 75–263') per isolate. Of the 56 samples sequenced, 27 had a mean read quality >30 before trimming; all samples had a mean read quality >24. We successfully assembled genomes for 45 study isolates, recovering a median of 59 contigs (IQR 55–62 contigs) and 2.388 Mbp (IQR 2.387–2.389 Mbp). We recovered 50% of each assembly length with contigs of 158.8 kbp (IQR 158.8–180.3 kbp) or longer. An additional 11 isolates had incomplete or fragmented assemblies, a median of 530 contigs (IQR 190–1,302 contigs), a length of 2.38 Mbp (IQR 2.086–2.398 Mbp), and an N50 (the length of the smallest contig among the set of the largest contigs that together cover >50% of the assembly) of 7.1 kbp (IQR 1.8–27.7 kbp).

In the fully assembled genomes, 39/45 isolates had the *sul1* gene, conferring sulfonamide resistance, 1 isolate had *tetO*, conferring tetracycline resistance, and 1 isolate had a plasmid harboring *ermX*, conferring macrolide and lincosamide resistance. The isolate carrying *ermX* exhibited phenotypic resistance to erythromycin and clindamycin during susceptibility testing with MIC of >256 mg/L (Figure, panel B). We detected *sul1* in 7/11 incomplete assemblies. None of the isolates we tested contained single-nucleotide variants in the CARD database, nor any markers of β -lactam resistance.

Discussion

Reports of *C. diphtheriae* outbreaks are becoming increasingly common. In particular, nontoxigenic strains pose a major threat to public health because they are not targeted by the current diphtheria toxoid vaccine and can cause invasive infections. Most (44/45) *C. diphtheriae* isolates collected at our institution during 2015–2018 were reported as penicillin-intermediate in accordance with the updated 2015 CLSI M45 breakpoints. However, this reclassification of susceptibility does not appear to be supported by evidence of resistance to penicillin at the phenotypic or genotypic level, at least within isolates identified from our institution's inner-city catchment area. In addition, the distribution of MICs for penicillin does not appear to have changed substantially over the 4-year study period.

Antimicrobial susceptibility testing revealed 2.2% of isolates in our study were erythromycin-resistant, but none were penicillin-resistant. These results are similar to those from a 2015 study in Canada by Bernard et al. (29) in which 32/195 (16.4%) isolates were erythromycin-resistant (MIC of >2 mg/L) by broth microdilution susceptibility testing, but none were penicillin-intermediate or penicillin-resistant (MIC of >1 mg/L).

Misclassification of penicillin susceptibility could have clinical implications. A preference for erythromycin over penicillin as a first-line therapy for *C. diphtheriae* infection could increase rates of inappropriate treatment because of greater rates of erythromycin resistance observed to date. Another disadvantage of using erythromycin for treating patients with cutaneous diphtheria is that wound cultures positive for *C. diphtheriae* often are concurrently positive for group A *Streptococcus*, for which penicillin is the optimal antimicrobial agent (13).

Maintaining effective antimicrobial options is essential to curtailing future outbreaks. The change in the breakpoint for penicillin susceptibility published in the CLSI M45 third edition in 2015 (27) could affect treatment decisions by clinicians. Clinical outcomes are unclear for patients with *C. diphtheriae* infection with MICs in the 0.12–1 mg/L range. A 2011 case report in Canada described a multidrug resistant *C. diphtheriae* isolate harboring the *ermX* gene and exhibiting resistance to erythromycin, clindamycin, and sulfonamide (25). The isolate had an MIC of penicillin of 0.25 mg/L and the patient ultimately was treated successfully with cephalexin (25).

Cases of penicillin treatment failure have been described in other reports. A case of *C. diphtheriae* endocarditis (MIC of >16 mg/L) was reported in a child who was refractory to initial therapy with penicillin G and whose condition did not improve until antimicrobial treatment was changed to meropenem and vancomycin (24). FitzGerald et al. (26) reported another case of penicillin G treatment failure in a child in the United Kingdom with cutaneous diphtheria. The patient's isolate was later found to be nonsusceptible to penicillin, but the MIC was not reported. The patient recovered shortly after a macrolide was administered, but the treatment caused intense gastrointestinal side effects (26).

Overall, our study and other reviews of *C. diphtheriae* susceptibility performed in Canada and globally suggest that elevated MICs of penicillin are rare (29,33). Despite clinical observation of isolated cases of penicillin resistance, one could argue that prematurely opting for erythromycin as first-line therapy for *C. diphtheriae* infection poses a greater risk than penicillin, given the aforementioned concerns of erythromycin resistance and potential for increased adverse effects.

Of note, WGS did not identify genetic markers of β -lactam resistance in the study isolates. However, WGS did detect *ermX* in the isolate phenotypically resistant to erythromycin; *ermX* is a gene encoding a 23S rRNA adenine N-6-methyltransferase known to

confer resistance to erythromycin and clindamycin in *C. diphtheriae* (25,34,35). Although the specific goal of sequencing was to detect β -lactam resistance mechanisms, WGS afforded the ability to identify other potential markers of antimicrobial resistance, namely *sul1* and *tet*. The *sul1* gene is known to encode dihydropteroate synthase, which can confer resistance to sulfonamides and the *tet* gene encodes a ribosomal protection protein that can mediate tetracycline resistance (36). A case of both *sul1* and *tet* genes in a non-diphtheria corynebacterial infection with corresponding elevated MICs of 32 mg/L was reported but has yet to be associated with *C. diphtheriae* (37).

Our laboratory does not routinely perform antimicrobial susceptibility testing for sulfonamides and tetracyclines because neither is recommended routinely for *C. diphtheriae* infections. The clinical relevance of detecting *sul1* and *tet* genes is unclear and requires further study. Additional susceptibility testing is needed to establish whether the study isolates carrying *sul1* and *tetO* exhibited corresponding phenotypic resistance to sulfonamides and tetracyclines.

ST76 was predominant in our study, exhibited in 93% of isolates. Further analysis confirmed that these isolates represented a single clonal strain (38), representing further clonal expansion compared with a previous study of *C. diphtheriae* isolates identified in Vancouver during 1998–2007. ST76 also was the dominant sequence type in that review but encompassed only 69% of isolates (13). ST76 has been reported elsewhere. An instance in an online MLST database, PubMLST.org (<https://pubmlst.org/cdiphtheriae>), lists 9 ST76 isolates submitted from St. Petersburg, Russia, during 2005–2010. All were nontoxicogenic by Elek test. In another report, 5 isolates from Belarus collected during 2004–2014 also were typed as ST76 and found to be nontoxicogenic (39). No epidemiologic links are apparent between our isolates and those described from Russia or Belarus.

We also noted ST5 and ST32 in our review from downtown Vancouver during 1998–2007 (13), although less frequently observed in our study. ST5 previously was recovered in Russia, the United States, and France, and ST32 is known to circulate in Europe and Australia (13,33,40,41). The novel sequence type related to ST441/442/444 identified in this study has not been described in other studies to date.

Among ST76 isolates, we noted heterogeneity in antimicrobial resistance marker carriage, specifically *sul1* and *ermX*, and 5/52 (9.6%) ST76 isolates had no identifiable markers. Patterns of resistance marker carriage also differed between isolates of other MLSTs, with no markers found in ST5 or ST32

isolates, *sul1* in the ST319 isolate, and *tetO* in the ST441/442/444 isolate. In addition to the 7 target regions used for routine MLST in our study, further assessment of intrastrain variability and the extent of clonality is warranted through analysis of additional genomic loci such as those performed in other studies of *C. diphtheriae* epidemiology (9).

Our study has some limitations. Our catchment area was small and limited to the persons from downtown Vancouver treated at St. Paul's Hospital, and MLST indicated highly clonal *C. diphtheriae* isolates. The generalizability of our results is limited and further study is needed to understand how our findings apply to other patient populations with different *C. diphtheriae* epidemiology (13). Nonetheless, several recent studies in multiple regions appear to support the notion that penicillin resistance remains scarce in circulating strains of *C. diphtheriae*. Other reviews of penicillin susceptibility in *C. diphtheriae*, 1 in Canada on 195 isolates and 1 in Algeria on 157 isolates, reported none that would be considered penicillin nonsusceptible by the 2010 CLSI breakpoints of MIC >1 mg/L (29,33).

Another limitation of our study is the lack of clinical outcomes to supplement our phenotypic and genotypic susceptibility data, precluding assessment of treatment success and failure rates on the basis of molecular and phenotypic characterization of isolates. Examination of clinical outcomes for patients treated for penicillin-intermediate diphtheria with MICs of 0.12–1 mg/L would be useful, as would an assessment of changes in prescribing practices for antimicrobial drugs related to increasing rates of isolates classified as penicillin-intermediate. Future studies are needed to explore clinical impacts of reclassification of isolates as penicillin-intermediate.

Last, any study using next-generation sequencing techniques to identify known resistance markers from databases, such as our study, has certain biases and limitations inherent to the sequencing and marker identification process. Current sequencing studies cannot account for markers not yet identified in databases, nor can such studies identify mixed or unspecified genetic effects. In addition, regions with poor assembly quality or with sequence or GC-content-dependent assembly gaps can preclude database matching.

In conclusion, we report the absence of penicillin nonsusceptibility in *C. diphtheriae* isolates collected during 2015–2018 and assessed phenotypically by susceptibility testing and genotypically by WGS. These results indicate that the 2015 CLSI M45 guidelines lowering the penicillin-susceptible breakpoint from an MIC of ≤ 1 mg/L to an MIC of ≤ 0.12 mg/L for *C. diphtheriae* might misclassify penicillin susceptibility

in isolates. Such misclassification could lead to shifts in prescribing practices toward less effective, less well-tolerated, and broader-spectrum antimicrobial drugs than penicillin. Further study is warranted to assess penicillin susceptibility in other contexts in which local strains and resistance patterns differ.

Acknowledgments

We thank the St. Paul's Hospital microbiology and virology laboratory staff for their commitment to quality testing and for identifying the isolates included in this study. We also thank the Canadian National Microbiology Laboratory for initial toxin testing of the bacterial isolates.

About the Author

Mr. Zou is a medical student at the University of Manitoba Max Rady College of Medicine in Winnipeg, Manitoba, Canada, undertaking a research elective at Providence Health Care, Vancouver, British Columbia, Canada. His research interests include diagnostic methods in medical microbiology and antimicrobial stewardship.

References

- Galazka A. The changing epidemiology of diphtheria in the vaccine era. *J Infect Dis.* 2000;181(Suppl 1):S2–9. <https://doi.org/10.1086/315533>
- Vitek CR, Wharton M. Diphtheria in the former Soviet Union: reemergence of a pandemic disease. *Emerg Infect Dis.* 1998;4:539–50. <https://doi.org/10.3201/eid0404.980404>
- Santos LS, Sant'anna LO, Ramos JN, Ladeira EM, Stavracakis-Peixoto R, Borges LL, et al. Diphtheria outbreak in Maranhão, Brazil: microbiological, clinical and epidemiological aspects. *Epidemiol Infect.* 2015;143:791–8. <https://doi.org/10.1017/S0950268814001241>
- Lodeiro-Colatosti A, Reischl U, Holzmann T, Hernández-Pereira CE, Rísquez A, Paniz-Mondolfi AE. Diphtheria outbreak in Amerindian communities, Wonken, Venezuela, 2016–2017. *Emerg Infect Dis.* 2018;24:1340–4. <https://doi.org/10.3201/eid2407.171712>
- Nanthavong N, Black AP, Nouanthong P, Souvannaso C, Vilivong K, Muller CP, et al. Diphtheria in Lao PDR: insufficient coverage or ineffective vaccine? *PLoS One.* 2015;10:e0121749. <https://doi.org/10.1371/journal.pone.0121749>
- du Plessis M, Wolter N, Allam M, de Gouveia L, Moosa F, Ntshoe G, et al. Molecular characterization of *Corynebacterium diphtheriae* outbreak isolates, South Africa, March–June 2015. *Emerg Infect Dis.* 2017;23:1308–15. <https://doi.org/10.3201/eid2308.162039>
- Mahomed S, Archary M, Mutevedzi P, Mahabeer Y, Govender P, Ntshoe G, et al. An isolated outbreak of diphtheria in South Africa, 2015. *Epidemiol Infect.* 2017;145:2100–8. <https://doi.org/10.1017/S0950268817000851>
- Meinel DM, Kuehl R, Zbinden R, Boskova V, Garzoni C, Fadini D, et al. Outbreak investigation for toxigenic *Corynebacterium diphtheriae* wound infections in refugees from Northeast Africa and Syria in Switzerland and Germany by whole genome sequencing. *Clin Microbiol Infect.* 2016;22:1003.e1–8. <https://doi.org/10.1016/j.cmi.2016.08.010>
- Dangel A, Berger A, Konrad R, Bischoff H, Sing A. Geographically diverse clusters of nontoxigenic *Corynebacterium diphtheriae* infection, Germany, 2016–2017. *Emerg Infect Dis.* 2018;24:1239–45. <https://doi.org/10.3201/eid2407.172026>
- Kantsone I, Lucenko I, Perevoscikovs J. More than 20 years after re-emerging in the 1990s, diphtheria remains a public health problem in Latvia. *Euro Surveill.* 2016;21:30414. <https://doi.org/10.2807/1560-7917.ES.2016.21.48.30414>
- Zasada AA, Rastawicki W, Rokosz N, Jagielski M. Seroprevalence of diphtheria toxoid IgG antibodies in children, adolescents and adults in Poland. *BMC Infect Dis.* 2013;13:551. <https://doi.org/10.1186/1471-2334-13-551>
- Swart EM, van Gageldonk PG, de Melker HE, van der Klis FR, Berbers GA, Mollema L. Long-term protection against diphtheria in the Netherlands after 50 Years of vaccination: results from a seroepidemiological study. *PLoS One.* 2016;11:e0148605. <https://doi.org/10.1371/journal.pone.0148605>
- Lowe CF, Bernard KA, Romney MG. Cutaneous diphtheria in the urban poor population of Vancouver, British Columbia, Canada: a 10-year review. *J Clin Microbiol.* 2011;49:2664–6. <https://doi.org/10.1128/JCM.00362-11>
- Romney MG, Roscoe DL, Bernard K, Lai S, Efstratiou A, Clarke AM. Emergence of an invasive clone of nontoxigenic *Corynebacterium diphtheriae* in the urban poor population of Vancouver, Canada. *J Clin Microbiol.* 2006;44:1625–9. <https://doi.org/10.1128/JCM.44.5.1625-1629.2006>
- Zasada AA. Nontoxigenic highly pathogenic clone of *Corynebacterium diphtheriae*, Poland, 2004–2012. *Emerg Infect Dis.* 2013;19:1870–2. <https://doi.org/10.3201/eid1911.130297>
- Gubler J, Huber-Schneider C, Gruner E, Altwegg M. An outbreak of nontoxigenic *Corynebacterium diphtheriae* infection: single bacterial clone causing invasive infection among Swiss drug users. *Clin Infect Dis.* 1998;27:1295–8. <https://doi.org/10.1086/514997>
- Farfour E, Badell E, Zasada A, Hotzel H, Tomaso H, Guillot S, et al. Characterization and comparison of invasive *Corynebacterium diphtheriae* isolates from France and Poland. *J Clin Microbiol.* 2012;50:173–5. <https://doi.org/10.1128/JCM.05811-11>
- DeWinter LM, Bernard KA, Romney MG. Human clinical isolates of *Corynebacterium diphtheriae* and *Corynebacterium ulcerans* collected in Canada from 1999 to 2003 but not fitting reporting criteria for cases of diphtheria. *J Clin Microbiol.* 2005;43:3447–9. <https://doi.org/10.1128/JCM.43.7.3447-3449.2005>
- Viguetti SZ, Pacheco LG, Santos LS, Soares SC, Bolt F, Baldwin A, et al. Multilocus sequence types of invasive *Corynebacterium diphtheriae* isolated in the Rio de Janeiro urban area, Brazil. *Epidemiol Infect.* 2012;140:617–20. <https://doi.org/10.1017/S0950268811000963>
- Zasada AA, Zaleska M, Podlasin RB, Seferynska I. The first case of septicemia due to nontoxigenic *Corynebacterium diphtheriae* in Poland: case report. *Ann Clin Microbiol Antimicrob.* 2005;4:8. <https://doi.org/10.1186/1476-0711-4-8>
- Tiley SM, Kociuba KR, Heron LG, Munro R. Infective endocarditis due to nontoxigenic *Corynebacterium diphtheriae*: report of seven cases and review. *Clin Infect Dis.* 1993;16:271–5. <https://doi.org/10.1093/clind/16.2.271>
- Griffith J, Bozio CH, Poel AJ, Fitzpatrick K, DeBolt CA, Cassidy P, et al. Imported toxin-producing cutaneous diphtheria—Minnesota, Washington, and New Mexico, 2015–2018. *MMWR Morb Mortal Wkly Rep.* 2019;68:281–4. <https://doi.org/10.15585/mmwr.mm6812a2>

23. Wilson AP. Treatment of infection caused by toxigenic and non-toxicogenic strains of *Corynebacterium diphtheriae*. *J Antimicrob Chemother*. 1995;35:717–20. <https://doi.org/10.1093/jac/35.6.717>
24. Fricchione MJ, Deyro HJ, Jensen CY, Hoffman JF, Singh K, Logan LK. Non-toxicogenic penicillin and cephalosporin-resistant *Corynebacterium diphtheriae* endocarditis in a child: a case report and review of the literature. *J Pediatric Infect Dis Soc*. 2014;3:251–4. <https://doi.org/10.1093/jpids/pit022>
25. Mina NV, Burdz T, Wiebe D, Rai JS, Rahim T, Shing F, et al. Canada's first case of a multidrug-resistant *Corynebacterium diphtheriae* strain, isolated from a skin abscess. *J Clin Microbiol*. 2011;49:4003–5. <https://doi.org/10.1128/JCM.05296-11>
26. FitzGerald RP, Rosser AJ, Perera DN. Non-toxicogenic penicillin-resistant cutaneous *C. diphtheriae* infection: a case report and review of the literature. *J Infect Public Health*. 2015;8:98–100. <https://doi.org/10.1016/j.jiph.2014.05.006>
27. Clinical and Laboratory Standards Institute. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria, 3rd edition (M45). Wayne (PA): The Institute; 2015.
28. Clinical and Laboratory Standards Institute. Antimicrobial Susceptibility Testing Workshop Presentations. "Unusual suspects" – resistance concerns and susceptibility testing among less common, but noteworthy bacteria. Wayne (PA): the Institute; 2016 [cited 2019 Oct 2]. https://s3.amazonaws.com/AST_Meeting/June_2016+_Unusual_Suspects_Resistance_Concerns_and_Susceptibility_Testing_Among_Less_Common_but_Noteworthy_Bacteria.zip
29. Bernard K, Pacheco AL. In vitro activity of 22 antimicrobial agents against *Corynebacterium* and *Microbacterium* species referred to the Canadian National Microbiology Laboratory. *Clin Microbiol NewsL*. 2015;37:187–98. <https://doi.org/10.1016/j.clinmicnews.2015.11.003>
30. Clinical and Laboratory Standards Institute. Methods for antimicrobial dilution and disk susceptibility testing of infrequently isolated or fastidious bacteria; approved guideline. 2nd edition (M45–A2). Wayne (PA): the Institute; 2010.
31. Larsen MV, Cosentino S, Rasmussen S, Friis C, Hasman H, Marvig RL, et al. Multilocus sequence typing of total-genome-sequenced bacteria. *J Clin Microbiol*. 2012;50:1355–61. <https://doi.org/10.1128/JCM.06094-11>
32. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res*. 2017;45:D566–73. <https://doi.org/10.1093/nar/gkw1004>
33. Benamrouche N, Hasnaoui S, Badell E, Guettou B, Lazri M, Guiso N, et al. Microbiological and molecular characterization of *Corynebacterium diphtheriae* isolated in Algeria between 1992 and 2015. *Clin Microbiol Infect*. 2016;22:1005.e1–7. <https://doi.org/10.1016/j.cmi.2016.08.013>
34. Tauch A, Bischoff N, Brune I, Kalinowski J. Insights into the genetic organization of the *Corynebacterium diphtheriae* erythromycin resistance plasmid pNG2 deduced from its complete nucleotide sequence. *Plasmid*. 2003;49:63–74. [https://doi.org/10.1016/S0147-619X\(02\)00115-4](https://doi.org/10.1016/S0147-619X(02)00115-4)
35. Coyle MB, Minshew BH, Bland JA, Hsu PC. Erythromycin and clindamycin resistance in *Corynebacterium diphtheriae* from skin lesions. *Antimicrob Agents Chemother*. 1979;16:525–7. <https://doi.org/10.1128/AAC.16.4.525>
36. Schröder J, Maus I, Meyer K, Wördemann S, Blom J, Jaenicke S, et al. Complete genome sequence, lifestyle, and multi-drug resistance of the human pathogen *Corynebacterium resistens* DSM 45100 isolated from blood samples of a leukemia patient. *BMC Genomics*. 2012;13:141. <https://doi.org/10.1186/1471-2164-13-141>
37. Fernández-Natal I, Rodríguez-Lázaro D, Marrodán-Ciordia T, Sáez-Nieto JA, Valdezate S, Rodríguez-Pollán H, et al. Characterization and antimicrobial susceptibility of one antibiotic-sensitive and one multidrug-resistant *Corynebacterium kroppenstedtii* strain isolated from patients with granulomatous mastitis. *New Microbes New Infect*. 2016;14:93–7. <https://doi.org/10.1016/j.nmni.2016.09.006>
38. Chorlton SD, Ritchie G, Lawson T, Romney MG, Lowe CF. Whole-genome sequencing of *Corynebacterium diphtheriae* isolates recovered from an inner-city population demonstrates the predominance of a single molecular strain. *J Clin Microbiol*. 2019 Nov 20 [Epub ahead of print]. <https://doi.org/10.1128/JCM.01651-19>
39. Grosse-Kock S, Kolodkina V, Schwalbe EC, Blom J, Burkovski A, Hoskisson PA, et al. Genomic analysis of endemic clones of toxigenic and non-toxicogenic *Corynebacterium diphtheriae* in Belarus during and after the major epidemic in 1990s. *BMC Genomics*. 2017;18:873. <https://doi.org/10.1186/s12864-017-4276-3>
40. Farfour E, Badell E, Dinu S, Guillot S, Guiso N. Microbiological changes and diversity in autochthonous non-toxicogenic *Corynebacterium diphtheriae* isolated in France. *Clin Microbiol Infect*. 2013;19:980–7. <https://doi.org/10.1111/1469-0691.12103>
41. Timms VJ, Nguyen T, Crighton T, Yuen M, Sintchenko V. Genome-wide comparison of *Corynebacterium diphtheriae* isolates from Australia identifies differences in the Pan-genomes between respiratory and cutaneous strains. *BMC Genomics*. 2018;19:869.d <https://doi.org/10.1186/s12864-018-5147-2>

Address for correspondence: Christopher F. Lowe, St. Paul's Hospital, Virology Laboratory, 1081 Burrard St, Vancouver, BC V6Z 1Y6, Canada; email: clowe@providencehealth.bc.ca

High Pathogenicity of Nipah Virus from *Pteropus lylei* Fruit Bats, Cambodia

Maria Gaudino, Noémie Aurine, Claire Dumont, Julien Fouret, Marion Ferren, Cyrille Mathieu, Olivier Reynard, Viktor E. Volchkov, Catherine Legras-Lachuer, Marie-Claude Georges-Courbot, Branka Horvat

We conducted an in-depth characterization of the Nipah virus (NiV) isolate previously obtained from a *Pteropus lylei* bat in Cambodia in 2003 (CSUR381). We performed full-genome sequencing and phylogenetic analyses and confirmed CSUR381 is part of the NiV-Malaysia genotype. In vitro studies revealed similar cell permissiveness and replication of CSUR381 (compared with 2 other NiV isolates) in both bat and human cell lines. Sequence alignments indicated conservation of the ephrin-B2 and ephrin-B3 receptor binding sites, the glycosylation site on the G attachment protein, as well as the editing site in phosphoprotein, suggesting production of nonstructural proteins V and W, known to counteract the host innate immunity. In the hamster animal model, CSUR381 induced lethal infections. Altogether, these data suggest that the Cambodia bat-derived NiV isolate has high pathogenic potential and, thus, provide insight for further studies and better risk assessment for future NiV outbreaks in Southeast Asia.

Nipah virus (NiV) is a zoonotic paramyxovirus that was first identified as the cause of an outbreak of encephalitis in humans in Malaysia and Singapore during 1998–1999 (1). Although NiV infection remains rare in humans, this virus has captured the attention of the public health community and scientists because of its high case-fatality rate, ranging from 40% in Malaysia to >90% in Bangladesh and

India (2,3). Its high pathogenicity, potential for interspecies transmission, and lack of validated medical countermeasures led to the classification of NiV as a Biosafety Level 4 (BSL-4) pathogen. In 2015, the World Health Organization (WHO) listed NiV as a priority pathogen because of its probability of causing severe outbreaks and subsequently placed NiV on the WHO Blueprint list of priority diseases (4). This designation was strengthened by the NiV outbreak in Kerala, India, where the virus had not previously been reported (3).

NiV is a member of the *Henipavirus* genus, together with Hendra virus, which first emerged in Brisbane, Queensland, Australia, in 1994 (5), and the nonpathogenic Cedar virus, which was discovered in Australia in 2009 (6). In addition, full-length henipalike viral sequences were found in fruit bats in Africa (7) and in rats in China (Mojjang virus) (8). As part of the Mononegavirales, NiV has a nonsegmented, negative-sense, single-stranded RNA genome. *Pteropus* fruit bats, commonly known as flying foxes, are considered the henipavirus natural reservoir; these bats, when infected with NiV, do not seem to display any apparent clinical signs of disease (9).

Only 2 NiV lineages are known to circulate in Asia and cause disease in humans: NiV-Malaysia and NiV-Bangladesh. In India and Bangladesh, NiV transmission from bats to humans was shown to occur through the consumption of raw date palm juice or fruits contaminated with the saliva or urine from fruit bats (10). In addition, as observed during the outbreaks in Malaysia in 1998 and 1999, transmission can occur via contact with infected domestic animals, such as pigs, that act as amplifying hosts of the virus (11). Interhuman transmission has been reported in Bangladesh and India in >50% of NiV outbreaks (12).

Clinical manifestations of NiV infection in humans can range from asymptomatic to acute

Author affiliations: Centre International de Recherche en Infectiologie, CIRI, INSERM U1111, CNRS, UMR5308, Univ Lyon, University Claude Bernard Lyon 1, École Normale Supérieure de Lyon, Lyon, France (M. Gaudino, N. Aurine, C. Dumont, J. Fouret, M. Ferren, C. Mathieu, O. Reynard, V.E. Volchkov, M.-C. Georges-Courbot, B. Horvat); ViroScan 3D, Trévoux, France (J. Fouret, C. Legras-Lachuer); University Claude Bernard Lyon 1, LEM, UMR5557, CNRS, INRA, VetAgro Sup, Lyon (C. Legras-Lachuer); Unité de Biologie des Infections Virales Emergentes, Institut Pasteur, INSERM P4, Jean Mérieux, Lyon (M.-C. Georges-Courbot)

DOI: <https://doi.org/10.3201/eid2601.191284>

respiratory syndrome, generalized vasculitis, and fatal encephalitis. Among the few survivors of NiV outbreaks, long-term neurologic problems have been reported; 20% of patients have residual neurologic sequelae (13), and NiV-Malaysia-infected patients experienced relapse and late-onset encephalitis (14).

NiV and henipa-like viruses have been detected molecularly or serologically in *Pteropus* bats in different countries of Asia (15) and Africa (7), Australia (16), and Brazil (17), and the worldwide distribution of fruit bats poses a continuous threat to another spillover with possible pandemic potential (18). However, since 1998, all NiV cases in humans have been identified in Malaysia, India, Bangladesh, and the Philippines (19). Human cases of NiV have not been reported in Cambodia or neighboring countries since the first serologic detection of NiV in Cambodia and isolation of CSUR381 in *Pteropus lylei* bats in Cambodia in 2003 (20,21). Initial phylogenetic analyses of the nucleoprotein and attachment glycoprotein of CSUR381 suggested the virus was part of the NiV-Malaysia genotype (21). However, a full-genome characterization and phylogenetic analysis have not been performed. In addition, the growth dynamics and virulence of this virus have not been analyzed, thus limiting more comprehensive evaluation of this virus's pathogenic potential. In this study, we performed an in-depth characterization of CSUR381, including its pathogenicity both in vitro and in vivo, ultimately to assess the outbreak risk that isolates circulating in Cambodia pose in Southeast Asia.

Materials and Methods

Viruses

In this study, we used 3 different NiV isolates: the NiV isolate CSUR381 from Cambodia (GenBank accession no. MK801755), NiV-Malaysia isolate UMMC1 (GenBank accession no. AY029767), and NiV-Bangladesh isolate SPB200401066 (GenBank accession no. AY988601). CSUR381 was isolated from *P. lylei* bat urine at the Institut Pasteur in Battambang, Cambodia, in 2003 (21), and the other 2 isolates were obtained from infected patients. We produced and titrated all viruses on Vero E6 cells.

Full-Genome Sequencing

We amplified and titrated the Cambodia NiV isolate on Vero cells and, after the second cell passage, extracted viral RNA from supernatant using the QIAamp Viral RNA Mini Kit (QIAGEN, <https://www.qiagen.com>) according to the manufacturer's instructions. We treated samples with DNase, purified and quantified

RNA using the QuantiFluor RNA System (Promega, <https://www.promega.com>), and analyzed using the AATI High Sensitivity Genomic DNA Analysis Fragment Analyzer (Advanced Analytical Technologies Inc., <https://www.agilent.com>). Then, we amplified viral RNA using the Single Primer Isothermal Amplification Kit (NuGEN, <https://www.nugen.com>). We prepared a library using Ovation Ultra Low (NuGen), which gave us average DNA fragment sizes of 382–426 bp. We then sequenced the whole genome of CSUR381 using MiSeq Nano v2 (Illumina, <https://www.illumina.com>), which produced read lengths of 2×150 nt.

We carried out genome assembly de novo using SPAdes (<http://cab.spbu.ru/software/spades>) and predicted open reading frames with Prodigal (<https://omictools.com/prodigal-tool>). We reconstructed the missing 5' and 3' extremities using the 5' RACE System for Rapid Amplification of cDNA Ends (Invitrogen, <https://www.thermofisher.com>) according to the manufacturer's recommendations. We performed reverse transcription with SuperScript II Reverse Transcriptase (Invitrogen) using the primer GSP1-leader (5'-GACCATTGATCCAACATC-3') to recover the viral leader sequence and GSP-trailer (5'-AAAGT-GATTGTCTACTACT-3') to recover the trailer sequence. After column purification, we tailed cDNA sequences with cytidine triphosphate and terminal deoxynucleotidyl transferase. Last, we amplified dC-tailed cDNA using the Abridged Anchor Primer provided in the 5' RACE System for Rapid Amplification of cDNA Ends Kit and primers nested-GSP2-leader (5'-TACAGCTTCAATGTCTGGGTCATT-3') to amplify the viral leader sequence, and nested-GSP2-trailer (5'-CAAGTTCAAGGACACCAAAAAGT-3') to amplify the viral trailer sequence. We sequenced PCR products using Sanger technology and submitted the complete genome sequence of CSUR381 to GenBank (accession no. MK801755).

Phylogenetic Analyses

Using the ClustalW algorithm (<http://www.clustal.org>), we performed multiple alignments for complete genomes and individual gene sequences. We implemented and manually checked the quality of alignments using BioEdit version 7.2.6 (22) and conducted genomic characterization and evolutionary analyses in MEGA version 7.0.26 (23). After determining the best DNA model to use for each alignment, we constructed maximum-likelihood phylogenetic trees for complete NiV genomes and all virus coding sequences. For statistical support, we used 500 bootstrap replicates for the analysis of the complete genome and 1,000 replicates for analyses of each gene.

Cell Lines and Infection

We cultured NCI-H358 (human bronchioalveolar carcinoma) and Vero E6 (African green monkey kidney) cells in Dulbecco's modified Eagle medium (DMEM) with GlutaMAX (Thermo Fisher Scientific, <https://www.thermofisher.com>) supplemented with 1% penicillin-streptomycin (10,000 U/mL), 1% L-glutamine, and 10% heat-inactivated (56°C for 30 minutes) fetal calf serum (FCS). We cultured human pulmonary microvascular endothelial cells (HPMECs) (24) in endothelial cell growth medium (Growth Medium MV 2 Kit; PromoCell, <https://www.promocell.com>). We incubated all these cell lines at 37°C with 5% carbon dioxide; all cell lines, including the *Pteropus* cell line described in the next paragraph, tested negative for *Mycoplasma* spp. by the MycoAlert kit (Lonza, <https://www.lonza.com>).

We generated a *Pteropus* flying fox cell line using a skin biopsy from the wing membrane of a female *P. giganteus* (also known as *P. medius* and flying fox) bat (25) of the order Yinpterochiroptera. Biopsies were collected from bats by Tiergarten Schönbrunn (Vienna, Austria) staff during regular veterinary checkups following appropriate guidelines to minimize animal stress. The biopsies were washed with sterile phosphate-buffered saline and transferred into Freezing Medium Cryo-SFM (PromoCell), and sample vials were put on dry ice for shipment to Centre International de Recherche en Infectiologie in Lyon, France. To obtain primary cell cultures, we fractionated the biopsies into petri dishes, harvested the homogenates, and incubated them at 37°C with 5% carbon dioxide in DMEM/F-12 (Gibco, <https://www.thermofisher.com>) supplemented with 10% fetal calf serum FCS, 1% L-glutamine (200 mM), 1,000 U/mL of penicillin, 1,000 U/mL of streptomycin, and 2.50 µg/mL amphotericin B (Gibco). We subsequently immortalized primary cells using the lentiviral vector SV40 large T-antigen produced at Genetic Analysis and Vectorology Platform (AniRA, École Normale Supérieure de Lyon, Lyon). We evaluated different clones on the basis of their morphologic stability and transfectability using jetPRIME kit (Polyplus, <https://www.polyplus-transfection.com>). We confirmed immortalization of clones by detecting large T-antigen inserts by reverse transcription PCR (RT-PCR). We cultured the final *Pteropus* cell line, which we designated PATGV1.12, in DMEM GlutaMAX supplemented with 10% heat-inactivated FCS. We additionally confirmed that this cell line was derived from *P. giganteus* bats by sequencing the mitochondrial region D-loop (26) and nuclear introns ACOX2, COPS7A, BGN, ROGD1, and STAT5A, which has

been suggested to be pertinent for distinguishing among closely related bat species (27).

We infected cells in 12-well plates at 80% confluence with a multiplicity of infection (MOI) of 0.3. For virus replication kinetics studies, we took 4 time points postinfection into consideration: 0 h, 24 h, 48 h, and 72 h. We performed infections in BLS-4 facility Jean Mérieux (Lyon). For each time point, we collected cell lysates according to validated BSL-4 procedures. We collected supernatants and kept them at -80°C until titration by plaque assay on Vero E6 cells.

Pseudotyping of Vesicular Stomatitis Virus and Evaluation of Cell Permissiveness

We used rVSVΔG-RFP (a recombinant vesicular stomatitis virus [VSV] in which the envelope glycoprotein G gene is replaced with the red fluorescent protein gene) (28,29) to generate pseudotyped VSVs harboring different combinations of NiV envelope glycoprotein G (attachment protein) and F (fusion protein) on their surfaces. Complementing rVSVΔG-RFP-infected cells with NiV glycoproteins expressed in trans, we were able to produce stocks of pseudotyped VSVs identical in their genetic background and differing only in the nature of their surface glycoproteins. Because the infectivity of rVSVΔG-RFP pseudotypes is restricted to a single round of replication, this tool is largely used for studying viral entry for a broad range of highly pathogenic viruses (30).

To create the pseudotypes, we cloned the NiV glycoprotein G and F genes from RNA isolated from CSUR381, UMMC1, and SPB200401066 into 6 separate pCAGGS plasmid vectors. We transfected these 3 plasmid pairs separately into BSR-T7 cells using TransIT-LT1 Transfection Reagent (Mirus Bio, <https://www.mirusbio.com>). We infected cells with rVSVΔG-RFP 16 h after transfection to produce a pseudotyped VSV for each NiV isolate. We collected supernatants at 24 h postinfection and concentrated pseudotyped VSVs by ultracentrifugation (28,000 rpm for 2 h at 4°C). We titrated these viruses on Vero cells. To evaluate viral entry into different cell lines, we performed infections in 24-well plates using 80% confluent, adherent cells and a 1-h contact between virus and cells. We determined the percentage of cells infected 6 h postinfection by quantifying cells expressing RFP via flow cytometry on a BD LSRFortessa (<https://www.bd.com>).

RNA Extraction and Real-Time RT-PCR

At the indicated time points, we collected cells and extracted RNA using the NucleoSpin RNA Kit (Macherey-Nagel, <https://www.mn-net.com>) according

to the manufacturer's instructions. We assessed the yield and purity of extracted RNA using the DS-11-FX spectrophotometer (DeNovix, <https://www.denovix.com>). We reverse transcribed extracted RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, <https://www.bio-rad.com>) and performed real-time PCR using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) on a StepOnePlus Real-Time PCR System (Applied Biosystems, <https://www.thermofisher.com>). As previously described (31), we amplified the NiV nucleoprotein gene and the *Pteropus* glyceraldehyde 3-phosphate dehydrogenase housekeeping gene using forward primer 5'-ATCATCCCTGCTTCTACT-3' and reverse primer 3'AGGTCAGATCCACAAC-5'. We analyzed quantitative RT-PCR results using StepOne version 2.3 (Applied Biosystems).

Experimental Infection of Hamsters

We obtained 2-month-old male golden hamsters (*Mesocricetus auratus*) from Janvier Labs (<https://www.janvier-labs.com>). We housed hamsters in a BSL-4 containment facility (INSERM P4, Jean Mérieux, Lyon) and handled them according to the regulations for animal maintenance of France. We treated hamsters with isoflurane anesthesia before manipulations. We subcutaneously infected 2 groups of 6 hamsters with a high dose (13,500 PFU/animal) of either the NiV-Malaysia or Cambodia NiV isolate and followed hamsters daily to record their body temperature and weight. The regional ethics committee for animal experimentation (Lyon) approved these animal experiments.

Results

Full-Genome Characterization and Phylogenetic Analyses

Analysis of the assembled viral sequence of CSUR381 showed a total genome length of 18,246 nt, similar to the lengths of NiV isolates reported in Malaysia. The nucleotide composition was 27.8% T or U, 18.4% C, 33.6% A, and 20.2% G; total GC content was 38.6%. To

Table 1. Whole-genome pairwise nucleotide identity comparisons between Nipah virus CSUR381, Cambodia, 2003, and other available henipaviruses

Henipavirus (GenBank accession no.)	Nucleotide identity, %*
Nipah/Malaysia/2000/human (NC002728.1)	97.7
Nipah/Malaysia/2001/human (AY029768.1)	97.7
Nipah/Malaysia/1999/swine (AJ627196.1)	97.6
Nipah/Bangladesh/2004/human (AY988601.1)	91.6
Nipah/India/2007/human (FJ513078.1)	91.4
Nipah/Bangladesh/2008/human (JN808863.1)	91.4
Nipah/India/2018/human (MH396625.1)	91.3
Hendra/Australia/2008/human (JN255805.1)	70.0
Hendra/Australia/2009/bat (JN255803.1)	69.9
Hendra/Australia/2007/horse (HM044321.1)	69.9
Cedar/Australia/2009/Ptalecto (JQ001776.1)	56.7
Paramyxo/Ghana/2009/Eidolonhelvum (HQ660129.1)	52.9
Mojiang/China/2012/Rattusflavipectus (NC025352.1)	48.9

*Calculated by using the p-distance method.

investigate genetic relationships between CSUR381 and other henipaviruses, we constructed distance matrices for the complete genome and for each gene using the p-distance method. When we compared the sequence of CSUR381 with those of other NiVs available in GenBank, the most similar sequences (with 97.7% nucleotide identity) were from NiV-Malaysia human isolates (GenBank accession nos. NC002728.1 and AY029768.1; Table 1). We also calculated nucleotide identity and amino acid homology for each of the 6 structural genes (Table 2). Genetic pairwise comparisons with other NiV isolates showed the lowest nucleotide identity and amino acid homology for phosphoprotein (087.1/82.7%) and the highest for matrix protein (98.9/99.4%).

Using the maximum-likelihood method, we constructed phylogenetic trees on the basis of the complete genome (Figure 1, panel A) and the nucleocapsid gene (Figure 1, panel B). The general time-reversible model for the complete genome and the Kimura 2-parameter model for the nucleocapsid gene were predicted to be the best for performing those particular phylogenetic analyses. CSUR381 clustered with the monophyletic group of the NiV-Malaysia genotype

Table 2. Pairwise comparison of NiV CSUR381, Cambodia, 2003, and other available NiVs, by NiV gene*

NiV (GenBank accession no.)	NiV gene, % nucleotide identity/% homology of deduced amino acid†					
	N	P	M	F	G	L
Nipah/Malaysia/2010/Pvampyrus (FN869553.1)	98.3/99.2	96.3/94.9	98.9/99.4	98.4/98.7	97.1/98.5	98.0/99.3
Nipah/Malaysia/2000/human (NC002728.1)	97.9/98.7	95.9/94.1	98.5/99.1	98.1/98.9	97.1/98.5	98.1/99.4
Nipah/Bangladesh/2004/human (AY988601.1)	93.8/98.5	87.8/84.8	93.0/99.1	93.0/98.2	88.3/95.5	91.7/98.2
Nipah/Bangladesh/2008/human (JN808863.1)	93.9/98.7	87.6/84.4	93.0/99.1	93.3/98.5	88.1/95.5	91.8/98.4
Nipah/India/2007/human (FJ513078.1)	93.5/98.5	87.4/84.3	92.9/98.9	93.1/98.4	88.0/95.3	91.9/98.4
Nipah/India/2018/human (MH396625.1)	93.3/98.5	87.1/82.7	92.6/99.1	93.0/98.5	87.2/95.3	91.7/98.5
Nipah/Thailand/2010/Plylei (KT163252.1)	93.7/98.7					
Nipah/Thailand/2010/Phyromelaneus (KT163247.1)	97.8/99.0					

*F, fusion protein; G, attachment protein; L, polymerase; M, matrix protein; N, nucleoprotein; NiV, Nipah virus; P, phosphoprotein.

†Both calculated by using the p-distance method.

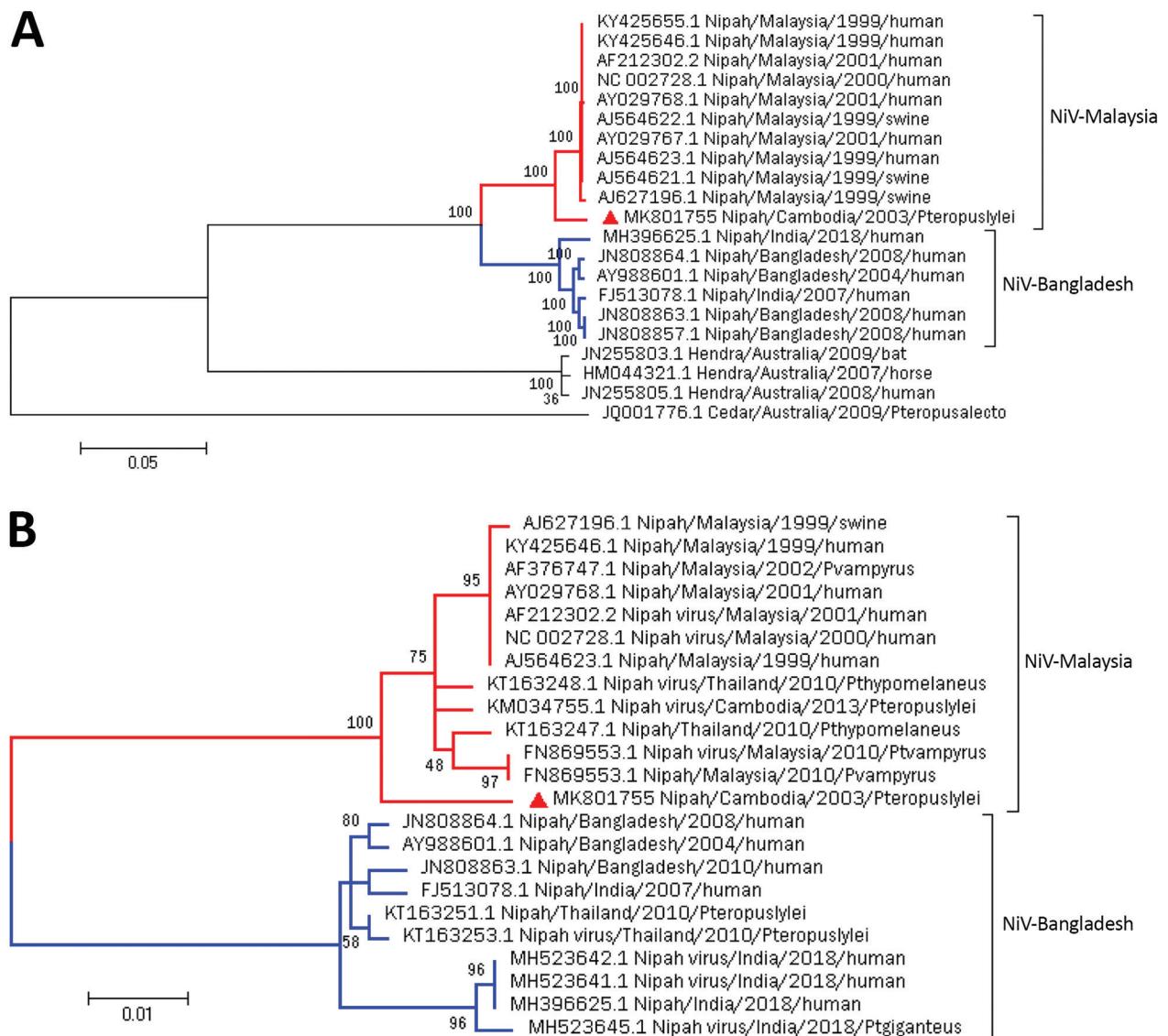


Figure 1. Maximum-likelihood phylogenetic analysis of NiV CSUR381, Cambodia, 2003 (red triangle), compared with other henipaviruses and NiVs. A) Phylogenetic tree constructed with complete genome sequences. A general time-reversible model was calculated as the best DNA model to conduct this analysis. B) Phylogenetic tree constructed by using the nucleocapsid gene. The Kimura 2-parameter model was calculated as the best DNA model to conduct this analysis. Bootstrap statistical support is marked on branch nodes. GenBank accession numbers of isolates are provided in branches, and NiV lineages of isolates are indicated. Phylogenetic trees are drawn to scale; scale bars represent branch lengths measured in the number of substitutions per site. NiV, Nipah virus.

for both the whole genome and nucleocapsid gene; bootstrap support was >98% in all cases, confirming the previous partial genomic characterization of CSUR381 (24). We then generated phylogenetic trees for each of the coding sequences of the 6 NiV structural proteins, which gave equivalent results (Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-1284-App1.pdf>).

Multiple alignment of the henipavirus phosphoprotein gene (Figure 2) revealed high conservation of

the editing site (5'-AAAAAGGG-3') in CSUR381, similar to other NiV and Hendra virus isolates and different from Cedar virus, a nonpathogenic virus isolated from a *P. alecto* bat in Australia (6). This finding suggests that CSUR381 might produce the nonstructural proteins V and W, capable of interacting with the host innate cellular immune response (32). Comparisons of the deduced V, W, and C amino acid homologies between CSUR381 and other known NiVs showed a variation of 88%–100% (Appendix Table).

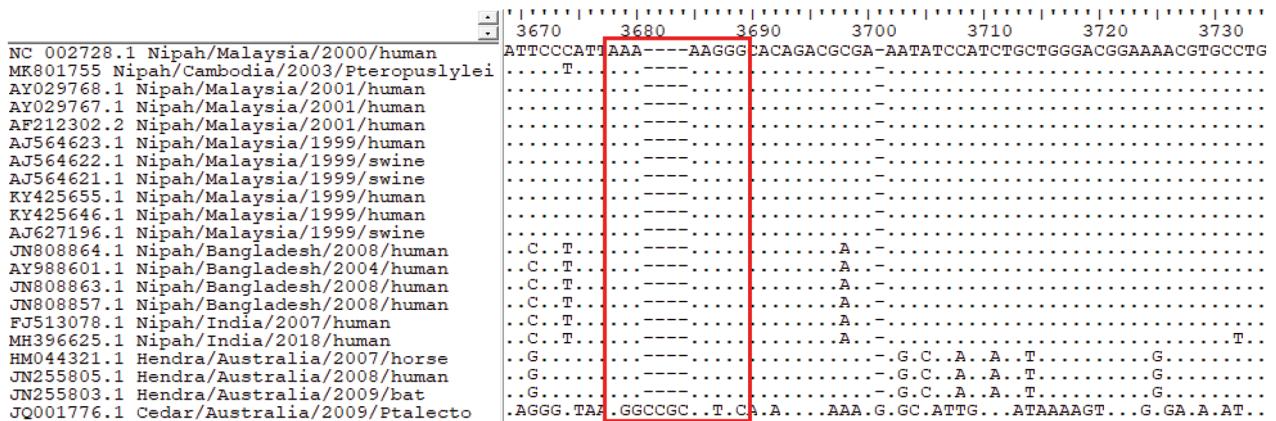


Figure 2. Multiple alignment of the phosphoprotein gene of Nipah virus CSUR381, Cambodia, 2003, and other henipavirus isolates. The highly conserved editing site (5'-AAAAGGG-3', red outline) is present in all Nipah and Hendra virus sequences but absent in the nonpathogenic Cedar virus sequence. GenBank accession numbers are provided for all isolates.

Evaluation of Virus Entry

We next determined the cellular permissiveness of a human endothelial cell line (HPMEC), a human respiratory epithelial cell line (NCI-H358), the newly generated *Pteropus* bat cell line (PAT-GV1.12), and Vero cells to CSUR381 compared with the NiV-Malaysia (UMMC1) and NiV-Bangladesh (SPB200401066) isolate using pseudotyped rVSVΔG-RFP viruses. Cell lines were infected for 1 h at an MOI of 0.3. The percentages of cells infected were analyzed by flow cytometry 6 h after infection (Figure 3), and results from HPMEC, NCI-H358, and PAT-GV1.12 were normalized to the findings from Vero. All tested cell lines were permissive to infection with all 3 viruses tested. Entry of NiV pseudotypes into the bat cell line PATGV1.12 and human respiratory epithelial cell line was similar. Compared with the NiV-Malaysia and NiV-Bangladesh pseudotypes, the CSUR381 pseudotyped virus showed higher but not significantly increased entry into the 3 tested cell lines (1-way analysis of variance).

We further analyzed the amino acid sequences of the F and G proteins of the 3 viruses by multiple alignment. The glycosylation site (N529/Q530/T531) (33) and ephrin-B2 and ephrin-B3 binding sites (34) in the G attachment protein were preserved (Appendix Figure 2). In addition, multiple alignments showed that the F cleavage site was preserved among all analyzed NiV isolates (Appendix Figure 3). Last, an analysis of the predicted N-terminal and C-terminal heptad-repeat regions within the F protein, which are needed for NiV fusion (35), showed high conservation, and compared with NiV-Malaysia and NiV-Bangladesh, only 1 aa difference (V159→I) was detected in CSUR381 (Appendix Figure 3). Altogether,

the high conservation of the NiV glycoproteins and results from pseudotype virus studies suggest that CSUR381 can enter target cells at least as well as NiV-Malaysia and NiV-Bangladesh.

Replication of NiV Isolates in Different Cell Types

To further evaluate the virulence of CSUR381, we compared the replication kinetics of this virus with those of the NiV-Malaysia and NiV-Bangladesh isolates.

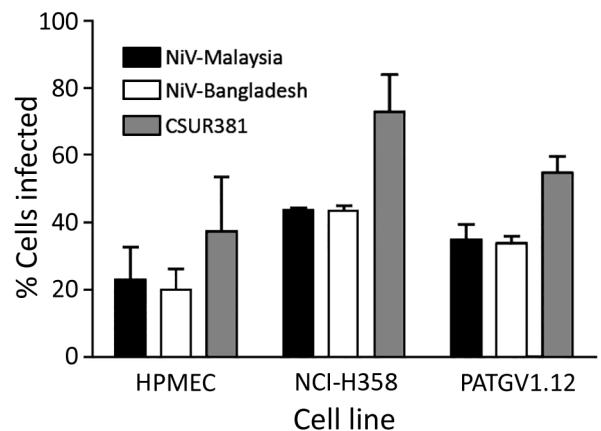


Figure 3. Evaluation of entry of VSVΔG-RFPs (vesicular stomatitis virus in which the envelope glycoprotein G gene is replaced with the red fluorescent protein gene) pseudotyped with the surface glycoproteins of NiVs CSUR381 (Cambodia 2003 isolate), UMMC1 (NiV-Malaysia isolate), and SPB200401066 (NiV-Bangladesh isolate) in different cell types. Infections of HPMEC, NCI-H358 (human bronchioalveolar cells), PATGV1.12 (bat cells), and Vero cells were performed at a multiplicity of infection of 0.3, and the percentages of infected cells were evaluated 6 hours postinfection by measuring RFP by flow cytometry and normalizing values to those from Vero cells. Histograms indicate the mean of 3 independent experiments, and error bars indicate upper half of SD. HPMEC, human pulmonary microvascular endothelial cell; NiV, Nipah virus.

We infected cell types known to be primary targets of NiV in humans, pulmonary endothelial (HPMEC) and bronchioalveolar epithelial (NCI-H358) cells, and the bat cell line PATGV1.12 at an MOI of 0.3 (Figure 4). NiV RNA synthesis was highest in HPMEC, where NiV-Bangladesh replicated the best, although a similar level of RNA and infectious virus particle production was observed for all 3 viruses (Figure 4, panel A). In accordance with virus entry studies (Figure 3), virus replication was also observed in PATGV1.12 (Figure 4, panels A and B). Differences among the 3

tested NiV isolates were observed only in NCI-H358, where NiV-Malaysia RNA synthesis was significantly increased ($p < 0.001$ by 2-way analysis of variance) compared with NiV-Bangladesh, provoking remarkable cytopathic effects (Figure 4, panel C). The formation of giant multinucleated cells, a hallmark of NiV infection, were already visible at 24 hours postinfection in all cell types and further developed during the course of the infection (Figure 4, panels C–E). Vero cells showed the most visible cytopathic effects, probably because of their interferon incompetence (36).

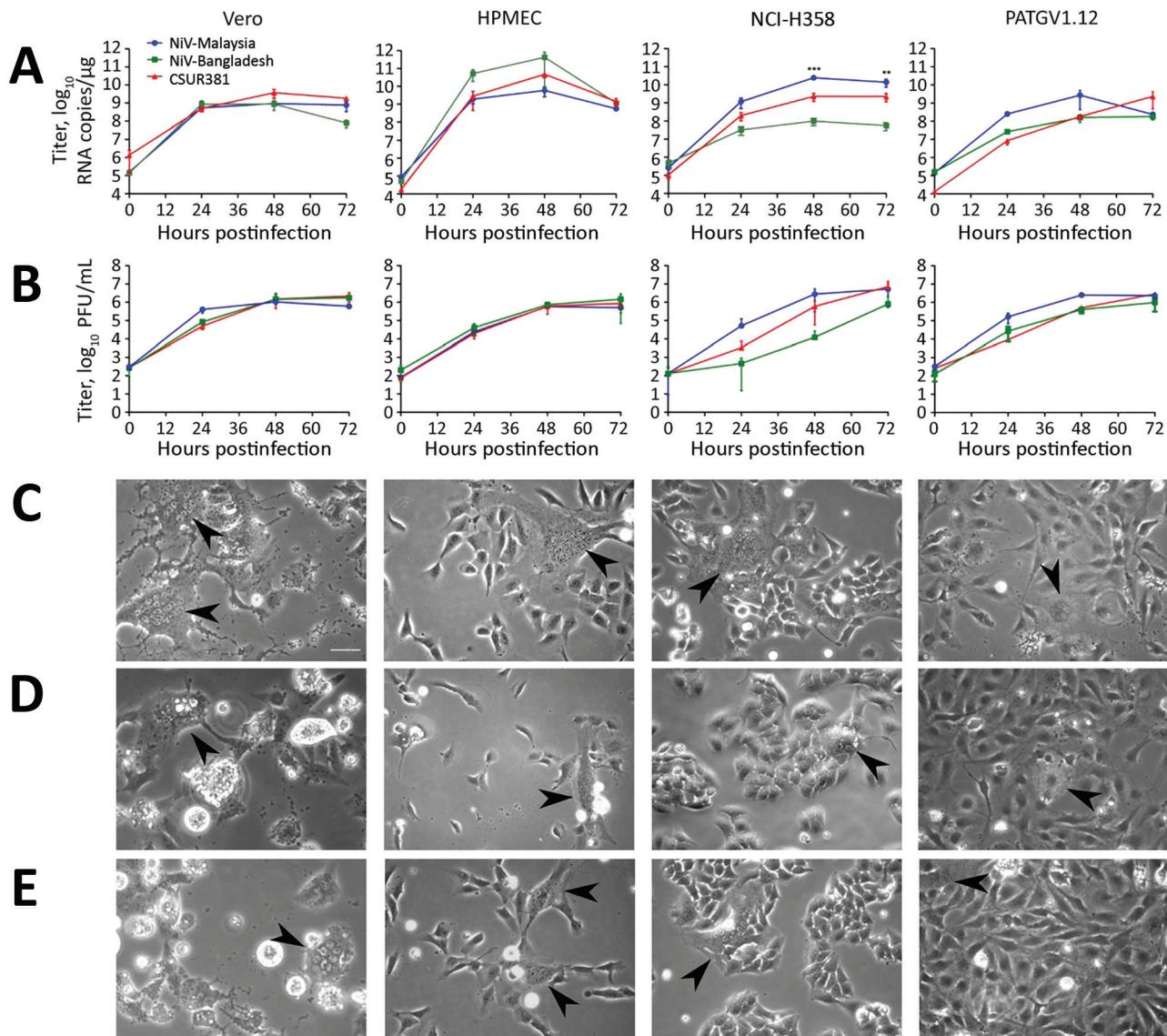


Figure 4. Replication of NiVs CSUR381 (Cambodia 2003 isolate), UMMC1 (NiV-Malaysia isolate), and SPB200401066 (NiV-Bangladesh isolate) in Vero, HPMEC, NCI-H358 (human bronchioalveolar cells), and PATGV1.12 (bat cells). A) Real-time reverse transcription PCR titer. Cells were infected at a multiplicity of infection of 0.3, and the production of the nucleocapsid gene was measured. Significance was measured by 2-way analysis of variance. B) Kinetics of infectious virus particle production in supernatant measured by Vero plaque assay. The average of 2 independent experiments is presented. C–E) Cytopathic effect of observed by light microscopy 48 h after infection with the NiV-Malaysia isolate (C), the NiV-Bangladesh isolate (D), and CSUR381 (E). Giant multinucleated cells are indicated with arrowheads. Scale bar indicates 25 μ m. HPMEC, human pulmonary microvascular endothelial cell; NiV, Nipah virus. ** $p < 0.01$; *** $p < 0.001$.

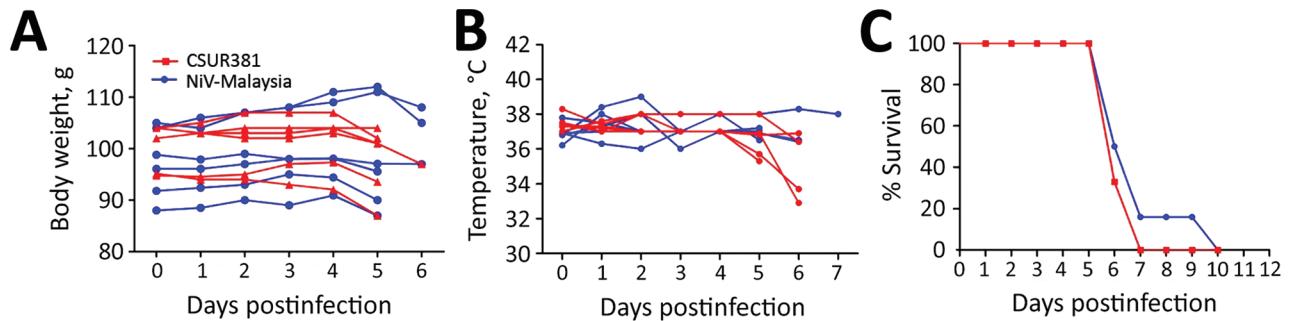


Figure 5. Pathogenicity of NiV CSUR381 (Cambodia 2003 isolate) and UMMC1 (NiV-Malaysia isolate) in golden hamsters (6 hamsters/group). A) Body weight. B) Body temperature. C) Survival. Survival between groups was not significantly different (Mantel-Cox test). NiV, Nipah virus.

Experimental Infection of Hamsters

We compared the pathogenicities of CSUR381 and the NiV-Malaysia isolate using the golden hamster animal model (37). We infected 6 hamsters with either CSUR381 or the NiV-Malaysia isolate and followed them for clinical signs of infection. At 6 days postinfection, the first neurologic signs (which included paralysis and trembling limbs) were observed in both groups; their presentation rapidly evolved toward breathing difficulties and prostration. Weight reductions were evident in several animals in the late stages of infection (Figure 5, panel A), and decreases in body temperature were found in a few hamsters (Figure 5, panel B). At 7 days postinfection, 100% lethality was observed in the CSUR381 group. In the Malaysia group, 1 animal survived until 10 days postinfection (Figure 5, panel C); however, the difference between the 2 groups was not significant. These results demonstrate similar lethality of the 2 analyzed NiV isolates, supporting our other data and suggesting CSUR381 has a high pathogenic potential.

Discussion

In this study, we performed a molecular and genetic characterization of CSUR381, a NiV isolated from *P. lylei* bats in Cambodia. Furthermore, we analyzed its pathogenicity compared with those of 2 other NiV isolates derived from human patients from the Malaysia and Bangladesh outbreaks. Our results highly suggest that CSUR381 is part of the NiV-Malaysia genotype. Further phylogenetic comparisons with other NiV isolates demonstrated 83%–99% amino acid homology for each of the 6 structural proteins. In addition, the editing site of the phosphoprotein gene was preserved, suggesting possible production of the nonstructural V and W proteins known to be involved in counteracting the host innate immune system and thus contributing to pathogenicity of CSUR381 (32).

Our virus entry studies showed highly similar results among the NiVs tested. All isolates

entered *Pteropus* bat and human cell lines at similar levels; high conservation of the NiV entry receptors (ephrin-B2 and ephrin-B3) (38) might be responsible for the observed results. Our data also indicate that CSUR381 enters all tested cell types as well as the other 2 NiV isolates tested, suggesting that virus entry is not a limiting factor preventing CSUR381 spillover from bats to humans. In addition, all 3 tested NiV isolates infected cells and replicated in bat and human cell lines at similar levels. Results of infections with CSUR381 in hamsters additionally strengthened the notion that CSUR381 is possibly similar pathogenically to the tested NiV-Malaysia strain, which caused fatal outbreaks in Malaysia (1).

Although NiV has been shown to circulate in Cambodia (20,21), Thailand (39), and Vietnam (40), transmission to humans or domestic animals has not been reported in these countries. According to our results, the absence of detected outbreaks in this region cannot be attributed to lower pathogenicity of the circulating NiVs; our results suggest that other factors probably contribute. However, the NiV isolate presented in this report has been the only live NiV isolated in this region, and the existence of other NiVs with different pathogenic potentials cannot be excluded.

In Cambodia, *P. lylei* bats were found to often forage in residential areas and visit palm trees used in the region as a source of date palm sap; thus, opportunities abound for bats to interact with humans and livestock in this country (41). Bat colony migration toward urban sites is further enhanced by the presence of hunters in rural areas (42) and deforestation (causing consequent damage to roosting trees and food sources) (43). Contamination of palm sap, which is consumed raw by persons in the region, with bat urine, saliva, or feces was found to be a major route of NiV transmission to humans during annual outbreaks in Bangladesh (10).

Diverse agricultural practices in Southeast Asia could also play a role in NiV regional ecodynamics, potentially favoring easier NiV spillover in some countries over others. High intensity pig farming

was recognized as a major risk factor for outbreaks in Malaysia during 1998–1999; because of the low-scale pig production ongoing in Cambodia (44), the risk for NiV transmission from *Pteropus* spp. to domestic animals and humans in this country might be reduced.

Unrecognized NiV outbreaks might have occurred in Cambodia and neighboring countries; hospital-based surveillance in Bangladesh was shown to have missed nearly half of the NiV outbreaks in that country since the first reported virus emergence (45). Interdisciplinary approaches are certainly required to identify these outbreaks and the drivers of NiV emergence (46), and regular testing of patients with encephalitis in Cambodia and neighboring countries could provide additional insight. Our study contributes to the assessment of the risk for NiV outbreaks in Asia. Our findings can be used to help target adequate preventive measures, which could ultimately help reduce the risk for NiV emergence.

Acknowledgments

We thank Jean-Marc Reynes and Pasteur Institute staff for providing us with the NiV Cambodia isolate, Pierre E. Rollin for the NiV Bangladesh isolate, and Doris Preininger, Anton Weissenbacher, and Tiergarten Schönbrunn for *P. giganteus* bat sampling. We thank Amelia Charlotte Coggon for English proofreading of the manuscript, and we also thank François Enchéry, Kévin Dhondt, Mathieu Iampietro, Sylvain Baize, and Géraldine Gourru-Lesimple for help initiating and finalizing this work.

This work was supported by LABEX ECOFECT (ANR-11-LABX-0048) of Lyon University, within the program Investissements d’Avenir (ANR-11-IDEX-0007) operated by the French National Research Agency, ANR-18-CE11-0014-02, Aviesan Sino-French Agreement on Nipah Virus Study, and the International Division of the Institut Pasteur in Paris (Actions Concertées Inter-Pasteurienne). J.F. was supported by the doctoral fellowship CIFRE-Défense operated by the Direction Générale de l’Armement.

About the Author

Ms. Gaudino is a graduate student at the International Centre for Infectiology Research in Lyon, France. Her main research interests include the study of mechanisms of pathogenicity and epidemiology of viral zoonoses.

References

1. Chua KB, Bellini WJ, Rota PA, Harcourt BH, Tamin A, Lam SK, et al. Nipah virus: a recently emergent deadly paramyxovirus. *Science*. 2000;288:1432–5. <https://doi.org/10.1126/science.288.5470.1432>
2. Arunkumar G, Chandni R, Mourya DT, Singh SK, Sadanandan R, Sudan P, et al.; Nipah Investigators People and Health Study Group. Outbreak investigation of Nipah virus disease in Kerala, India, 2018. *J Infect Dis*. 2019;219:1867–78. <https://doi.org/10.1093/infdis/jiy612>
3. Luby SP, Gurley ES, Hossain MJ. Transmission of human infection with Nipah virus. *Clin Infect Dis*. 2009;49:1743–8. <https://doi.org/10.1086/647951>
4. Sweileh WM. Global research trends of World Health Organization’s top eight emerging pathogens. *Global Health*. 2017;13:9. <https://doi.org/10.1186/s12992-017-0233-9>
5. Murray K, Selleck P, Hooper P, Hyatt A, Gould A, Gleeson L, et al. A morbillivirus that caused fatal disease in horses and humans. *Science*. 1995;268:94–7. <https://doi.org/10.1126/science.7701348>
6. Marsh GA, de Jong C, Barr JA, Tachedjian M, Smith C, Middleton D, et al. Cedar virus: a novel henipavirus isolated from Australian bats. *PLoS Pathog*. 2012;8:e1002836. <http://dx.doi.org/10.1371/journal.ppat.1002836>
7. Drexler JF, Corman VM, Müller MA, Maganga GD, Vallo P, Binger T, et al. Bats host major mammalian paramyxoviruses. *Nat Commun*. 2012;3:796. <https://doi.org/10.1038/ncomms1796>
8. Wu Z, Yang L, Yang F, Ren X, Jiang J, Dong J, et al. Novel henipa-like virus, Mojiang paramyxovirus, in rats, China, 2012. *Emerg Infect Dis*. 2014;20:1064–6. <https://doi.org/10.3201/eid2006.131022>
9. Middleton DJ, Morrissy CJ, van der Heide BM, Russell GM, Braun MA, Westbury HA, et al. Experimental Nipah virus infection in pteropid bats (*Pteropus poliocephalus*). *J Comp Pathol*. 2007;136:266–72. <https://doi.org/10.1016/j.jcpa.2007.03.002>
10. Luby SP, Rahman M, Hossain MJ, Blum LS, Husain MM, Gurley E, et al. Foodborne transmission of Nipah virus, Bangladesh. *Emerg Infect Dis*. 2006;12:1888–94. <https://doi.org/10.3201/eid1212.060732>
11. Middleton DJ, Westbury HA, Morrissy CJ, van der Heide BM, Russell GM, Braun MA, et al. Experimental Nipah virus infection in pigs and cats. *J Comp Pathol*. 2002;126:124–36. <https://doi.org/10.1053/jcpa.2001.0532>
12. Luby SP, Gurley ES, Hossain MJ. Transmission of human infection with Nipah virus. *Clin Infect Dis*. 2009;49:1743–8. <https://doi.org/10.1086/647951>
13. Sejvar JJ, Hossain J, Saha SK, Gurley ES, Banu S, Hamadani JD, et al. Long-term neurological and functional outcome in Nipah virus infection. *Ann Neurol*. 2007; 62:235–42. <https://doi.org/10.1002/ana.21178>
14. Tan CT, Goh KJ, Wong KT, Sarji SA, Chua KB, Chew NK, et al. Relapsed and late-onset Nipah encephalitis. *Ann Neurol*. 2002;51:703–8. <https://doi.org/10.1002/ana.10212>
15. Enchéry F, Horvat B. Understanding the interaction between henipaviruses and their natural host, fruit bats: paving the way toward control of highly lethal infection in humans. *Int Rev Immunol*. 2017;36:108–21. <https://doi.org/10.1080/08830185.2016.1255883>
16. Halpin K, Young PL, Field HE, Mackenzie JS. Isolation of Hendra virus from pteropid bats: a natural reservoir of Hendra virus. *J Gen Virol*. 2000;81:1927–32. <https://doi.org/10.1099/0022-1317-81-8-1927>
17. de Araujo J, Lo MK, Tamin A, Ometto TL, Thomazelli LM, Nardi MS, et al. Antibodies against henipa-like viruses in Brazilian bats. *Vector Borne Zoonotic Dis*. 2017;17:271–4. <https://doi.org/10.1089/vbz.2016.2051>
18. Luby SP. The pandemic potential of Nipah virus. *Antiviral Res*. 2013;100:38–43. <https://doi.org/10.1016/j.antiviral.2013.07.011>

19. Mathieu C, Horvat B. Henipavirus pathogenesis and antiviral approaches. *Expert Rev Anti Infect Ther.* 2015;13:343–54. <https://doi.org/10.1586/14787210.2015.1001838>
20. Olson JG, Rupprecht C, Rollin PE, An US, Niezgodna M, Clemins T, et al. Antibodies to Nipah-like virus in bats (*Pteropus lylei*), Cambodia. *Emerg Infect Dis.* 2002;8:987–8. <https://doi.org/10.3201/eid0809.010515>
21. Reynes J-M, Cournor D, Ong S, Faure C, Seng V, Molia S, et al. Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis.* 2005;11:1042–7. <https://doi.org/10.3201/eid1107.041350>
22. Thomas HA. BioEdit, a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser.* 1999;41:95–8.
23. Kumar S, Stecher G, Tamura K. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Mol Biol Evol.* 2016;33:1870–4. <https://doi.org/10.1093/molbev/msw054>
24. Krump-Konvalinkova V, Bittinger F, Unger RE, Peters K, Lehr HA, Kirkpatrick CJ. Generation of human pulmonary microvascular endothelial cell lines. *Lab Invest.* 2001;81:1717–27. <https://doi.org/10.1038/labinvest.3780385>
25. Mlíkovský J. Correct name for the Indian flying fox (*Pteropodidae*). *Vespertilio.* 2012;16:203–4.
26. Saccone C, Attimonelli M, Sbisà E. Structural elements highly preserved during the evolution of the D-loop-containing region in vertebrate mitochondrial DNA. *J Mol Evol.* 1987;26:205–11. <https://doi.org/10.1007/BF02099853>
27. Dool SE, Puechmaile SJ, Foley NM, Allegrini B, Bastian A, Mutumi GL, et al. Nuclear introns outperform mitochondrial DNA in inter-specific phylogenetic reconstruction: lessons from horseshoe bats (*Rhinolophidae*: Chiroptera). *Mol Phylogenet Evol.* 2016;97:196–212. <https://doi.org/10.1016/j.ympev.2016.01.003>
28. Reynard O, Volchkov VE. Characterization of a novel neutralizing monoclonal antibody against Ebola virus GP. *J Infect Dis.* 2015;212(Suppl 2):S372–8. <https://doi.org/10.1093/infdis/jiv303>
29. Talekar A, Pessi A, Glickman F, Sengupta U, Briese T, Whitt MA, et al. Rapid screening for entry inhibitors of highly pathogenic viruses under low-level biocontainment. *PLoS One.* 2012;7:e30538. <https://doi.org/10.1371/journal.pone.0030538>
30. Whitt MA. Generation of VSV pseudotypes using recombinant Δ G-VSV for studies on virus entry, identification of entry inhibitors, and immune responses to vaccines. *J Virol Methods.* 2010;169:365–74. <http://dx.doi.org/10.1016/j.jviromet.2010.08.006>
31. Mathieu C, Guillaume V, Sabine A, Ong KC, Wong KT, Legras-Lachuer C, et al. Lethal Nipah virus infection induces rapid overexpression of CXCL10. *PLoS One.* 2012;7:e32157. <https://doi.org/10.1371/journal.pone.0032157>
32. Satterfield BA, Cross RW, Fenton KA, Agans KN, Basler CF, Geisbert TW, et al. The immunomodulating V and W proteins of Nipah virus determine disease course. *Nat Commun.* 2015;6:7483. <https://doi.org/10.1038/ncomms8483>
33. Guillaume V, Aslan H, Ainouze M, Guerbois M, Wild TF, Buckland R, et al. Evidence of a potential receptor-binding site on the Nipah virus G protein (NiV-G): identification of globular head residues with a role in fusion promotion and their localization on an NiV-G structural model. *J Virol.* 2006;80:7546–54. <https://doi.org/10.1128/JVI.00190-06>
34. Xu K, Broder CC, Nikolov DB. Ephrin-B2 and ephrin-B3 as functional henipavirus receptors. *Semin Cell Dev Biol.* 2012;23:116–23. <https://doi.org/10.1016/j.semcdb.2011.12.005>
35. Porotto M, Rockx B, Yokoyama CC, Talekar A, Devito I, Palermo LM, et al. Inhibition of Nipah virus infection in vivo: targeting an early stage of paramyxovirus fusion activation during viral entry. *PLoS Pathog.* 2010;6:e1001168. <https://doi.org/10.1371/journal.ppat.1001168>
36. Desmyter J, Melnick JL, Rawls WE. Defectiveness of interferon production and of rubella virus interference in a line of African green monkey kidney cells (Vero). *J Virol.* 1968;2:955–61.
37. Wong KT, Grosjean I, Brisson C, Blanquiere B, Fevre-Montange M, Bernard A, et al. A golden hamster model for human acute Nipah virus infection. *Am J Pathol.* 2003;163:2127–37. [https://doi.org/10.1016/S0002-9440\(10\)63569-9](https://doi.org/10.1016/S0002-9440(10)63569-9)
38. Bossart KN, Tachedjian M, McEachern JA, Cramer G, Zhu Z, Dimitrov DS, et al. Functional studies of host-specific ephrin-B ligands as henipavirus receptors. *Virology.* 2008;372:357–71. <https://doi.org/10.1016/j.virol.2007.11.011>
39. Wacharapluesadee S, Lumlerdacha B, Boongird K, Wanghongsa S, Chanhom L, Rollin P, et al. Bat Nipah virus, Thailand. *Emerg Infect Dis.* 2005;11:1949–51. <https://doi.org/10.3201/eid1112.050613>
40. Hasebe F, Thuy NTT, Inoue S, Yu F, Kaku Y, Watanabe S, et al. Serologic evidence of Nipah virus infection in bats, Vietnam. *Emerg Infect Dis.* 2012;18:536–7. <https://doi.org/10.3201/eid1803.111121>
41. Choden K, Ravon S, Epstein JH, Hoem T, Furey N, Gely M, et al. *Pteropus lylei* primarily forages in residential areas in Kandal, Cambodia. *Ecol Evol.* 2019;9:4181–91. <https://doi.org/10.1002/ece3.5046>
42. Thanapongtharm W, Linard C, Wiriyarat W, Chinsorn P, Kanchanasaka B, Xiao X, et al. Spatial characterization of colonies of the flying fox bat, a carrier of Nipah virus in Thailand. *BMC Vet Res.* 2015;11:81. <http://dx.doi.org/10.1186/s12917-015-0390-0>
43. Thanapongtharm W, Paul MC, Wiratsudakul A, Wongphruksasoong V, Kalpravidh W, Wongsathapornchai K, et al. A spatial assessment of Nipah virus transmission in Thailand pig farms using multi-criteria decision analysis. *BMC Vet Res.* 2019;15:73. <https://doi.org/10.1186/s12917-019-1815-y>
44. Huynh TTT, Aarnink AJA, Drucker A, Verstegen MWA. Pig production in Cambodia, Laos, Philippines, and Vietnam: a review. *Asian J Agric Dev.* 2006;3:69–90.
45. Hegde ST, Salje H, Sazzad HMS, Hossain MJ, Rahman M, Daszak P, et al. Using healthcare-seeking behaviour to estimate the number of Nipah outbreaks missed by hospital-based surveillance in Bangladesh. *Int J Epidemiol.* 2019;48:1219–27. <https://doi.org/10.1093/ije/dyz057>
46. Daszak P, Zambrana-Torrel C, Bogich TL, Fernandez M, Epstein JH, Murray KA, et al. Interdisciplinary approaches to understanding disease emergence: the past, present, and future drivers of Nipah virus emergence. *Proc Natl Acad Sci U S A.* 2013;110(Suppl 1):3681–8. <https://doi.org/10.1073/pnas.1201243109>

Address for correspondence: Branka Horvat, CIRI, INSERM U1111, 21 Ave Tony Garnier, 69007, Lyon, France; email: branka.horvat@inserm.fr

Varicella in Adult Foreigners at a Referral Hospital, Central Tokyo, Japan, 2012–2016

Saho Takaya, Satoshi Kutsuna, Yuichi Katanami, Kei Yamamoto, Nozomi Takeshita, Kayoko Hayakawa, Yasuyuki Kato, Shuzo Kanagawa, Norio Ohmagari

We report a case series of varicella among adult foreigners at a referral hospital in central Tokyo, Japan, during 2012–2016. This series highlights differences in varicella vaccination schedules by country and epidemiology by climate and identifies immigrants and international students as high-risk populations for varicella.

Varicella is a benign disease with fever and rash caused by primary infection with varicella zoster virus (VZV) (1). Varicella is usually self-limiting but can sometimes be life-threatening, especially in adults (1). The World Health Organization estimates that 140 million varicella cases and 4,200 related deaths occur each year (1). In Japan, 1 million persons, primarily young children, have varicella every year. Of these patients, 4,000 require hospitalization, and adults make up ≈60% of this population (2).

Varicella vaccine is a live attenuated vaccine and is effective against varicella in healthy children (1). One dose of varicella vaccine reduces the number of varicella cases but can also reduce opportunities for exposure to the virus in the community and result in breakthrough varicella. Therefore, many industrialized countries now recommend 2 doses of varicella vaccine (2). In Japan, the 2-dose varicella vaccination became part of the childhood immunization schedule in October 2014; the number of varicella cases has drastically decreased since implementation (3).

We report cases of varicella in adult foreigners who were given a diagnosis at the National Center for Global Health and Medicine (NCGM) Medical Center in central Tokyo, Japan. These cases highlight major health issues, including differences in varicella vaccination programs in countries and VZV epidemiology in temperate and tropical regions, and identify

immigrants and international students as high-risk populations for varicella.

The Study

We reviewed 22 varicella cases in adult foreigners diagnosed during January 2012–December 2016 at the NCGM. During the same period, 7 cases of varicella were diagnosed in Japanese adults at the NCGM. We compiled basic demographic information for the patients (Table). Eleven case-patients were from Vietnam, 5 from China, and 1 each from 6 other countries (Cambodia, New Zealand, Belgium, Italy, Uzbekistan, and Nepal). Among 21 patients who resided in central Tokyo, 18 were international students. Fourteen patients were male, and the median age was 19 years (range 18–35 years).

The patients sought medical care on median day 2 of illness (range days 1–5). Most diagnoses of varicella were made clinically; for some case-patients, varicella was confirmed by serologic testing or PCR or antigen detection testing of skin lesions. Twenty patients received antiviral therapy (acyclovir or valacyclovir). Five case-patients were hospitalized because of severe malaise and for infection control to prevent secondary cases.

Nine patients from Vietnam and 1 from China (case-patients 3–12) were students at the same language school who lived together in the school dormitories. During this outbreak, varicella developed in 9 students during a 2-month period after the index case (case 3). Information about previous vaccination and past infection could not be obtained for most patients in this outbreak because of their inability to communicate in Japanese or English and our inability to organize translation in the first language of the patients immediately at their visits. Overall, we found that there were 81 international students in 3 dormitories: 30 (37.0%) reported previous varicella and 6 (7.4%) claimed to have been

Author affiliation: National Center for Global Health and Medicine, Tokyo, Japan

DOI: <https://doi.org/10.3201/eid2601.170565>

Table. Characteristics for 22 case-patients (adult foreigners) with varicella, National Center for Global Health and Medicine, Tokyo, Japan, 2012–2016*

Case-patient no.	Time of onset	Days after onset of index case	Age, y/sex	Country of origin	Status or occupation	Exposure source	History of varicella	Previous varicella vaccination	Hospitalization (reason)
1	2012 Apr	Not applicable	19/M	Cambodia	Student	Sibling	NA	NA	Yes (infection control)
2	2012 Jul	Not applicable	32/F	China	Housewife	Own child	No	NA	No
3	2014 Apr	Index case	18/M	Vietnam	Student	UNK	No	UNK	No
4	2014 Apr	15	18/M	Vietnam	Student	School dormitory	No	UNK	No
5	2014 Apr	15	18/M	Vietnam	Student	School dormitory	NA	NA	No
6	2014 May	19	18/M	Vietnam	Student	School dormitory	NA	NA	No
7	2014 May	35	19/M	Vietnam	Student	School	NA	NA	No
8	2014 May	41	19/F	Vietnam	Student	School dormitory	NA	UNK	No
9	2014 May	42	18/F	Vietnam	Student	School dormitory	NA	UNK	No
10	2014 Jun	52	19/M	Vietnam	Student	School	NA	NA	No
11	2014 Jun	55	23/M	Vietnam	Student	School dormitory	NA	NA	No
12	2014 Jun	56	19/M	China	Student	School dormitory	NA	NA	No
13	2016 Mar	Not applicable	18/F	Vietnam	Student	UNK	No	No	No
14	2016 Mar	Not applicable	19/F	New Zealand	Student	UNK	No	No	Yes (malaise)
15	2016 Apr	Index case	35/F	China	Housewife	UNK	No	No	Yes (malaise)
16	2016 Apr	12	34/M	China	Unemployed	Patient 15 (wife)	No	Yes	No
17	2016 May	Not applicable	19/M	China	Student	UNK	No	No	Yes (malaise, headache)
18	2016 May	Not applicable	33/M	Belgium	Tourist	UNK	UNK	NA	No
19	2016 Jul	Not applicable	21/F	Italy	Student	UNK	No	No	Yes (infection control)
20	2016 Aug	Not applicable	20/M	Uzbekistan	Student	UNK	Yes	No	No
21	2016 Sep	Not applicable	23/M	Vietnam	Student	UNK	No	UNK	No
22	2016 Oct	Not applicable	25/F	Nepal	Sales clerk	UNK	No	No	No

*Case-patients 3–12 were from an outbreak in the same language school. Not applicable indicates sporadic cases, not cluster cases. NA, not available; UNK, unknown.

vaccinated at least once. Although we contacted the school to provide postexposure prophylaxis, none of 41 students without previous VZV infection had received it.

Conclusions

Japan has >2 million foreign residents; most are from China (30%), South Korea (20%), and the Philippines (10%) (4). However, Japan does not have any vaccination requirements for foreign residents. Because varicella is not a target of the World Health Organization Expanded Program on Immunization, the vaccine is not included in the national immunization schedules of many countries.

In China, from which Japan receives most of its immigrants and tourists, varicella vaccine was approved in the late 1990s and has been given on a voluntary basis (5). Although 80% of children in kindergartens and primary schools in Beijing were reported to have received the varicella vaccine, most

had only received 1 dose, and breakthrough varicella has become a public health concern (5). In South Korea, the single-dose varicella vaccination was included in the national immunization program in 2005 and is recommended for 12–15-month-old children (6). Despite high use of the vaccine (>95%), varicella outbreaks continuously occur (6). The varicella vaccine has not been introduced into the national vaccination program of the Philippines (7).

The epidemiology of varicella differs between temperate and tropical regions. In temperate areas, most of the population contracts VZV before adolescence. In tropical areas, childhood varicella is much less common, and many persons stay susceptible to VZV until adulthood (1,8). This difference is attributed to changes in VZV transmissibility by temperature and humidity, population density, and sociologic factors, such as nursery school attendance (8).

Previous research has shown immigrants and refugees to be at high risk for varicella. A study in

Denmark during the 1980s investigated an epidemic of varicella among refugees during a preasylum phase (9). The study showed that 44% of Tamil refugees from Sri Lanka had varicella infections within 1 week to 5 months after arrival in Denmark. Considering that many immigrants contracted varicella shortly after their arrival, it was deemed necessary to check VZV immunity for immigrants before they entered that country, rather than after entry.

In an outbreak study of 18 varicella cases among Mexican-born workers in Alabama, USA (10), Mexican-born workers were 5 times more likely to be susceptible to VZV than US-born workers. Although postexposure prophylaxis vaccine was provided to susceptible workers, a second dose was not administered to many of those who needed it. This finding shows the difficulty in reaching these populations because of their lack of stable access to healthcare. Faced with a rapidly aging population, Japan has also been accepting nurses and caregivers from countries in Southeast Asia as medical personnel in healthcare facilities and nursing homes (11). Thus, nosocomial infection with VZV is also a matter of concern.

Japan had 208,000 international students during 2015 (12). More than 90% of these students were from countries in Asia; most were from China (45%), Vietnam (19%), and Nepal (8%). The demographics of the case-patients in our study were consistent with these data. In general, colleges are suitable environments for communicable disease outbreaks because of highly dense interactions among students and staff, crowded living conditions, and an influx of students from many countries (13). Because 10%–20% of persons in Japan 15–30 years of age are susceptible to VZV (14), varicella outbreaks among international students might spread into the local student population. Despite these facts, Japan does not have any regulations or recommendations regarding the health of international students. In the United States, the American College Health Association strongly recommends institutional prematriculation immunizations (15). These guidelines recommend that all college students should have 2 doses of varicella vaccine unless they have other evidence of VZV immunity.

This varicella case series occurred in multifactorial contexts. However, little is known about the immunity against VZV among foreign residents in Japan. Educational institutions that receive international students need to consider varicella as a major preventable health issue among their students. Healthcare providers for immigrants and

international students should also be aware of the risk for varicella and should verify VZV immunity or varicella vaccination status for students.

This study was supported by a grant (27-6001) from the National Center for Global Health and Medicine, Tokyo, Japan.

About the Author

Dr. Takaya is a physician at the Disease Control and Prevention Center, National Center for Global Health and Medicine, Tokyo, Japan. Her primary research interest is tropical medicine.

References

1. World Health Organization. Varicella and herpes zoster vaccines: WHO position paper; 2014 [cited 2016 Jun 2]. http://www.who.int/immunization/position_papers/WHO_pp_varicella_herpes_zoster_june2014_presentation.pdf
2. National Institute of Infectious Diseases. Japan. Varicella vaccine fact sheet; 2010 [cited 2016 Nov 4]. <http://www.mhlw.go.jp/stf2/shingi2/2r9852000000bx23-att/2r9852000000bxqx.pdf>
3. National Institute of Infectious Diseases. Japan. Varicella situation update after the introduction of two doses of varicella vaccine into the routine vaccination, from Infectious Diseases Weekly Report (IDWR), a third report [cited 2018 Apr 20]. <https://www.niid.go.jp/niid/ja/varicella-m/varicella-idwrs/7620-varicella-20171020.html>
4. Japanese Ministry of Justice. The number of foreign residents in Japan as of December 2015–2016 [cited 2016 Dec 7]. http://www.moj.go.jp/nyuukokukanri/kouhou/nyuukokukanri04_00057.html
5. Zhang X, Yu Y, Zhang J, Huang S, Wang Z, Zhang J, et al. The epidemiology of varicella cases among children in Beijing's Fengtai District from 2008 to 2012. *Vaccine*. 2014; 32:3569–72. <https://doi.org/10.1016/j.vaccine.2014.04.069>
6. Oh SH, Choi EH, Shin SH, Kim YK, Chang JK, Choi KM, et al. Varicella and varicella vaccination in South Korea. *Clin Vaccine Immunol*. 2014;21:762–8. <https://doi.org/10.1128/CVI.00645-13>
7. World Health Organization. WHO vaccine-preventable diseases: monitoring system; 2017 global summary [cited 2018 Apr 20]. http://apps.who.int/immunization_monitoring/globalsummary
8. Lolekha S, Tanthiphabha W, Sornchai P, Kosuwan P, Sutra S, Warachit B, et al. Effect of climatic factors and population density on varicella zoster virus epidemiology within a tropical country. *Am J Trop Med Hyg*. 2001;64: 131–6. <https://doi.org/10.4269/ajtmh.2001.64.131>
9. Kjersem H, Jepsen S. Varicella among immigrants from the tropics, a health problem. *Scand J Soc Med*. 1990;18:171–4. <https://doi.org/10.1177/140349489001800303>
10. Danovaro-Holliday MC, Gordon ER, Jumaan AO, Woernle C, Judy RH, Schmid DS, et al. High rate of varicella complications among Mexican-born adults in Alabama. *Clin Infect Dis*. 2004;39:1633–9. <https://doi.org/10.1086/425613>
11. Ministry of Health, Labour, and Welfare. Acceptance of foreign nurses and carers from Indonesia, the Philippines, and Vietnam [cited 2016 Dec 5]. http://www.mhlw.go.jp/stf/seisakunitsuite/bunya/koyou_roudou/koyou/gaikokujin/other22/index

12. Japan Student Services Organization. International students in Japan; 2015 [cited 2016 Nov 5]. http://www.jasso.go.jp/en/about/statistics/intl_student/data2015.html
13. Jewett A, Bell T, Cohen NJ, Buckley K, Leino EV, Even S, et al. US college and university student health screening requirements for tuberculosis and vaccine-preventable diseases, 2012. *J Am Coll Health*. 2016;64:409–15. <https://doi.org/10.1080/07448481.2015.1117465>
14. National Institute of Infectious Diseases. Japan. Immunity status for varicella-zoster virus between 2014 and 2016 [cited 2018 Apr 20]. <https://www.niid.go.jp/niid/ja/y-graphs/7200-varicella-yosoku-year2016.html>
15. American College Health Association. ACHA guidelines: recommendations for institutional prematriculation immunizations; 2014 [cited 2018 Apr 20]. https://www.acha.org/documents/resources/guidelines/ACHA_RIPI_April_2014.pdf

Address for correspondence: Satoshi Kutsuna, Disease Control and Prevention Center, National Center for Global Health and Medicine, 1-21-1, Toyama, Shinjuku, Tokyo 162-8655, Japan; email: sonare.since1192@gmail.com



EMERGING INFECTIOUS DISEASES®

January 2018

High-Consequence Pathogens

- Zika Virus Testing and Outcomes during Pregnancy, Florida, USA, 2016
- Sensitivity and Specificity of Suspected Case Definition Used during West Africa Ebola Epidemic
- Nipah Virus Contamination of Hospital Surfaces during Outbreaks, Bangladesh, 2013–2014
- Detection and Circulation of a Novel Rabbit Hemorrhagic Disease Virus, Australia
- Drug-Resistant Polymorphisms and Copy Numbers in *Plasmodium falciparum*, Mozambique, 2015
- Increased Severity and Spread of *Mycobacterium ulcerans*, Southeastern Australia
- Emergence of Vaccine-Derived Polioviruses during Ebola Virus Disease Outbreak, Guinea, 2014–2015
- Characterization of a Feline Influenza A(H7N2) Virus
- Japanese Encephalitis Virus Transmitted Via Blood Transfusion, Hong Kong, China
- Changing Geographic Patterns and Risk Factors for Avian Influenza A(H7N9) Infections in Humans, China
- Pneumonic Plague in Johannesburg, South Africa, 1904
- Dangers of Noncritical Use of Historical Plague Databases
- Recognition of Azole-Resistant Aspergillosis by Physicians Specializing in Infectious Diseases, United States
- Melioidosis, Singapore, 2003–2014
- Serologic Evidence of Fruit Bat Exposure to Filoviruses, Singapore, 2011–2016
- Expected Duration of Adverse Pregnancy Outcomes after Zika Epidemic
- Seroprevalence of Jamestown Canyon Virus among Deer and Humans, Nova Scotia, Canada
- Postmortem Findings for a Patient with Guillain-Barré Syndrome and Zika Virus Infection
- Rodent Abundance and Hantavirus Infection in Protected Area, East-Central Argentina
- Two-Center Evaluation of Disinfectant Efficacy against Ebola Virus in Clinical and Laboratory Matrices
- Phylogeny and Immunoreactivity of Human Norovirus GII.P16-GII.2, Japan, Winter 2016–17
- Mammalian Pathogenesis and Transmission of Avian Influenza A(H7N9) Viruses, Tennessee, USA, 2017
- Whole Genome Analysis of Recurrent *Staphylococcus aureus* t571/ST398 Infection in Farmer, Iowa, USA
- Visceral Leishmaniasis in Traveler to Guyana Caused by *Leishmania siamensis*, London
- Investigation of Canine-Mediated Human Rabies Death, Haiti, 2015
- Epidemiology of Cutaneous Leishmaniasis Outbreak, Waziristan, Pakistan
- Ocular Vaccinia Infection in Dairy Worker, Brazil
- Estimation of Undiagnosed *Naegleria fowleri* Primary Amebic Meningoencephalitis, United States
- Leprosy in Nonimmigrant Canadian Man without Travel

To revisit the January 2018 issue, go to:

<https://wwwnc.cdc.gov/eid/articles/issue/24/1/table-of-contents>

Geographic Distribution and Incidence of Melioidosis, Panama¹

Ana B. Araúz,² Katiana Castillo,² Erika Santiago, Yarineth Quintero,³ Enrique Adames, Boris Castillo, Amalia Rodríguez-French, German Henostroza

Melioidosis is an infection caused by *Burkholderia pseudomallei*. Most cases occur in Southeast Asia and northern Australia; <100 cases have been reported in the Americas. We conducted a retrospective study and identified 12 melioidosis cases in Panama during 2007–2017, suggesting possible endemicity and increased need for surveillance.

Burkholderia pseudomallei, a gram-negative bacillus found in the environment of some tropical and subtropical regions, is the etiologic agent of melioidosis (1–3). Most melioidosis cases in the world are reported from Southeast Asia and northern Australia; only sporadic cases are reported from other regions (4–6). In the Americas, <100 acquired cases were identified from 1947 through June 2015. Only 3 cases were reported from Panama, 1 each in 1947, 1948, and 2011. However, cases were reported in Antioquia, Colombia (1,7,8), near the border with Panama. Melioidosis might be misdiagnosed and underreported because of the lack of diagnostic resources in the rural areas where cases are most likely to occur (9,10).

People become infected with *B. pseudomallei* through inoculation in compromised derma, inhalation, or ingestion. Some evidence suggests ingestion is associated with bacteremia, even though ingestion is considered an uncommon pathway (2,3,11). Clinical manifestations of melioidosis are diverse and may include localized cutaneous infection, pneumonia, involvement of bones and joints, intraabdominal abscesses, sepsis, and even death (2,12). Diagnosis is usually made through blood cultures, but the

bacterium often is misidentified as *B. thailandensis* or *B. cepacia* (10,13).

Current treatment for melioidosis includes an induction phase of 2–6 weeks with intravenous ceftazidime (or carbapenem for more severe cases), followed by a 2–6-month eradication phase using oral trimethoprim/sulfamethoxazole (TMP/SMX) or doxycycline. Doxycycline previously has been used for eradication, but recent studies suggest TMP/SMX is more effective (2,3).

During the previous 10 years, cases of melioidosis have been identified in different regions of Panama. The aim of this study is to describe the clinical signs and symptoms and geographic distribution of melioidosis in Panama to elucidate the current status of the disease in the Americas.

The Study

We conducted a retrospective review of medical records from 2007–2017 from 2 national tertiary level hospitals in Panama City. Hospital Santo Tomás and Complejo Hospitalario Metropolitano Dr. Arnulfo Arias Madrid (CHMDrAAM) are the 2 main referral hospitals for Panama and are in the capital city.

We reviewed specimen registries from the microbiology laboratories at each institution; we also identified 1 case from a poster presented at a national scientific meeting. We included patients who had a culture-positive report for *B. pseudomallei* and a clinical diagnosis of melioidosis at discharge. We excluded 2 patients with culture-positive results for *B. pseudomallei* because their clinical diagnoses were not related to their test results.

The microbiology laboratories of Hospital Santo Tomás and CHDrAAM identified *B. pseudomallei*

Author affiliations: Hospital Santo Tomás, Panama City, Panama (A.B. Araúz, K. Castillo, E. Santiago, Y. Quintero, E. Adames, A. Rodríguez-French); Universidad de Panamá, Panama City (A.B. Araúz, E. Adames, A. Rodríguez-French); Complejo Hospitalario Metropolitano Dr. Arnulfo Arias Madrid, Panama City (B. Castillo); University of Alabama at Birmingham, Birmingham, Alabama, USA (G. Henostroza)

DOI: <https://doi.org/10.3201/eid2601.180870>

¹Preliminary results from this study were presented at IDWeek, October 26–30, 2016, New Orleans, Louisiana, USA.

²These authors contributed equally to this article.

³Current affiliation: Hospital del Niño, Panama City, Panama.

strains from blood culture by using BacT/ALERT 3D Microbial Identification System (bioMérieux, <https://www.biomerieux.com>). Both laboratories also obtained isolates of *B. pseudomallei* from clinical specimens inoculated in Columbia agar prepared with 5% sheep blood and in MacConkey agar. Both laboratories used the VITEK 2 (bioMérieux) system to identify strains, which were then sent to the national reference laboratory at Instituto Conmemorativo Gorgas de Estudios de la Salud in Panama City, Panama, for microbiology confirmation and antimicrobial susceptibility testing.

We used a standardized form to collect data and then entered data into an Excel (Microsoft, <https://www.microsoft.com>) database for descriptive analysis. The Institutional Review Board of Hospital Santo Tomás reviewed and approved this study.

We identified 12 cases that occurred during 2007–2017: 8 in Hospital Santo Tomás and 4 in CHM-DrAAM. We obtained medical records for all but 1 case, for which we obtained data from a poster presented at the 37th American College of Physicians Annual Central America Chapter Meeting in Panama City, Panama, in 2015 (14).

The mean age of cases was 50.3 years (SD ±12 years); most (9/12) patients were male. We noted bacteremia and sepsis in most (8/12) cases, pneumonia in 6 cases, and intraabdominal abscesses in 4 cases. Other signs and symptoms included endocarditis, meningitis, osteomyelitis, and septic arthritis (Table). Diabetes mellitus was the predominant risk factor. Most patients came from rural areas or suburbs of Panama City (Figure), and none reported travel outside of Panama.

Table. Clinical and epidemiologic characteristics of patients with melioidosis, Panama, 2007–2017*

Characteristics	Patient no.											
	1	2	3	4	5†	6	7	8	9	10	11	12
Age, y/sex	29/M	72/F	31/M	42/F	47/M	47/M	54/M	60/M	61/M	59/M	42/F	60/M
Area of origin	Oeste Pmá	Darién	Pmá	Darién	Oeste Pmá	Darién	Oeste Pmá	Coclé	Darién	Pmá	Colón	Pmá
Date of illness onset	Oct 2007	Aug 2009	Oct 2009	Nov 2009	NA	May 2012	Jul 2014	Aug 2015	Dec 2015	Aug 2016	Oct 2016	Jun 2017
Occupation	NA	NA	NA	NA	NA	NA	NA	Farmer	Farmer	Driver	Housewife	Retired
Risk factors	DM	DM	DM	DM	DM	CKD	DM	DM	DM	DM	DM	None
Duration of symptoms, wks	1	4	4	2	4	NA	NA	1	2	1	3	4
Symptoms												
Fever	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y	Y
Cough	Y	N	N	N	N	N	Y	Y	Y	Y	Y	N
Dyspnea	Y	N	Y	N	N	N	Y	Y	Y	Y	Y	N
Abdominal pain	N	Y	Y	Y	Y	N	Y	N	N	Y	N	N
Jaundice	N	N	N	Y	N	N	N	N	N	N	N	N
Seizures	N	N	N	N	N	N	N	Y	N	N	N	N
Joint pain	N	N	N	N	N	N	N	N	Y	N	N	Y
Diagnosis												
Bacteremia	Y	Y	N	N	Y	N	Y	Y	Y	Y	Y	N
Septic shock	N	N	N	Y	Y	N	Y	Y	Y	Y	Y	N
Pneumonia	Y	N	N	N	N	N	Y	Y	Y	Y	Y	N
UTI	Y	N	N	N	N	N	N	N	N	Y	N	N
Spleen abscess	N	Y	N	Y	N	N	N	N	N	N	N	N
Pancreatic abscess	N	N	N	N	Y	N	N	N	N	N	N	N
Liver abscess	N	N	Y	Y	N	N	N	N	N	N	N	N
Endocarditis	N	N	N	N	Y	N	N	N	N	N	N	N
Osteomyelitis	N	N	N	N	N	Y	N	N	N	N	N	N
Septic arthritis	N	N	N	N	N	N	N	N	Y	N	N	Y
Meningitis	N	N	N	N	N	N	N	Y	N	N	N	N
Positive culture	Blood, urine	Blood	LA	LA	Blood	TA	Blood	Blood, sputum, CSF	Blood, joint fluid	Blood, sputum, urine	Blood	SCA
Treatment	IPM, CAZ, TMP/SMX	MEM, TMP/SMX	IPM, TMP/SMX	IPM, FEP, TMP/SMX	CAR	IPM	MEM	MEM, CAZ	MEM	MEM, CAZ	MEM, CAZ	MEM
Outcome	Rec	Alive	Alive	Alive	Alive	Alive	Died	Died	Died	Died	Died	Alive

*CAR, carbapenem; CAZ, ceftazidime; CKD, chronic kidney disease; CSF, cerebrospinal fluid; DM, diabetes mellitus; FEP, cefepime; IPM, imipenem; LA, liver abscess; MEM, meropenem; N, no; NA, not available; Pmá, Panamá; SCA, sternoclavicular abscess; TA, tibial abscess; TMP/SMX, trimethoprim/sulfamethoxazol; UTI, urinary tract infection; Y, yes.

†Information adapted from abstract of poster presented by E. Brid at American College of Physicians Central America Chapter Scientific Meeting, Panama City, Panama, 2015 Feb 27 (14).

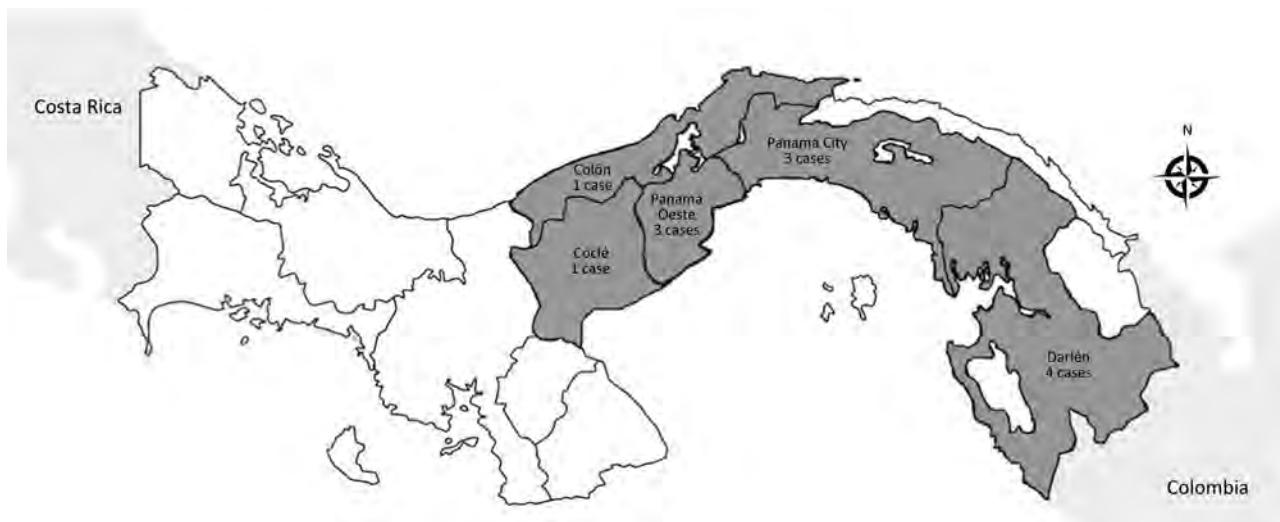


Figure. Regional distribution of melioidosis cases in Panama, 2007–2017.

All cases occurred during the rainy season, which is May–November in Panama. Five patients (41.7%) died while hospitalized; these patients had the most severe clinical manifestations of the disease, bacteremia, pneumonia, and septic shock, similar to cases reported from Central America (15).

Rapid microbiologic identification of *B. pseudomallei* is necessary to initiate appropriate, life-saving treatments. However, laboratory results can take >48 hours, delaying appropriate antimicrobial drug therapy. Of the 7 patients in this study who survived, records showed they were treated with TMP/SMX or doxycycline, but the length of antimicrobial drug treatments were not noted in the records.

Conclusions

The increase in reports of melioidosis in the Americas requires greater awareness of this disease among clinicians, especially those caring for patients with diabetes. Melioidosis often is misdiagnosed as pulmonary tuberculosis and scrofula (10); we found 2 misidentified clinical cases in our study. More studies are needed to identify specific high-risk areas and transmission routes in the Americas. Such insights can inform earlier clinical suspicion and guide the formulation of prevention strategies.

Because the clinical signs and symptoms of melioidosis are nonspecific, microbiologic identification is crucial to diagnosis. Thus, improved laboratory capacity is critical to improve patient outcomes in affected areas to aid epidemiologic and antibiotic susceptibility surveillance efforts. Collaboration among countries in the region could drive efforts to describe the origins of this disease and the actual prevalence in the Americas.

Our study has limitations because we collected data retrospectively and only included the most severe cases in Panama. Melioidosis occurs more frequently in rural areas, and cases might not be identified because of the lack of laboratory or diagnostic tools. We provide a perspective on the processes that hinder our knowledge of this disease in Panama, such as lack of surveillance data and inadequate laboratory capacity. Our data justify the need for increased surveillance for melioidosis and reinforce the need for complete epidemiologic data and adequate strain storage for further genetic analysis. Epidemiologic studies of seroprevalence, environmental sampling, and increased access to PCR techniques and broth microdilution testing are needed to determine whether *B. pseudomallei* is endemic to Panama and to improve treatment outcomes.

Acknowledgments

We thank the infectious diseases, pulmonology, intensive care, and internal medicine departments of Hospital Santo Tomás for their collaboration. We thank the microbiology laboratories of Hospital Santo Tomás and CHMDrAAM, and the National Public Health Reference Central Laboratory of Instituto Conmemorativo Gorgas de Estudios de la Salud for information related to the strains. We also thank Edgardo Brid for facilitating information related to his poster presentation on a case of melioidosis.

About the Author

Dr. Araúz is an infectious disease specialist in Hospital Santo Tomás and professor of internal medicine at Universidad de Panama. Her primary research interest is in tropical diseases with emphases on melioidosis, HIV, histoplasmosis, and tuberculosis.

References

1. Benoit TJ, Blaney DD, Doker TJ, Gee JE, Elrod MG, Rolim DB, et al. A review of melioidosis cases in the Americas. *Am J Trop Med Hyg*. 2015;93:1134-9. <https://doi.org/10.4269/ajtmh.15-0405>
2. Currie BJ. Melioidosis: evolving concepts in epidemiology, pathogenesis, and treatment. *Semin Respir Crit Care Med*. 2015;36:111-25. <https://doi.org/10.1055/s-0034-1398389>
3. Wiersinga WJ, Virk HS, Torres AG, Currie BJ, Peacock SJ, Dance DAB, et al. Melioidosis. *Nat Rev Dis Primers*. 2018;4:17107. <https://doi.org/10.1038/nrdp.2017.107>
4. Perumal Samy R, Stiles BG, Sethi G, Lim LHK. Melioidosis: clinical impact and public health threat in the tropics. *PLoS Negl Trop Dis*. 2017;11:e0004738. <https://doi.org/10.1371/journal.pntd.0004738>
5. Chewapreecha C, Holden MT, Vehkala M, Välimäki N, Yang Z, Harris SR, et al. Global and regional dissemination and evolution of *Burkholderia pseudomallei*. *Nat Microbiol*. 2017;2:16263. <https://doi.org/10.1038/nmicrobiol.2016.263>
6. Currie B, Dance D, Cheng A. The global distribution of *Burkholderia pseudomallei* and melioidosis: an update. *Trans R Soc of Trop Med Hyg*. 2008;102:S1-4. [https://doi.org/10.1016/S0035-9203\(08\)70002-6](https://doi.org/10.1016/S0035-9203(08)70002-6)
7. Montúfar FE, Ochoa JE, Ortega H, Franco L, Montúfar MC, Monsalve A, et al. Melioidosis in Antioquia, Colombia: an emerging or endemic disease? a cases series. *Int J Infect Dis*. 2015;37:50-7. <https://doi.org/10.1016/j.ijid.2015.05.023>
8. Adames E, Mendez A, Barrios J. Hepatic and skin abscess by *Burkholderia pseudomallei* [in Spanish]. *Rev méd cient*. 2013;26:34-44.
9. Limmathurotsakul D, Golding N, Dance DAB, Messina JP, Pigott DM, Moyes CL, et al. Predicted global distribution of *Burkholderia pseudomallei* and burden of melioidosis. *Nat Microbiol*. 2016;1:15008. <https://doi.org/10.1038/nmicrobiol.2015.8>
10. Kingsley PV, Arunkumar G, Tipre M, Leader M, Sathiakumar N. Pitfalls and optimal approaches to diagnose melioidosis. *Asian Pac J Trop Med*. 2016;9:515-24. <https://doi.org/10.1016/j.apjtm.2016.04.003>
11. Lim C, Peacock SJ, Limmathurotsakul D. Association between activities related to routes of infection and clinical manifestations of melioidosis. *Clin Microbiol Infect*. 2016;22:79.e1-3. <https://doi.org/10.1016/j.cmi.2015.09.016>
12. Currie BJ, Ward L, Cheng AC. The epidemiology and clinical spectrum of melioidosis: 540 cases from the 20 year Darwin prospective study. *PLoS Negl Trop Dis*. 2010;4:e900. <https://doi.org/10.1371/journal.pntd.0000900>
13. Lau SK, Sridhar S, Ho CC, Chow WN, Lee KC, Lam CW, et al. Laboratory diagnosis of melioidosis: past, present and future. *Exp Biol Med (Maywood)*. 2015;240:742-51. <https://doi.org/10.1177/1535370215583801>
14. Brid E. Melioidosis: pancreatitis y endocarditis; presentación poco común. In: Abstracts of the XXXVII American College of Physicians Annual Central America Chapter Meeting; Panama; 2015 February 27-28.
15. Sanchez-Villamil JI, Torres AG. Melioidosis in Mexico, Central America, and the Caribbean. *Trop Med Infect Dis*. 2018;3:24. <https://doi.org/10.3390/tropicalmed3010024>

Address for correspondence: Ana B. Araúz, Hospital Santo Tomás, Avenida Balboa y Calle Justo Arosemena. Panama City, Panama; email: anabelenarauz@gmail.com



**EMERGING
INFECTIOUS DISEASES®**

March 2018

Mycobacteria

- Coccidioidomycosis Outbreaks, United States and Worldwide, 1940–2015
- Multistate Epidemiology of Histoplasmosis, United States, 2011–2014
- Epidemiology of Recurrent Hand, Foot and Mouth Disease, China, 2008–2015
- Capsule Typing of *Haemophilus influenzae* by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry
- Emergence of *Streptococcus pneumoniae* Serotype 12F after Sequential Introduction of 7- and 13-Valent Vaccines, Israel
- Major Threat to Malaria Control Programs by *Plasmodium falciparum* Lacking Histidine-Rich Protein 2, Eritrea
- Use of Influenza Risk Assessment Tool for Prepandemic Preparedness
- Use of Verbal Autopsy to Determine Underlying Cause of Death during Treatment of Multidrug-Resistant Tuberculosis, India
- Increasing Prevalence of Nontuberculous Mycobacteria in Respiratory Specimens from US-Affiliated Pacific Island Jurisdictions
- Use of Genome Sequencing to Define Institutional Influenza Outbreaks, Toronto, Ontario, Canada, 2014–15
- Influenza Vaccination and Incident Tuberculosis among Elderly Persons, Taiwan
- Epidemiology and Molecular Identification and Characterization of *Mycoplasma pneumoniae*, South Africa, 2012–2015
- Prospective Observational Study of Incidence and Preventable Burden of Childhood Tuberculosis, Kenya
- Acquired Resistance to Antituberculosis Drugs in England, Wales, and Northern Ireland, 2000–2015
- Characteristics Associated with Negative Interferon- γ Release Assay Results in Culture-Confirmed Tuberculosis Patients, Texas, USA, 2013–2015
- Genetic Spatiotemporal Anatomy of *Plasmodium vivax* Malaria Episodes in Greece, 2009–2013

**To revisit the March 2018 issue, go to:
[https://wwwnc.cdc.gov/eid/articles/
issue/24/3/table-of-contents](https://wwwnc.cdc.gov/eid/articles/issue/24/3/table-of-contents)**

Shigella Bacteremia, Georgia, USA, 2002–2012¹

Melissa Tobin-D'Angelo, Nadine Oosmanally, Siri N. Wilson, Evan J. Anderson,
Suzanne Segler, Lynett Poventud

Shigella commonly causes gastroenteritis but rarely spreads to the blood. During 2002–2012, we identified 11,262 *Shigella* infections through population-based active surveillance in Georgia; 72 (0.64%) were isolated from blood. Bacteremia was associated with age ≥ 18 years, black race, and *S. flexneri*. More than half of patients with bacteremia were HIV-infected.

Shigella is among the more common bacterial causes of diarrhea. In the United States, $\approx 500,000$ illnesses occur annually, and prevalence ranges from 3.8 to 5 cases per 100,000 population (1,2). Transmission occurs not only through contaminated food and water but also through fecal–oral transmission; ingestion of only 10–100 *Shigella* organisms can cause disease (1,3). Risk factors for *Shigella* include daycare attendance, international travel, and men having sex with men (1,4). Although 4 main serogroups exist, *S. sonnei* accounts for $\approx 70\%$ of US isolates, and *S. flexneri* accounts for $\approx 24\%$ (1).

Shigella bacteremia is uncommon, and risk factors are not well described in the United States. Internationally, many patients with *Shigella* bacteremia are HIV infected (5,6). In the United States, case series describe *Shigella* bacteremia in children < 1 year of age and in adults with malnutrition, HIV infection, and other immunocompromising conditions (e.g., diabetes mellitus, malignancy) (7,8). Data collected by the Georgia Department of Public Health as part of the Foodborne Disease Active Surveillance Network (FoodNet) provided an opportunity to better understand the epidemiology of *Shigella* bacteremia.

Author affiliations: Georgia Department of Public Health, Atlanta, Georgia, USA (M. Tobin-D'Angelo, N. Oosmanally, S.N. Wilson, L. Poventud); Georgia Emerging Infections Program, Atlanta (M. Tobin-D'Angelo, N. Oosmanally, E.J. Anderson, S. Segler); Emory University School of Medicine, Atlanta (E.J. Anderson); Veterans Administration Medical Center, Atlanta (S. Segler)

DOI: <https://doi.org/10.3201/eid2601.181698>

The Study

The Georgia Department of Public Health participates in FoodNet as part of the Emerging Infections Program, a collaboration between 10 US sites and the Centers for Disease Control and Prevention. FoodNet conducts active surveillance for laboratory-confirmed infections of 7 bacterial and 2 parasitic pathogens commonly transmitted through food (1). Georgia residents who had laboratory-confirmed *Shigella* were identified through FoodNet surveillance data during 2002–2012. We excluded duplicates, defined as an additional positive laboratory test within 30 days of the original diagnosis with the same *Shigella* serogroup. We also excluded isolates from nonblood and nonfecal sources or unknown sources from the analyses. In addition, after initial case-counts, we excluded *S. boydii* and *S. dysenteriae* because of small numbers. For patients with *Shigella*, we obtained HIV status from the Georgia Department of Public Health HIV surveillance data collected in the Enhanced HIV/AIDS Reporting System, which included the year of diagnosis, AIDS status, and route of HIV transmission (if known). We compared case-patient information between patients with fecal versus blood isolates and described factors associated with HIV among patients with bacteremia. We used ArcView GIS version 10.3 (<https://www.esri.com>) to characterize case-patients by county.

The Georgia Public Health Laboratory performed *Shigella* identification, serogroup determination, serotyping, and pulse-field gel electrophoresis. Detailed serogroup and serotype data were available for 2005–2012.

We analyzed data using SAS version 9.3 (<https://www.sas.com>). We created logistic regression models using significant variables and possible confounders and identified the final model using Score Selection.

¹The findings of this study were presented in part at IDWeek 2013, October 2–6, 2013, San Francisco, CA, USA.

For 2002–2012, we identified 11,262 *Shigella* infections among Georgia residents. During this time, 10,806 (96.0%) of cultures were isolated from feces, and 72 (0.66%) were isolated from blood. We excluded 13 *S. dysenteriae* (1 blood isolate) and 31 *S. boydii* (all fecal isolates) from further analysis. Patients with *Shigella* bacteremia were concentrated in the Atlanta metropolitan area (Table). Fifty-three (74%) blood isolates versus 3,089 (29%) fecal isolates were from patients ≥ 18 years of age. No bacteremia cases were outbreak-associated. Only 1 (0.87%) of 114 patients with documented international travel had bacteremia. Demographic variables significantly associated with bacteremia on bivariate analysis included male sex, black race, and residence in the Atlanta metropolitan area. Analysis of clinical variables demonstrated that patients with bacteremia were more likely to be hospitalized (61% vs. 17%; $p < 0.001$) and to die (Table). Male sex, age ≥ 18 years, and *S. flexneri* serotypes remained significant on multivariate analysis.

Thirty-seven (51%) of the 72 patients with bacteremia were known to be HIV-infected. All but 3 HIV-infected patients resided in the Atlanta metropolitan area. Among those known to be HIV-infected, 92% were black, 97% had a known AIDS diagnosis, 97% were male, and 68% were known to be men who have sex with men (MSM), a risk factor for HIV acquisition.

Among *Shigella* fecal isolates typed from 2005 to 2012, *S. sonnei* predominated (6,017 [89%] vs. 710 [10%] *S. flexneri*). In contrast, equal numbers of *S. sonnei* and *S. flexneri* (26 cases each) were identified from blood ($p < 0.01$ for the difference between proportions in the blood vs. feces). *S. flexneri* serotype 2a comprised 5 isolates, serotype 3 comprised 6 isolates, and serotype 4a comprised 9 isolates, for a total of 85% of these 26

isolates. No serogroup trend over time was apparent with *S. flexneri*; the number of bacteremia cases ranged from 1 to 8 per year, peaking in 2009, with a single case each in 2002, 2006, and 2008. For *S. sonnei*, 2–6 bacteremia cases were identified per year, peaking in 2003, with a single case each in 2009 and 2010. Among bacteremia patients with serogroup determination, factors associated with HIV infection were black race (odds ratio 5.2 [95% CI 1.3–20.6]) and *S. flexneri* infection (odds ratio 40.4 [95% CI 8.0–204.9]).

Conclusions

The relative predominance of *S. flexneri* among blood isolates in comparison to fecal isolates in this analysis is noteworthy. In other large international series, *S. flexneri* has been identified in most bacteremia patients (5,8,9). More than half of the bacteremia patients in our study were known to be HIV-infected. Other researchers have noted the prominence of *S. flexneri* among MSM and HIV-infected persons (1,4,10,11). In data from South Africa, *S. flexneri* serotype 2a was also identified in 30% of invasive isolates (6). It is unclear whether these serotypes might have increased virulence or might be more common because of transmission networks, particularly among the HIV-infected patients in this study. It is clear, however, that HIV infection correlates with the epidemiology of *Shigella* bacteremia, particularly in the Atlanta area. Some of the demographic factors associated with bacteremia (e.g., black race, identification of *S. flexneri*) also were associated with HIV infection within the subset of patients with bacteremia. The predominance of the MSM risk factor among HIV-infected patients, along with the low infectious dose and possibility of sexual transmission of *Shigella*, make it possible that

Table. Epidemiologic and clinical characteristics of shigellosis cases, Georgia, USA, 2002–2012

Characteristic	Isolates, no. positive/total no. (%) [*]			Odds ratio (95% CI)	
	All <i>Shigella</i>	Feces	Blood	Univariate [†]	Multivariate [‡]
Outbreak-associated case [‡]	591/3,681 (16.1)	586/3,509 (16.7)	0/38	15.46 (0.95–251.97)	
International travel-acquired case [§]	114/3,681 (8.3)	113/1,315 (8.6)	1/17 (5.9)	1.51 (0.20–11.5)	
Hospitalized patient	1,940/10,443 (18.6)	1,826/10,804 (16.9)	44/72 (61.1)	0.10 (0.06–0.18)	
Died	35/11,262 (0.31)	29/8,823 (0.3)	2/66 (3.0)	0.11 (0.25–0.45)	
Atlanta resident [¶]	3,793/11,262 (33.7)	3,647/10,794 (33.8)	50/72 (69.4)	4.46 (2.70–7.38)	
Patient race/ethnicity					
White	4,396/9,502 (46.3)	4,782/9,026 (53.0)	12/71 (16.9)	7.36 (4.10–13.20)	
Other	484/9,502 (5.1)	364/9,026 (4.0)	1/71 (1.4)	6.50 (0.40–105.15)	
Hispanic	874/7,545 (11.6)	844/6,405 (13.2)	1/63 (1.6)	8.18 (1.13–59.04)	
Black	4,082/9,502 (43.0)	3,880/9,026 (43.0)	58/71 (81.7)	6.03 (3.30–11.03)	5.40 (2.94–9.89)
Male sex	5,500/11,196 (49.1)	5,348/10,744 (49.8)	51/72 (70.8)	2.45 (1.47–4.08)	1.50 (0.83–2.68)
Age ≥ 18 y	3,321/11,262 (29.5)	3,089/10,773 (28.7)	53/72 (73.6)	6.83 (4.04–11.56)	4.61 (2.55–8.34)
<i>S. flexneri</i> serogroup [§]	755/6,960 (10.8)	710/6,742 (10.5)	26/52 (50.0)	8.50 (4.91–14.72)	1.96 (1.06–3.61)

^{*}Unknown results were excluded.

[†]Odds ratio in comparison to isolation from feces. Blank cells indicate variables were not included in the final model.

[‡]Final logistic regression model results (cases with missing data were excluded from the multivariate analysis).

[§]Reported only for 2005–2012.

[¶]Atlanta resident is a patient residing in the 20-county metropolitan statistical area.

we could be missing outbreaks within this population (7). Clinicians caring for HIV-infected patients should be aware of the possibility of *Shigella* bacteremia. Additionally, identification of *Shigella* bacteremia in an adult should prompt HIV testing unless another immunocompromising condition exists.

Limitations of our study include the unavailability of epidemiologic and clinical data for all patients in the study. We had information about HIV status only for patients with *Shigella* bacteremia. Other clinical characteristics that might be associated with *Shigella* bacteremia were not collected and could not be analyzed (e.g., malignancy, transplantation) (4,10). Finally, some epidemiologic information and detailed identification of *Shigella* serogroups and serotypes was not available until 2005.

In summary, although *S. sonnei* predominated among fecal isolates in this study, similar numbers of *S. sonnei* and *S. flexneri* were identified in blood cultures. *Shigella* bacteremia, particularly when caused by *S. flexneri*, should prompt evaluation for a concomitant HIV infection among certain adult populations.

Acknowledgments

We thank Rodriques Lambert for linking of HIV data. We also thank Monica M. Farley and Wendy Baughman for oversight of collection of *Shigella* surveillance data.

This study was funded through the Centers for Disease Control and Prevention cooperative agreement 1U50CK000196-01.

Conflict of interest: E.J.A. received funding outside of this current study from Abbvie for consulting; MedImmune, Regeneron, and NovaVax for funding to institutions for clinical trials; and MedImmune for editorial assistance.

About the Author

Dr. Tobin-D'Angelo is the team lead for foodborne and waterborne diseases and outbreaks at the Georgia Department of Public Health and the Georgia representative to FoodNet. Her primary research interests include foodborne and waterborne infections and the impact of changes in laboratory testing on the surveillance of infectious diseases.

References

- Shiferaw B, Shallow S, Marcus R, Segler S, Soderlund D, Hardnett FP, et al.; Emerging Infections Program FoodNet Working Group. Trends in population-based active surveillance for shigellosis and demographic variability in FoodNet sites, 1996–1999. *Clin Infect Dis*. 2004;38 (Suppl 3):S175–80. <https://doi.org/10.1086/381584>
- Centers for Disease Control and Prevention. Vital signs: incidence and trends of infection with pathogens transmitted commonly through food—Foodborne Diseases Active Surveillance Network, 10 U.S. sites, 1996–2010. *MMWR Morb Mortal Wkly Rep*. 2011;60:749–55.
- DuPont HL, Levine MM, Hornick RB, Formal SB. Inoculum size in shigellosis and implications for expected mode of transmission. *J Infect Dis*. 1989;159:1126–8. <https://doi.org/10.1093/infdis/159.6.1126>
- Tauxe RV, McDonald RC, Hargrett-Bean N, Blake PA. The persistence of *Shigella flexneri* in the United States: increasing role of adult males. *Am J Public Health*. 1988;78:1432–5. <https://doi.org/10.2105/AJPH.78.11.1432>
- Davies NE, Karstaedt AS. *Shigella bacteraemia* over a decade in Soweto, South Africa. *Trans R Soc Trop Med Hyg*. 2008;102:1269–73. <https://doi.org/10.1016/j.trstmh.2008.04.037>
- Keddy KH, Sooka A, Crowther-Gibson P, Quan V, Meiring S, Cohen C, et al.; Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA). Systemic shigellosis in South Africa. *Clin Infect Dis*. 2012;54:1448–54. <https://doi.org/10.1093/cid/cis224>
- Hawkins C, Taiwo B, Bolon M, Julka K, Adewole A, Stosor V. *Shigella sonnei* bacteremia: two adult cases and review of the literature. *Scand J Infect Dis*. 2007;39:170–3. <https://doi.org/10.1080/00365540600786580>
- Struelens MJ, Patte D, Kabir I, Salam A, Nath SK, Butler T. *Shigella* septicemia: prevalence, presentation, risk factors, and outcome. *J Infect Dis*. 1985;152:784–90. <https://doi.org/10.1093/infdis/152.4.784>
- Greenberg D, Marcu S, Melamed R, Lifshitz M. *Shigella* bacteremia: a retrospective study. *Clin Pediatr (Phila)*. 2003;42:411–5. <https://doi.org/10.1177/000992280304200504>
- Aragón TJ, Vugia DJ, Shallow S, Samuel MC, Reingold A, Angulo FJ, et al. Case-control study of shigellosis in San Francisco: the role of sexual transmission and HIV infection. *Clin Infect Dis*. 2007;44:327–34. <https://doi.org/10.1086/510593>
- Baer JT, Vugia DJ, Reingold AL, Aragon T, Angulo FJ, Bradford WZ. HIV infection as a risk factor for shigellosis. *Emerg Infect Dis*. 1999;5:820–3. <https://doi.org/10.3201/eid0506.990614>

Address for correspondence: Melissa Tobin-D'Angelo, Georgia Department of Public Health, 2 Peachtree St NW, #14-243, Atlanta, GA 30303, USA; email: melissa.tobin-dangelo@dph.ga.gov

Distribution of Japanese Encephalitis Virus, Japan and Southeast Asia, 2016–2018

Ryusei Kuwata,¹ Shun Torii,¹ Hiroshi Shimoda, Supriyono, Thanmaporn Phichitraslip, Noppadol Prasertsincharoen, Hitoshi Takemae, Reu Caesar James Taga Bautista, Valeen Drex Bendette Mendio Ebor, Jose Alexander Cabiling Abella, Alan Payot Dargantes, Upik Kesumawati Hadi, Agus Setiyono, Emmanuel Tugbang Baltazar, Luzviminda Tadeja Simborio, Srihadi Agungpriyono, Sathaporn Jittapalpong, Worawut Rerkamnuaychoke, Eiichi Hondo, Ken Maeda²

During 2016–2018, we conducted surveillance for Japanese encephalitis virus (JEV) in mosquitoes and pigs in Japan, Thailand, the Philippines, and Indonesia. Phylogenetic analyses demonstrated that our isolates (genotypes Ia, Ib, III, IV) were related to JEV isolates obtained from the same regions many years ago. Indigenous JEV strains persist in Asia.

The locations of epidemics of arthropodborne viruses (arboviruses) are strongly associated with the distribution of their vectors. In general, the distribution of arboviruses can expand through the dispersal, transfer, and migration of their vector arthropods and reservoir animals. Mosquitoes transmit a variety of viral pathogens (e.g., dengue, Zika, and chikungunya viruses) and have caused a number of arboviral epidemics throughout the world (1). Japanese encephalitis virus (JEV; family *Flaviviridae*, genus *Flavivirus*) is a mosquito-borne arbovirus that causes a severe form of encephalitis in humans. JEV is distributed across most of Asia, the western Pacific, and northern Australia (2). The World Health Organization has estimated that the annual number

of Japanese encephalitis cases worldwide exceeds 60,000 (2). JEV is transmitted primarily by mosquitoes of the *Culex vishnui* subgroup, principally *Cx. tritaeniorhynchus* Giles; pigs and wading ardeid birds, such as egrets and herons, are known to be the major amplifying hosts (3).

On the basis of their genome sequences, JEVs are classified into 5 genotypes (4). JEV genotype I (GI), which has been further classified into subgenotypes GIa and GIb, and JEV GIII are the dominant lineages and have been detected widely throughout Asia. JEV GII is the third most common lineage and has been found in Indonesia, Singapore, South Korea, Malaysia, and Australia. JEV GIV and GV are rare lineages; only a few viruses of these genotypes have been isolated from Indonesia, Malaysia, and China as of October 2019. Over the past 30 years, JEV GIa has displaced GIII as the dominant lineage in many countries of Asia (5). Although the origin and spreading pattern of JEV genotypes across the world have been investigated in some reports (6,7), the exact mechanisms of JEV genotype shift remain unclear.

The Study

To study the epidemiology of arbovirus infection, we, an international team of researchers in Japan, Thailand, the Philippines, and Indonesia, conducted arbovirus surveillance in our respective countries during 2016–2018 with the support of our governments. In each country, we collected mosquitoes in and around cattle or pig housing using sweeping

Author affiliations: Yamaguchi University, Yamaguchi, Japan (R. Kuwata, S. Torii, H. Shimoda, Supriyono, K. Maeda); Kasetsart University, Bangkok, Thailand (T. Phichitraslip, N. Prasertsincharoen, S. Jittapalpong); Nagoya University, Nagoya, Japan (H. Takemae, E. Hondo); Central Mindanao University, Musuan, the Philippines (R.C.J.T. Bautista, V.D.B.M. Ebor, J.A.C. Abella, A.P. Dargantes, E.T. Baltazar, L.T. Simborio); Bogor Agricultural University, Bogor, Indonesia (U.K. Hadi, A. Setiyono, S. Agungpriyono); Rajamangala University of Technology, Chonburi, Thailand (W. Rerkamnuaychoke)

DOI: <https://doi.org/10.3201/eid2601.190235>

¹These authors contributed equally to this article.

²Current affiliation: National Institute of Infectious Diseases, Tokyo, Japan.

nets and aspirators. We collected mosquitoes that had digested blood in their midguts. We identified their species and sorted them into pools, which we used for virus isolation. We also collected serum samples from pigs and wild boars from each country to use for virus isolation.

We passed homogenized mosquito or serum samples through 0.45- μ m filters (Corning Inc., <https://www.corning.com>) and inoculated filtrates onto monolayers of 3 culture cell lines (mammalian cell lines Vero9013 and BHK-21 and mosquito cell line C6/36). We assessed cytopathic effect (CPE) daily and collected supernatants from cells that exhibited CPE. If we observed no CPE, we passaged the cells 5 times for 7 days each, after which, if virus was present, CPE should have become apparent. We extracted RNA from culture supernatants using the QIAamp Viral RNA Mini Kit (QIAGEN, <https://www.qiagen.com>) and subjected the resulting RNA to reverse transcription PCR (RT-PCR) using the QIAGEN One-Step RT-PCR Kit and 2 universal flavivirus-specific primer sets (MAMD and cFD2 or FU2 and cFD3) (8,9) to screen for flaviviruses. To determine genome sequences, we used the QIAGEN One-Step RT-PCR Kit, TaKaRa LA RT-PCR Kit version 2.1 (Takara Bio, <https://www.takarabio.com>), and Invitrogen 5' RACE System for Rapid Amplification of cDNA Ends version 2.0 (<https://www.thermofisher.com>) as needed in combination with several JEV-specific primers (Appendix Table 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-0235-App1.pdf>).

Of 945 pig serum samples, we selected 56 candidate samples for virus isolation on the basis of their RT-PCR results with the MAMD and cFD2 primers, and from these samples, we obtained the full or partial genome sequences of 5 JEV isolates (Appendix Table 2). Out of a total of 22,277 mosquitoes comprising ≥ 16 species, we obtained the full or partial genome sequences of only 2 JEV isolates (Appendix Table 3). Overall, we obtained the full-genome sequence of 4 of the 7 JEV isolates and the partial genome sequence

(envelope gene) of the remaining 3 isolates (DDBJ accession nos. LC461956–62; Table).

A preliminary study we performed showed that many pigs in these countries possessed antibodies against JEV (K. Maeda, unpub. data). We found that JEV was more often isolated from serum samples from JEV antibody-negative pigs in farms where JEV seroprevalence was low. Because JEV isolation seems to be difficult in endemic regions, we suggest selecting younger pigs, which are less likely to be JEV antibody positive, for virus isolation studies to increase the chances of success.

The 2 JEV isolates we recovered in Japan, JEV/MQ/Yamaguchi/803/2016 and JEV/MQ/Yamaguchi/804/2016, were GIa and not GIII. In a phylogenetic analysis, these viruses clustered with JEV isolates recovered from Japan (in 2013 and 2009), China (in 2007), and South Korea (in 2010) (Figure, panel A). The 2 viruses collected in Japan in 2016 were most closely related to viruses recovered in 2013 from the same site that had been sampled in our previous study (10), suggesting that this strain has been maintained in the Yamaguchi area of Japan since at least 2013.

The JEV isolate we obtained from Thailand, JEV/sw/Thailand/185/2017, was GIb and clustered with other JEV isolates detected in Thailand during 1985–2005 (11), as well as isolates from Myanmar in 2010, Cambodia in 2014 and 2015, and Singapore in 2014 (Figure, panel B). Thus, these JEVs have been maintained in Thailand and other parts of the southern peninsula of continental Asia for >30 years.

JEV is distributed extensively throughout the Philippines (12). However, only 3 JEV GIII isolates from the Philippines (which were obtained from pigs during 1984–1986) were available for genetic analysis. Our 2 Philippines-derived JEV isolates, JEV/sw/Mindanao/K3/2018 and JEV/sw/Mindanao/K4/2018, clustered with these isolates (Figure, panel C). Our 2 Indonesia JEV isolates, JEV/sw/Bali/93/2017 and JEV/sw/Bali/94/2017, were obtained from the island of Bali, where a JEV vaccination program began in March 2018

Table. JEV isolates, Japan and Southeast Asia, 2016–2018*

Virus isolate	Country	Province or city, island	Source	Collection date	DDBJ accession no.
JEV/MQ/Yamaguchi/803/2016	Japan	Yamaguchi	<i>Culex tritaeniorhynchus</i> mosquito	2016 Sep 8	LC461956†
JEV/MQ/Yamaguchi/804/2016	Japan	Yamaguchi	<i>C. tritaeniorhynchus</i> mosquito	2016 Sep 8	LC461957‡
JEV/sw/Thailand/185/2017	Thailand	Nakornnayok	Pig serum sample	2017 Aug 14	LC461958‡
JEV/sw/Mindanao/K3/2018	The Philippines	Butuan, Mindanao	Pig serum sample	2018 Mar 9	LC461959‡
JEV/sw/Mindanao/K4/2018	The Philippines	Butuan, Mindanao	Pig serum sample	2018 Mar 9	LC461960†
JEV/sw/Bali/93/2017	Indonesia	Denpasar, Bali	Pig serum sample	2017 Oct 17	LC461961‡
JEV/sw/Bali/94/2017	Indonesia	Denpasar, Bali	Pig serum sample	2017 Oct 17	LC461962‡

*JEV, Japanese encephalitis virus.

†Envelope gene sequence.

‡Full-genome sequence.

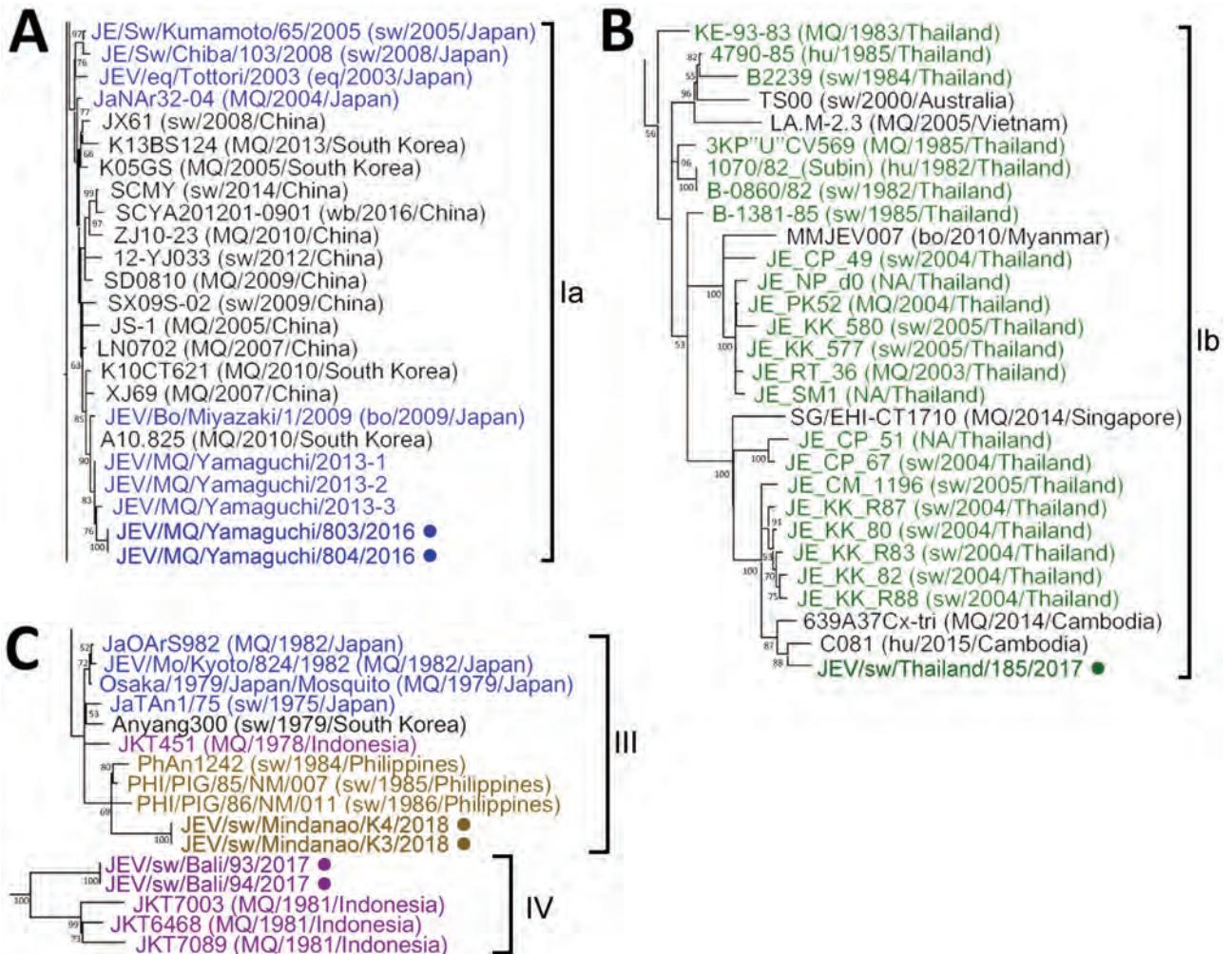


Figure. Maximum-likelihood phylogeny of JEV isolates, Japan and Southeast Asia, 2016–2018 (circles), and reference isolates constructed on the basis of the envelope gene sequence (1,500 nt). A) Genotype Ia (Gla); B) genotype GIIb; C) genotypes GIII and GIV. Tree sections were reconstructed by using MEGA6 (<https://megasoftware.net>) with 100 bootstraps under the general time-reversible model. JEVs isolated in Japan (blue), Thailand (green), the Philippines (yellow), and Indonesia (maroon) are indicated. Origin, year, and country of isolation are provided in parentheses. bo, bovine; eq, equine; hu, human; JEV, Japanese encephalitis virus; MQ, mosquito; NA, data not available; sw, swine; wb, wild boar. An expanded version of this figure showing the parent tree for the phylogenetic tree sections, along with DDBJ and GenBank accession numbers, is available (<https://wwwnc.cdc.gov/EID/article/26/11/19-0235-F1.htm>).

(13). These JEV isolates clustered with other Indonesia isolates obtained in the 1980s (Figure, panel C) (14).

Conclusions

In summary, our phylogenetic analysis revealed that the JEV isolates we obtained from Japan were GIa, the isolate from Thailand was GIb, the isolates from the Philippines were GIII, and the isolates from Indonesia were GIV (Figure, panels A–D; Appendix Figure). These results indicated that JEV GIII and GIV are still active and being maintained in parts of Asia.

Our data demonstrate that a number of the JEV isolates we obtained in select countries of Southeast Asia

during 2016–2018 were phylogenetically related to isolates reported in the same country in the 1980s, suggesting that some JEV strains have been maintained in their corresponding regions. Contrary to our expectation, the JEV transmission cycle seems to have been maintained indigenously. JEV strains are presumed to be transferred between JEV-endemic regions by movement of arthropod vectors and bird reservoirs. Nonetheless, we infer that fixation of an invading JEV strain into a new region is difficult unless the new strain possesses properties advantageous for virus growth and expansion (15). However, the genotype shift from GIII to GIa has occurred in East Asia since the 1990s, indicating that

G1a must have had some sort of growth advantage over G1II that permitted its spreading to and expansion in these countries. Our findings that JEV strain invasion in Asia is infrequent could assist in public health decision-making regarding vaccine formulation and campaign strategies.

This work was supported in part by grants-in-aid from the following funders: the Japanese Ministry of Health, Labour, and Welfare (grant no. H30-shokuhin-ippan-004); Ministry of Education, Culture, Sports, Science, and Technology and Japan Society for the Promotion of Science through the KAKENHI program (grant nos. JP15H05262 and JP15K19084); Japan Agency for Medical Research and Development through the Asia Project; and Department of Science and Technology–Philippine Council for Health Research and Development.

About the Author

Dr. Kuwata is a microbiologist at Yamaguchi University, Yamaguchi, Japan. His research interests are epidemiology and vectorborne diseases.

References

1. Arrigo NC, Weaver SC, Calisher CH. The taxonomy of arboviruses. In: Vasilakis N, Gubler DJ, editors. *Arboviruses: molecular biology, evolution and control*. Poole, UK: Caister Academic Press; 2016. p. 9–29.
2. Campbell GL, Hills SL, Fischer M, Jacobson JA, Hoke CH, Hombach JM, et al. Estimated global incidence of Japanese encephalitis: a systematic review. *Bull World Health Organ*. 2011;89:766–74E. <https://doi.org/10.2471/BLT.10.085233>
3. Samy AM, Alkhishe AA, Thomas SM, Wang L, Zhang W. Mapping the potential distributions of etiological agent, vectors, and reservoirs of Japanese encephalitis in Asia and Australia. *Acta Trop*. 2018;188:108–17. <https://doi.org/10.1016/j.actatropica.2018.08.014>
4. Nabeshima T, Morita K. Phylogeographic analysis of the migration of Japanese encephalitis virus in Asia. *Future Virol*. 2010;5:343–54. <https://doi.org/10.2217/fvl.10.21>
5. Nga PT, del Carmen Parquet M, Cuong VD, Ma SP, Hasebe F, Inoue S, et al. Shift in Japanese encephalitis virus (JEV) genotype circulating in northern Vietnam: implications for frequent introductions of JEV from Southeast Asia to East Asia. *J Gen Virol*. 2004;85:1625–31. <https://doi.org/10.1099/vir.0.79797-0>
6. Han N, Adams J, Chen P, Guo ZY, Zhong XF, Fang W, et al. Comparison of genotypes I and III in Japanese encephalitis virus reveals distinct differences in their genetic and host diversity. *J Virol*. 2014;88:11469–79. <https://doi.org/10.1128/JVI.02050-14>
7. Gao X, Liu H, Li X, Fu S, Cao L, Shao N, et al. Changing geographic distribution of Japanese encephalitis virus genotypes, 1935–2017. *Vector Borne Zoonotic Dis*. 2019;19:35–44. <https://doi.org/10.1089/vbz.2018.2291>
8. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB. Phylogeny of the genus *Flavivirus*. *J Virol*. 1998;72:73–83.
9. Scaramozzino N, Crance JM, Jouan A, DeBriel DA, Stoll F, Garin D. Comparison of flavivirus universal primer pairs and development of a rapid, highly sensitive heminested reverse transcription-PCR assay for detection of flaviviruses targeted to a conserved region of the NS5 gene sequences. *J Clin Microbiol*. 2001;39:1922–7. <http://dx.doi.org/10.1128/JCM.39.5.1922-1927.2001>
10. Kuwata R, Sugiyama H, Yonemitsu K, Van Dung N, Terada Y, Taniguchi M, et al. Isolation of Japanese encephalitis virus and a novel insect-specific flavivirus from mosquitoes collected in a cowshed in Japan. *Arch Virol*. 2015;160:2151–9. <https://doi.org/10.1007/s00705-015-2488-x>
11. Nitatpattana N, Dubot-Pères A, Gouilh MA, Souris M, Barbazan P, Yoksan S, et al. Change in Japanese encephalitis virus distribution, Thailand. *Emerg Infect Dis*. 2008;14:1762–5. <https://doi.org/10.3201/eid1411.080542>
12. Lopez AL, Aldaba JG, Roque VG Jr, Tandoc AO III, Sy AK, Espino FE, et al. Epidemiology of Japanese encephalitis in the Philippines: a systematic review. *PLoS Negl Trop Dis*. 2015;9:e0003630. <https://doi.org/10.1371/journal.pntd.0003630>
13. Im J, Balasubramanian R, Yastini NW, Suwarba IGN, Andayani AR, Bura V, et al. Protecting children against Japanese encephalitis in Bali, Indonesia. *Lancet*. 2018;391:2500–1. [https://doi.org/10.1016/S0140-6736\(18\)31191-7](https://doi.org/10.1016/S0140-6736(18)31191-7)
14. Garjito TA, Widiarti, Anggraeni YM, Alfiah S, Tunggul Satoto TB, Farchanny A, et al. Japanese encephalitis in Indonesia: An update on epidemiology and transmission ecology. *Acta Trop*. 2018;187:240–7. <https://doi.org/10.1016/j.actatropica.2018.08.017>
15. Obara M, Yamauchi T, Watanabe M, Hasegawa S, Ueda Y, Matsuno K, et al. Continuity and change of Japanese encephalitis virus in Toyama Prefecture, Japan. *Am J Trop Med Hyg*. 2011;84:695–708. <https://doi.org/10.4269/ajtmh.2011.10-0188>

Address for correspondence: Ken Maeda, Department of Veterinary Science, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan; email: kmaeda@nih.go.jp

Novel Reassortant Highly Pathogenic Avian Influenza A(H5N2) Virus in Broiler Chickens, Egypt

Kareem E. Hassan, Jacqueline King, Magdy El-Kady, Manal Affi, Hassanein H. Abozeid, Anne Pohlmann, Martin Beer, Timm Harder

We detected a novel reassortant highly pathogenic avian influenza A(H5N2) virus in 3 poultry farms in Egypt. The virus carried genome segments of a pigeon H9N2 influenza virus detected in 2014, a nucleoprotein segment of contemporary chicken H9N2 viruses from Egypt, and hemagglutinin derived from the 2.3.4.4b H5N8 virus clade.

Since 2006, Egypt's poultry industry has been plagued by endemic infections with highly pathogenic avian influenza (HPAI) virus, subtype H5N1, clade 2.2.1, of the goose/Guangdong (gs/GD) lineage (1). In addition, low pathogenicity avian influenza (LPAI) virus of subtype H9N2, G1 lineage, introduced in 2011 (2), and HPAI H5N8 (gs/GD clade 2.3.4.4b) introduced in 2016, have become entrenched in local poultry populations (3). Despite ongoing control measures, respiratory disease with increased mortality rates is endemic in poultry farms in Egypt.

The zoonotic nature of HPAI H5N1 2.2.1 viruses has caused in Egypt the highest number of human infection cases per country worldwide; a low level of sporadic benign human cases of H9N2 viral infection has also been reported from Egypt (4). Continued adaptation by point mutations, but not reassortment, to enhance replication in mammalian hosts has been repeatedly reported in avian influenza in Egypt (5). Here, we describe the detection of a new reassortant HPAI virus in commercial chicken holdings in Egypt. This virus carries the hemagglutinin (HA) gene of

HPAI clade 2.3.4.4b H5N8 virus and 7 genome segments derived from Egyptian H9N2 viruses (6).

The Study

During January–April 2019, we examined samples from 11 commercial broiler farms reporting respiratory clinical signs among chickens by using the Riems Influenza A Typing Assay (7). We detected co-presence of avian influenza viruses subtypes H5 and H9 with N2 (8 farms) as well as H5N2 only (3 farms).

We selected 8 samples representing H5N8, H9N2, and H5N1 from 2017–2018, plus 1 positive H5N2 sample from 2019, for full-genome sequencing (Table 1; Appendix 1 Table, <https://wwwnc.cdc.gov/EID/article/26/1/19-0570-App1.pdf>). Sanger- and next-generation sequencing results identified various reassortants new to Egypt (Figure 1). All H5 HA segments encoded a polybasic cleavage site, PLREKRRKR-GLF (H5 clade 2.3.4.4b) or PQGEKRRKRR-GLF (H5 clade 2.2.1.2), thus classifying those viruses as highly pathogenic. We identified the closest related sequences by BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) homology searches in the GISAID (<http://platform.gisaid.org>) and International Nucleotide Sequence Data Collaboration (<http://www.insdc.org>) databases. Phylogenetic analyses of each genome segment aided in clustering sequences (Appendix 1 Figure). We delineated the putative origin of each of the genome segments (Figure 1). The HA segment of the novel HPAI H5N2 reassortant virus was derived from clade 2.3.4.4b viruses with closest homology to viruses circulating in ducks in Egypt in 2017 (Figure 2), whereas ≥ 4 additional genome segments (polymerase basic 1, polymerase basic 2, polymerase, and nonstructural protein) originated from novel reassortant H9N2 viruses first detected in pigeons

Author affiliations: Friedrich-Loeffler-Institut, Greifswald-Riems, Germany (K.E. Hassan, J. King, A. Pohlmann, M. Beer, T. Harder); Beni Suef University, Beni Suef, Egypt (K.E. Hassan, M. El-Kady); Cairo University, Cairo, Egypt (M. Affi, H.H. Abozeid)

DOI: <https://doi.org/10.3201/eid2601.190570>

Table. Characteristics of avian influenza viruses in samples from diseased poultry, Egypt*

Strain	Subtype	Collection date	Host species	Governorate	Flock size	Mortality rate, %	Genome sequence	Clade
A/duck/Egypt/AR518/2017	H5N8	2017 Mar 15	Duck	Giza	3,000	40	Full	2.3.4.4b
A/duck/Egypt/AR560/2018	H5N8	2018 May 20	Duck	Giza	5,000	35	Full	2.3.4.4b
A/turkey/Egypt/AR550/2018	H5N8	2018 Mar 2	Turkey	Beni-Suef	5,000	100	Full	2.3.4.4b
A/duck/Egypt/AR526/2017	H5N1	2017 Mar 20	Duck	Beni-Suef	3,000	15	Full	2.2.1.2
A/chicken/Egypt/AR528/2017	H5N1	2017 Mar 22	Chicken, layer	Beni-Suef	5,000	30	Full	2.2.1.2
A/chicken/Egypt/AR544/2018	H9N2	2018 Jan 20	Chicken, broiler	Giza	10,000	25	Full	G1.B
A/chicken/Egypt/AR545/2018	H9N2	2018 Mar 25	Chicken, broiler	Qualiobia	5,000	25	Full	G1.B
A/chicken/Egypt/AR546/2018	H9N2	2018 Mar 22	Chicken, broiler	El-Menia	8,000	10	Full	G1.B
A/chicken/Egypt/AI00994/2019	H5N2	2019 Jan 19	Chicken, broiler	Beheira	17,000	47	Full	2.3.4.4b
A/chicken/Egypt/AI00986/2019	H5N2	2019 Jan 5	Chicken, broiler	Fayoum	10,000	5	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00987/2019	H5, H9, N2	2019 Jan 9	Chicken, broiler	Beheira	7,000	15	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00988/2019	H5, H9, N2	2019 Jan 19	Chicken, broiler	Beheira	8,000	20	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00989/2019	H5, H9, N2	2019 Jan 27	Chicken, broiler	El-Menia	6,000	14	HA (partial)	2.3.4.4b
A/chicken/Egypt/AI00991/2019	H5, H9, N2	2019 Feb 16	Chicken, broiler	Beni-Suef	74,000	16	NA*	NA
A/chicken/Egypt/AI00992/2019	H5N2	2019 Mar 3	Chicken, broiler	Beheira	5,000	15	HA (partial)	2.3.4.4b
A/duck/Egypt/AI00993/2019	H5, N8, N2	2019 Jan 14	Chicken, broiler	Giza	4,000	15	NA	NA
A/chicken/Egypt/AI00995/2019	H5, H9, N2	2019 Jan 14	Chicken, broiler	Beheira	33,000	33.3	NA	NA
A/chicken/Egypt/AI00996/2019	H5, H9, N2	2019 Jan 15	Chicken, broiler	Beheira	10,000	60	NA	NA
A/chicken/Egypt/AI00997/2019	H5, N8, H9, N2	2019 Mar 9	Chicken, broiler	Dakhalia	40,000	7.5	NA	NA

*HA, hemagglutinin; NA, not available.

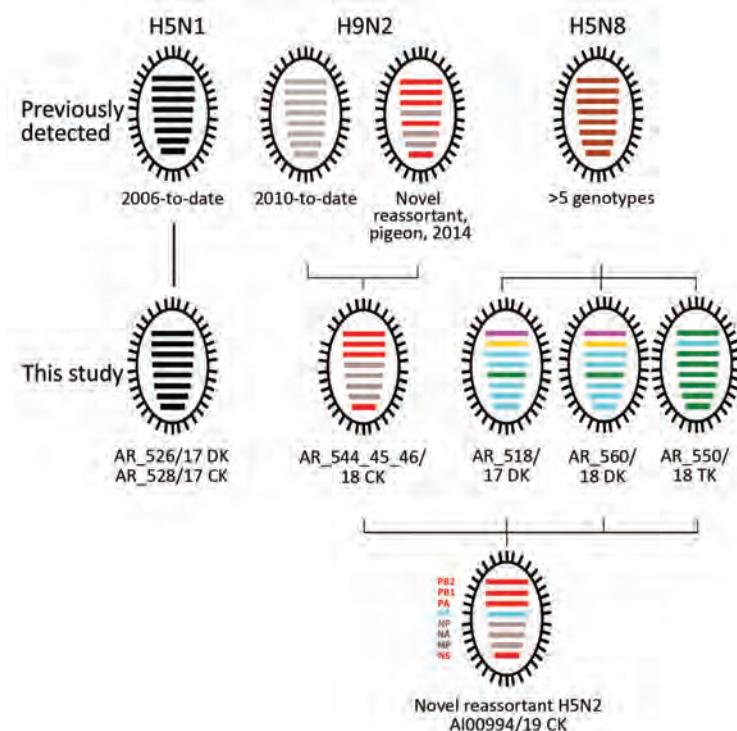


Figure 1. Genotype and reassortment analyses based on full-length genome sequences of avian influenza viruses in Egypt previously detected and those identified in this study. Colors indicate grouping of segment origin according to phylogenetic analyses (Appendix 1 Figure, <https://wwwnc.cdc.gov/EID/article/26/1/19-0570-App1.pdf>): highly pathogenic avian influenza (HPAI) H5N1 2.2.1.2 virus from Egypt (black); H9N2 subtype from Egypt circulating in chickens since 2010 (gray); H9N2 subtype from Egypt first detected in pigeons in 2014 (red); HPAI H5N8 viruses previously detected and circulating in Egypt (brown; different genotypes); Polymerase basic (PB) 2 segment most closely related to an H3N6 virus from Bangladesh (purple); PB1 segment most closely related to an H7N7 virus from Georgia (yellow); segments most closely related to H5N8 viruses from China (blue) or Russia (green).

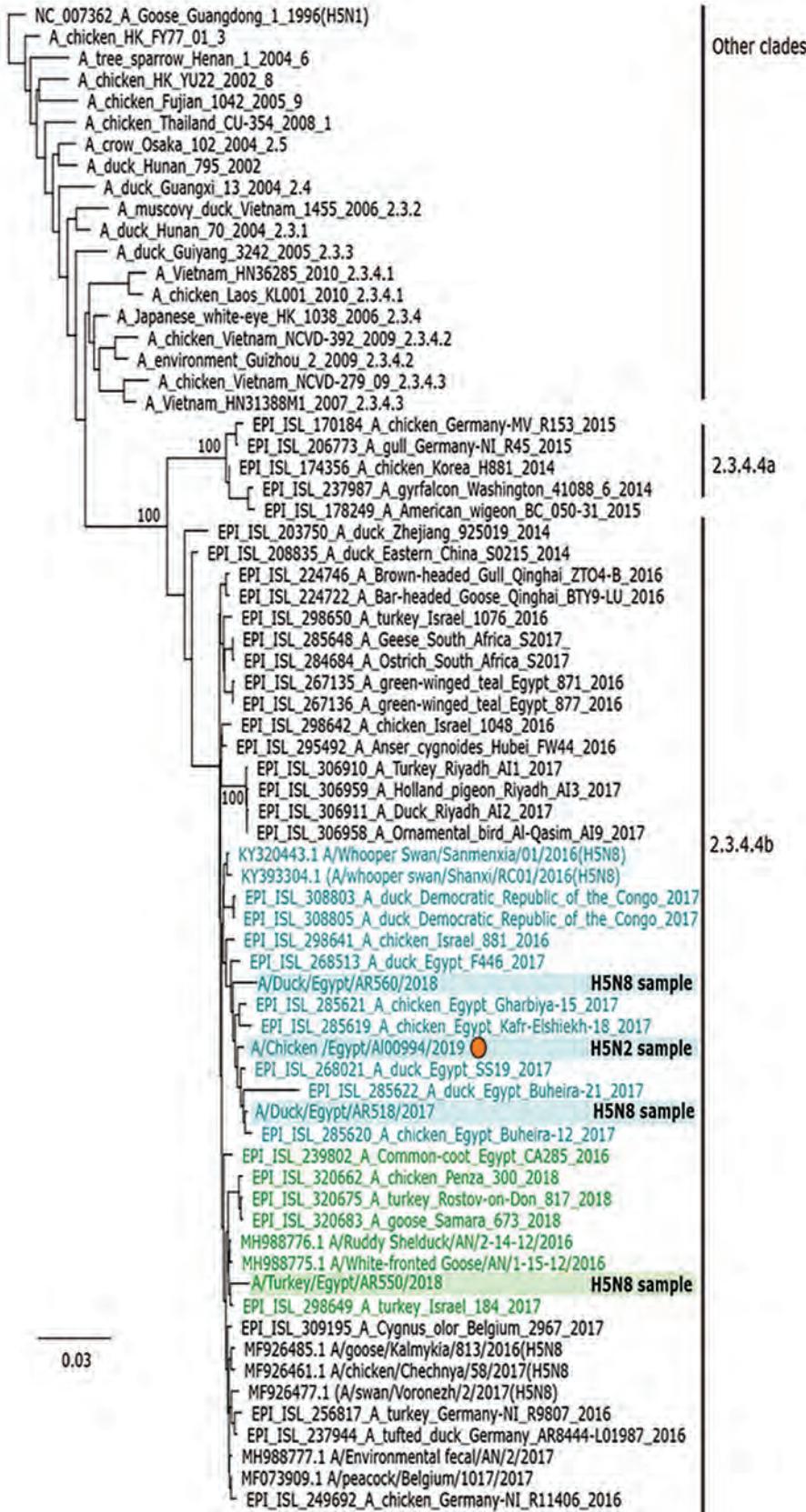


Figure 2. Phylogenetic analysis of the hemagglutinin segments of reassortant highly pathogenic avian influenza H5N2 and H5N8 viruses belonging to clade 2.3.3.4b from Egypt and reference viruses. Sequence analysis was based on alignment analyses by MAFFT version 7.450 embedded in the Geneious software suite, version 11.1.7 (<https://www.geneious.com>), with manual editing. We performed maximum-likelihood calculations using PhyML version 3.0 (<http://www.atgc-montpellier.fr/phyml/>); we chose the best-fit model according to the Bayesian selection criterion using Model Finder embedded in Geneious. Colors indicate grouping of segment origin and match those shown in Figure 1: blue, most closely related to H5N8 viruses from China; green, most closely related to H5N8 viruses from Russia and Europe. GenBank or GISAID accession numbers (<http://www.gisaid.org>) are provided for reference sequences. Scale bar indicates nucleotide substitutions per site.

in Egypt during 2014 (6). The nucleoprotein segment and perhaps others were acquired from H9N2 viruses circulating in chickens in Egypt since 2010. Matrix and neuraminidase segments are identical in the pigeon and chicken H9N2 viruses. We identified no new mutations in the genome of reassortant H5N2 that would suggest increased adaptation to mammalian hosts. In addition, we observed 2 previously undescribed genotypes of HPAI H5N8 with distinct polymerase basic 1 and 2 segment origins (Figure 1). The composition of HPAI H5N1 viruses phylogenetically assigned to clade 2.2.1.2 (Figure 1; Appendix 1 Figure) was unaltered compared with other HPAI H5N1 viruses isolated since 2015.

Natural reassortants between H5 HPAI of the gs/GD lineage and H9N2 viruses, including subtype H5N2, have repeatedly emerged in Southeast Asia (8). So far, both the HPAI H5N1 2.2.1.2 and the co-circulating H9N2-G1 viruses appeared to be genotypically stable in poultry in Egypt. Successful forced reassortment of these viruses by co-cultivation and serum selection in embryonated chicken eggs ruled out a principal incompatibility between their genome segments (5); however, Naguib et al. did not rescue an H5N2 reassortant.

We and others have shown that HPAI H5 viruses of clade 2.3.4.4 have a high tendency to reassort with various influenza A viruses of wild birds or poultry (9). Thus, the incursion of clade 2.3.4.4b viruses into Egypt in 2016 not only added another antigenically distinct HPAI virus, but also signaled an increased reassortment risk. In fact, the 2.3.4.4b H5N8 virus proved to be a parent of the newly emerged H5N2 reassortant. Likewise, the second parental virus, an influenza A(H9N2) virus first detected in pigeons, was not described in Egypt before 2014. Genotypically, this H9N2 virus is distinct from the third parental virus, that is, the original H9N2 virus introduced to poultry in Egypt in 2010 (Figure 1). Infection of pigeons with clade 2.3.4.4b H5N8 HPAI virus has been described in Egypt, although pigeons are believed to be less susceptible to avian influenza infections (10). Although we cannot attribute the origin of the current HPAI H5N2 reassortant to a single host species, we cannot exclude pigeons as a possible host.

In March 2019, Egypt's Ministry of Agriculture announced the detection of a new influenza A(H5N2) virus from seemingly healthy ducks in the Dakahlia governorate (11); recently published information on this reassortant indicated the presence of a neuraminidase N2 segment of chicken H9N2 viruses in the background of an HPAI H5 clade 2.3.4.4b virus (11). Our data confirm the presence of a different H5N2 reassortant and its occurrence in chickens in different

geographic regions of Egypt (Table 1). We detected the current reassortant HPAI H5N2 viruses in 2 different broiler farms in Beheira (January and March 2019) and 1 broiler farm in Fayoum (January 2019) governorates (Table 1). The HA amino acid sequence of these reassortants does not signal antigenic variation compared with parent HPAI H5 subtype of clade 2.3.4.4b. Antigenic and further phenotypic properties, such as host specificity, require investigation as soon as isolates are available. For the H5N2-positive samples, only FTA card material was available at Friedrich-Loeffler-Institut. However, H5N2 isolates were successfully generated at the Beni-Suef University, Egypt, but were currently not available for further antigenic and phenotypic analyses (M. El-Kady, unpub. data).

Intensified targeted surveillance in poultry and pigeons is urgently required and may lead to detection of additional reassortants. However, co-detection in a sample of H5N8 and H9N2 subtypes by reverse transcription quantitative PCR may blur the identification of H5N2 reassortants; plaque purification of such samples would aid in separating subtypes but cannot currently be used in routine diagnostics.

There is a risk for transboundary spread of HPAI A(H5N2) virus in northern Africa and the Middle East, and similar reassortment events are to be expected in regions where clade 2.3.4.4 HPAI and H9N2 viruses are co-circulating. Long-term solutions in combating avian influenza virus infections in poultry are sorely needed and would help to lower risks of human exposure to zoonotic avian influenza viruses such as the highly zoonotic H7N9 viruses in China that carry a full set of internal genes of an H9N2-G1-like avian influenza virus (12).

Acknowledgments

We acknowledge the originating and submitting laboratories that provided sequences available in the EpiFlu database (<http://www.gisaid.org>) (Appendix 2, <https://wwwnc.cdc.gov/EID/26/1/19-0570-App2.xlsx>).

K.E.H. received a grant from the Egyptian Ministry of Higher Education (Mission sector). This work was financed in part by the European Union Horizon 2020 program grant agreement DELTA-FLU (no. 727922).

About the Author

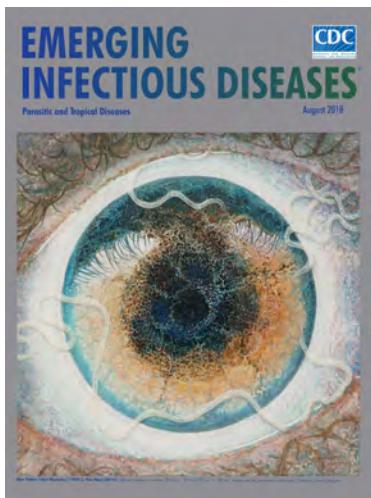
Dr. Hassan is a poultry veterinary specialist at Beni-Suef University, currently on leave to complete his PhD studies at the Friedrich-Loeffler-Institut, Isle of Riems, Germany. His primary research interest is focused on respiratory infectious diseases in poultry with an emphasis on avian influenza.

References

1. Peyre M, Samaha H, Makonnen YJ, Saad A, Abd-Elnabi A, Galal S, et al. Avian influenza vaccination in Egypt: limitations of the current strategy. *J Mol Genet Med*. 2009;3:198–204.
2. El-Zoghby EF, Arafa AS, Hassan MK, Aly MM, Selim A, Kilany WH, et al. Isolation of H9N2 avian influenza virus from bobwhite quail (*Colinus virginianus*) in Egypt. *Arch Virol*. 2012;157:1167–72. <https://doi.org/10.1007/s00705-012-1269-z>
3. Salaheldin AH, El-Hamid HS, Elbestawy AR, Veits J, Hafez HM, Mettenleiter TC, et al. Multiple introductions of influenza A(H5N8) virus into poultry, Egypt, 2017. *Emerg Infect Dis*. 2018;24:943–6. <https://doi.org/10.3201/eid2405.171935>
4. World Health Organization. Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to WHO, 2003–2019. 2019 [cited 2019 Mar 4]. https://www.who.int/influenza/human_animal_interface/2019_02_12_tableH5N1.pdf
5. Naguib MM, Ulrich R, Kasbohm E, Eng CLP, Hoffmann D, Grund C, et al. Natural reassortants of potentially zoonotic avian influenza viruses H5N1 and H9N2 from Egypt display distinct pathogenic phenotypes in experimentally infected chickens and ferrets. *J Virol*. 2017;91:e01300–17. <https://doi.org/10.1128/JVI.01300-17>
6. Kandeil A, El-Shesheny R, Maatouq A, Moatasim Y, Cai Z, McKenzie P, et al. Novel reassortant H9N2 viruses in pigeons and evidence for antigenic diversity of H9N2 viruses isolated from quails in Egypt. *J Gen Virol*. 2017;98:548–62. <https://doi.org/10.1099/jgv.0.000657>
7. Hoffmann B, Hoffmann D, Henritzi D, Beer M, Harder TC. Riems influenza A typing array (RITA): an RT-qPCR-based low density array for subtyping avian and mammalian influenza A viruses. *Sci Rep*. 2016;6:27211. <https://doi.org/10.1038/srep27211>
8. Kim HR, Park CK, Oem JK, Bae YC, Choi JG, Lee OS, et al. Characterization of H5N2 influenza viruses isolated in South Korea and their influence on the emergence of a novel H9N2 influenza virus. *J Gen Virol*. 2010;91:1978–83. <https://doi.org/10.1099/vir.0.021238-0>
9. Pohlmann A, Starick E, Grund C, Höper D, Strebelow G, Globig A, et al. Swarm incursions of reassortants of highly pathogenic avian influenza virus strains H5N8 and H5N5, clade 2.3.4.4b, Germany, winter 2016–17. *Sci Rep*. 2018;8:15. <https://doi.org/10.1038/s41598-017-16936-8>
10. Abolnik C, Stutchbury S, Hartman MJ. Experimental infection of racing pigeons (*Columba livia domestica*) with highly pathogenic clade 2.3.4.4 subgroup B H5N8 avian influenza virus. *Vet Microbiol*. 2018;227:127–32. <https://doi.org/10.1016/j.vetmic.2018.10.028>
11. Hagag NM, Erfan AM, El-Husseiny M, Shalaby AG, Saif MA, Tawakol MM, et al. Isolation of a novel reassortant highly pathogenic avian influenza (H5N2) virus in Egypt. *Viruses*. 2019;11:565. <https://doi.org/10.3390/v11060565>
12. Lam TT-Y, Wang J, Shen Y, Zhou B, Duan L, Cheung C-L, et al. The genesis and source of the H7N9 influenza viruses causing human infections in China. *Nature*. 2013;502:241–4. <https://doi.org/10.1038/nature12515>

Address for correspondence: Timm Harder, Federal Research Institute for Animal Health, Friedrich-Loeffler-Institut, Institute of Diagnostic Virology, Suedufer 10, Greifswald-Insel Riems 17493, Germany; email: timh.harder@fli.de

EID Podcast: A Worm's Eye View



Seeing a several-centimeters-long worm traversing the conjunctiva of an eye is often the moment when many people realize they are infected with *Loa loa*, commonly called the African eyeworm, a parasitic nematode that migrates throughout the subcutaneous and connective tissues of infected persons. Infection with this worm is called loiasis and is typically diagnosed either by the worm's appearance in the eye or by a history of localized Calabar swellings, named for the coastal Nigerian town where that symptom was initially observed among infected persons. Endemic to a large region of the western and central African rainforests, the *Loa loa* microfilariae are passed to humans primarily from bites by flies from two species of the genus *Chrysops*, *C. silacea* and *C. dimidiata*. The more than 29 million people who live in affected areas of Central and West Africa are potentially at risk of loiasis.

Ben Taylor, cover artist for the August 2018 issue of EID, discusses how his personal experience with the *Loa loa* parasite influenced this painting.

Visit our website to listen:
<https://tools.cdc.gov/medialibrary/index.aspx#/media/id/392605>

**EMERGING
INFECTIOUS DISEASES**

Infectivity of Norovirus GI and GII from Bottled Mineral Water during a Waterborne Outbreak, Spain

Susana Guix, Cristina Fuentes, Rosa M. Pintó, Albert Blanco, Aurora Sabrià, Eduard Anfruns-Estrada, Virginia Rodríguez Garrido, Manuel Alonso, Rosa Bartolomé, Thais Cornejo, Tomàs Pumarola, Albert Bosch

During a waterborne outbreak of norovirus in Spain, we estimated 50% illness doses for a group of exposed (secretor) persons to be 556 (95% CI 319–957) genome copies/day for norovirus GI and 2,934 (95% CI 1,683–5,044) genome copies/day for norovirus GII. Use of a propidium monoazide viability assay reduced these values.

Human noroviruses are a major agent of acute gastroenteritis, are distributed worldwide, and affect all age groups (1). One of the largest outbreaks of infection with norovirus, caused by consumption of contaminated bottled spring water, occurred in Spain during 2016 and affected >4,100 persons (2). Multiple genotypes (GI.2, GII.2, GII.4, and GII.17) were identified among patients, and high levels of norovirus genomes were quantified in contaminated water coolers.

Differences in susceptibility to infections with different genotypes have been described and depend on expression of histoblood group antigens (3), for which expression is determined primarily by the *FUT2* gene. Although secretor-negative persons are resistant to several norovirus genotypes (3), symptomatic infections in nonsecretors have been documented for GI.3, GII.1, GII.2, GII.3, GII.6, GII.7, GII.4, and GII.17 (3). These differences, which are based on host genetic susceptibility, partially hamper development of dose-response models.

Noroviruses are highly infectious, although infectivity might vary between genotypes, and data on

their infectivity are still scarce. Reported doses causing infection in 50% of exposed persons (ID_{50}), determined from volunteer secretor adults challenged with a GI.1 from a stool specimen, range from 18 (95% CI 1–4,350) (4,5) to 2,800 (95% CI, 290–25,000) genome equivalents (6).

Using human intestinal organoids, Costantini et al. estimated the ID_{50} for stool specimens containing GII as 440–4,000 genome copies/mL (7). Because genome to infectious virus ratios might be sample specific, especially in environmental samples, determining norovirus infectivity from common-source outbreaks and naturally contaminated samples is essential. Data reported for oyster-related norovirus outbreaks by Thebault et al. inferred higher infectivity estimates: secretors had an ID_{50} of 7.1 (95% CI 0.73 to >10⁶) virus genomes/oyster consumed and a dose causing illness in 50% of exposed persons of 32 (95% CI 1.32 to >10⁶) virus genomes/oyster consumed for norovirus GI and an ID_{50} of 1.6 (95% CI 0.74 to >10⁶) virus genomes/oyster consumed and a dose causing illness in 50% of exposed persons of 4.86 (1.24 to >10⁶) virus genomes/oyster consumed for norovirus GII (8).

In this study, we estimated the 50% illness dose in conditions of natural exposure to contaminated water. These persons were selected from a group of exposed persons during a large waterborne outbreak in Spain in 2016 (2).

The Study

This study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of the Hospital Universitari Vall d'Hebron (PR[AG]211/2016). Informed written consent was obtained from all persons.

We provided a questionnaire on water consumption, occurrence, type and duration of gastroenteritis

Author affiliations: University of Barcelona, Barcelona, Spain (S. Guix, C. Fuentes, R.M. Pintó, A. Blanco, A. Sabrià, E. Anfruns-Estrada, A. Bosch); Vall d'Hebron University Hospital, Barcelona (V. Rodríguez Garrido, M. Alonso, R. Bartolomé, T. Cornejo, T. Pumarola)

DOI: <https://doi.org/10.3201/eid2601.190778>

symptoms, and blood type (ABO) to 26 persons who had been exposed to drinking water from a water cooler in Spain during 2016. Acute gastroenteritis was defined as vomiting or diarrhea (≥ 3 loose stools within 24 hours) and ≥ 2 of the following: nausea, abdominal pain, or fever (temperature $\geq 37.8^\circ\text{C}$). We collected saliva samples to determine secretor status by genotyping the *FUT2* gene (9). We collected stool specimens during the symptomatic phase from 13 of 15 symptomatic patients and screened for norovirus by real-time quantitative PCR as described (10). We also performed genotype analysis for polymerase and capsid genes (11) and used probit analysis to determine the 50% illness dose.

A total of 69% of persons were secretors and 31% were nonsecretors (Table 1). The overall attack rate for symptomatic infection was 58%: 67% for secretors and 38% for nonsecretors. Percentages of secretors were 80% for symptomatic persons and 55% for asymptomatic persons. According to water intake, 62% reported consuming 200–600 mL/day, 23% reported consuming 601–1,000 mL/day, and 15% reported consuming $>1,000$ mL/day. We found no major differences in water consumption between secretors and nonsecretors. An increase in the proportion of symptomatic persons and the amount of water consumed daily was observed

only for secretors (Figure). Gastroenteritis developed in 3 of 8 nonsecretors even though they had not ingested the largest amount of water.

Of 8 exposed nonsecretor persons, 3 (38%) showed development of acute gastroenteritis and only GI viruses were detected in their stool. Although we could not completely rule out that GII viruses might have also infected these persons but were not detected, it is plausible that gastroenteritis in these persons was related to the GI infection. Although binding of GI.2 to nonsecretor histoblood group antigens has been demonstrated in vitro (12–14), we report clinical infections in nonsecretors. The observed attack rate for a symptomatic GI infection was similar between secretors (50%) and nonsecretors (38%).

We used data for quantification of norovirus in the contaminated water cooler to which the subjects were exposed (2) to estimate the average 50% illness dose. High levels of genome copies per liter of water for GI (1.1×10^3) and GII (5.8×10^3) had been detected. Intact virions, estimated by using a viability real-time quantitative PCR, represented $<4.4\%$ to 5.6% of the total number of genomes. We estimated 50% illness doses according to the percentages of persons with gastroenteritis and the median water volumes recorded for each group (Table 2). We calculated 95% CIs by using the scenarios corresponding

Table 1. Characteristics of 26 exposed persons during a waterborne outbreak of norovirus, Spain*

Person no.	Age, y	Duration of symptoms, days	Daily water intake, mL†	Secretor status	ABO blood type	Virus genogroup	Virus genotype
1	46	2	200–600	+	NA	I	GI.P2_GI.2
2	25	2	200–600	+	NA	II	GII.P17_GII.17
3	58	4	200–600	+	NA	II	GII.P17_GII.17
4	45	5	200–600	–	O	I	GI.P2_GI.2
5‡	29	3	200–600	+	O	NA	NA
6‡	31	1	200–600	+	O	NA	NA
7	34	2	200–600	–	O	I	GIP2_GI.2
8	42	4	200–600	+	O	I + II	GI.P2_GI.2
9	45	5	601–1,000	–	NA	I	NA
10	32	3	601–1,000	+	A	I + II	GI.P2_GI.2 and GII.P17_GII.17
11	29	4	601–1,000	+	B	I + II	GII.P17_GII.17
12	26	6	601–1,000	+	O	I + II	GI.P2_GI.2
13	41	9	601–1,000	+	O	I + II	NA
14	25	4	$>1,000$	+	NA	I + II	GII.P2_GII.2
15	55	2	$>1,000$	+	A	I + II	GI.P2_GI.2
16‡	27	0	200–600	–	NA	NA	NA
17‡	52	0	200–600	–	AB	NA	NA
18‡	54	0	200–600	+	NA	NA	NA
19‡	47	0	200–600	+	O	NA	NA
20‡	59	0	200–600	+	O	NA	NA
21‡	30	0	200–600	+	O	NA	NA
22‡	59	0	200–600	–	O	NA	NA
23‡	NA	0	200–600	+	O	NA	NA
24‡	33	0	601–1,000	+	NA	NA	NA
25‡	38	0	$>1,000$	–	NA	NA	NA
26‡	42	0	$>1,000$	–	NA	NA	NA

*NA, not available; –, negative; +, positive.

†Plastic glasses used by persons to drink water had a volume of 200 mL.

‡These persons did not provide a stool sample.

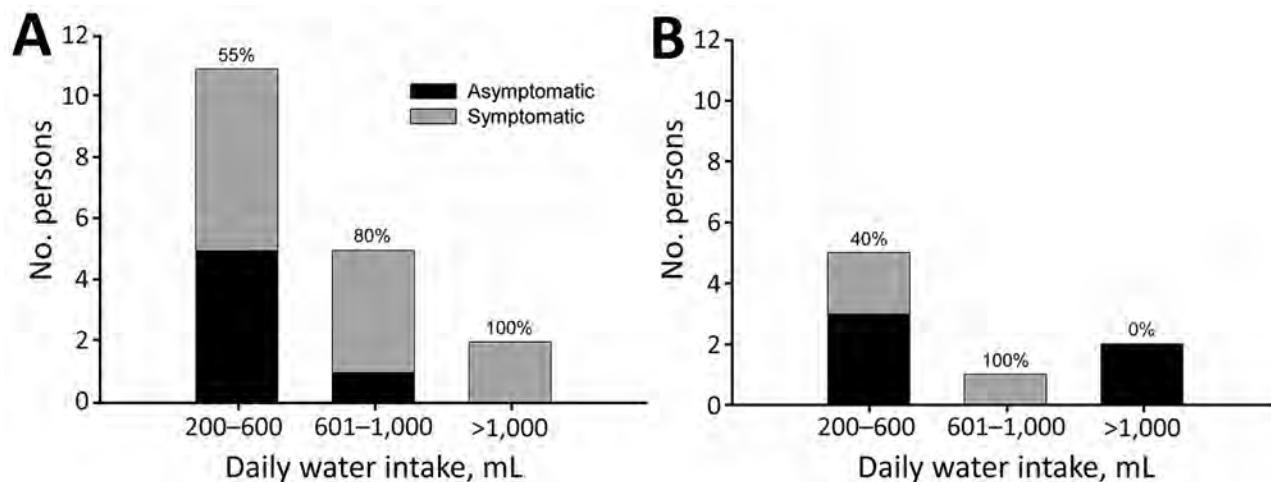


Figure. Distribution of A) secretor and B) nonsecretor persons by level of daily water intake and the occurrence of symptoms during a waterborne outbreak of norovirus, Spain. Numbers at the top of each bar indicate percentages of symptomatic persons.

to the minimum and maximum water volumes from each group. The 50% illness dose estimates for secretors were 556 (95% CI, 319–957) genome copies/day for GI exposures and 2,934 (95% CI, 1,683–5,044) genome copies/day for GII exposures. For nonsecretors, the number of infected persons was too low to calculate 50% illness doses.

Conclusions

Our data showed differences in infectivity between norovirus GI and GII, which differs from what was previously observed in oyster-related outbreaks (8). Because the number of persons studied was low in both studies, factors that might modify how a particular person responds to virus exposure might partially explain these different observations. In addition, factors related to particular genotypes involved in the outbreaks might also influence infectivity. Although values estimated in this study fall within the range of ID_{50} and 50% illness dose data obtained by other investigators (4,6,8), we observed that median values extrapolated from oyster-related outbreaks (8) are the lowest values. This trend might be caused by natural variability, different preimmune status

of exposed persons, or strain differences, or the proportion of noninfectious genomes with respect to total genome copies might be lower within contaminated oysters.

Because of the interaction of norovirus with specific carbohydrates found in oyster intestinal tissues (15), these animals might specifically bioaccumulate intact infectious virions. In this regard, in our study, after we considered only those genomes incorporated in undamaged capsids, resulting 50% illness doses for secretors were only 25 (95% CI 15–42) for GI and 165 (95% CI 95–284) for GII.

Because the proportion of infectious genomes might vary in each particular environmental scenario, infectivity data for norovirus relating to total and infectious genomes should be consistently investigated whenever possible for a better refinement of risk assessment approaches. Outbreaks caused by consumption of contaminated water and shellfish are often caused by multiple virus strains. Although this factor might hamper infectivity estimates, these types of studies are nevertheless valuable to risk managers and regulators for future formulation of guidelines on acceptance levels for norovirus in specific matrices.

Table 2. Illness status observed for secretors and nonsecretors of norovirus, according to different daily doses of ingested virus genomes, Spain*

Virus genotype	Ingested genome copies/d	Ingested infectious genome copies/d	Secretors		Nonsecretors	
			No. exposed persons	% Symptomatic persons	No. exposed persons	% Symptomatic persons
GI	220–660	10–29	9	22.2	5	40.0
	661–1,100	30–49	5	80.0	1	100.0
	>1,100	>49	2	100.0	2	0.0
GII	1,160–3,480	65–196	9	33.3	5	0.0
	3,481–5,800	197–327	5	80.0	1	0.0
	>5,800	>327	2	100.0	2	0.0

*n = 13. Persons who did not provide a stool sample were excluded from the analysis.

This study was supported in part by projects Food-FP7-311846 (European Union) and XRB-Biotechnology Reference Network (Generalitat de Catalunya).

About the Author

Dr. Guix is an associate professor in the Department of Genetics, Microbiology and Statistics, and academic secretary of the Research Institute of Nutrition and Food Safety, University of Barcelona, Barcelona, Spain. Her research interests are human foodborne enteric viruses, such as norovirus, hepatitis A, and astrovirus; molecular and clinical aspects; and the role of these viruses as food and environmental contaminants; and virus inactivation studies.

References

- Lopman BA, Steele D, Kirkwood CD, Parashar UD. The vast and varied global burden of norovirus: prospects for prevention and control. *PLoS Med*. 2016;13:e1001999. <https://doi.org/10.1371/journal.pmed.1001999>
- Blanco A, Guix S, Fuster N, Fuentes C, Bartolomé R, Cornejo T, et al. Norovirus in bottled water associated with gastroenteritis outbreak, Spain, 2016. *Emerg Infect Dis*. 2017;23:1531-4. <https://doi.org/10.3201/eid2309.161489>
- Nordgren J, Svensson L. Genetic susceptibility to human norovirus infection: an update. *Viruses*. 2019;11:E226. <https://doi.org/10.3390/v11030226>
- Teunis PF, Moe CL, Liu P, Miller SE, Lindesmith L, Baric RS, et al. Norwalk virus: how infectious is it? *J Med Virol*. 2008;80:1468-76. PubMed <https://doi.org/10.1002/jmv.21237>
- Kirby AE, Teunis PF, Moe CL. Two human challenge studies confirm high infectivity of Norwalk virus. *J Infect Dis*. 2015;211:166-7. <https://doi.org/10.1093/infdis/jiu385>
- Atmar RL, Opekun AR, Gilger MA, Estes MK, Crawford SE, Neill FH, et al. Determination of the 50% human infectious dose for Norwalk virus. *J Infect Dis*. 2014;209:1016-22. PubMed <https://doi.org/10.1093/infdis/jit620>
- Costantini V, Morantz EK, Browne H, Ettayebi K, Zeng XL, Atmar RL, et al. Human norovirus replication in human intestinal enteroids as model to evaluate virus inactivation. *Emerg Infect Dis*. 2018;24:1453-64. <https://doi.org/10.3201/eid2408.180126>
- Thebault A, Teunis PF, Le Pendu J, Le Guyader FS, Denis JB. Infectivity of GI and GII noroviruses established from oyster related outbreaks. *Epidemics*. 2013;5:98-110. <https://doi.org/10.1016/j.epidem.2012.12.004>
- Marionneau S, Airaud F, Bovin NV, Le Pendu J, Ruvoën-Clouet N. Influence of the combined ABO, FUT2, and FUT3 polymorphism on susceptibility to Norwalk virus attachment. *J Infect Dis*. 2005;192:1071-7. <https://doi.org/10.1086/432546>
- Pérez-Sautu U, Sano D, Guix S, Kasimir G, Pintó RM, Bosch A. Human norovirus occurrence and diversity in the Llobregat River catchment, Spain. *Environ Microbiol*. 2012;14:494-502. <https://doi.org/10.1111/j.1462-2920.2011.02642.x>
- van Beek J, de Graaf M, Smits S, Schapendonk CM, Verjans GM, Vennema H, et al. Whole-genome next-generation sequencing to study within-host evolution of norovirus (NoV) among immunocompromised patients with chronic NoV infection. *J Infect Dis*. 2017;216:1513-24. <https://doi.org/10.1093/infdis/jix520>
- Kubota T, Kumagai A, Ito H, Furukawa S, Someya Y, Takeda N, et al. Structural basis for the recognition of Lewis antigens by genogroup I norovirus. *J Virol*. 2012;86:11138-50. <https://doi.org/10.1128/JVI.00278-12>
- Shanker S, Czako R, Sankaran B, Atmar RL, Estes MK, Prasad BV. Structural analysis of determinants of histo-blood group antigen binding specificity in genogroup I noroviruses. *J Virol*. 2014;88:6168-80. <https://doi.org/10.1128/JVI.00201-14>
- Shirato H, Ogawa S, Ito H, Sato T, Kameyama A, Narimatsu H, et al. Noroviruses distinguish between type 1 and type 2 histo-blood group antigens for binding. *J Virol*. 2008;82:10756-67. PubMed <https://doi.org/10.1128/JVI.00802-08>
- Le Guyader F, Loisy F, Atmar RL, Hutson AM, Estes MK, Ruvoën-Clouet N, et al. Norwalk virus-specific binding to oyster digestive tissues. *Emerg Infect Dis*. 2006;12:931-6. PubMed <https://doi.org/10.3201/eid1206.051519>

Address for correspondence: Albert Bosch, Department of Genetics, Microbiology and Statistics, School of Biology, University of Barcelona, Diagonal 643, 08028, Barcelona, Spain; email: abosch@ub.edu

Effect of Pediatric Influenza Vaccination on Antibiotic Resistance, England and Wales

Chungman Chae,¹ Nicholas G. Davies,¹ Mark Jit, Katherine E. Atkins

Vaccines against viral infections have been proposed to reduce prescribing of antibiotics and thereby help control resistant bacterial infections. However, by combining published data sources, we predict that pediatric live attenuated influenza vaccination in England and Wales will not substantially reduce antibiotic consumption or adverse health outcomes associated with antibiotic resistance.

Antibiotic use drives the spread of antibiotic resistance. A considerable proportion of antibiotic prescriptions are prescribed unnecessarily for conditions that are either self-limiting or nonbacterial in etiology (1). Because influenza is often treated inappropriately with antibiotics, expanding access to influenza vaccines has been proposed as a means of reducing unnecessary prescribing and preventing resistant infections (2).

In 2013, England and Wales began rolling out the live attenuated influenza vaccine (LAIV) for 2–16-year-old children (3). Here, we estimate the potential effect on antibiotic prescribing and antibiotic resistance.

The Study

We assumed that some influenza cases lead to general practitioner (GP) consultations and some GP consultations lead to antibiotic prescriptions. Our age-stratified analysis focused on community antibiotic use as the driver of resistance, because hospitalizations for influenza are rare relative to GP consultations (4).

To estimate the influenza-attributable consultation rate, we used a previous time-series statistical attribution covering the 1995–2009 influenza

seasons in the United Kingdom (5), yielding a population-wide average of 14.7 influenza-attributable GP consultations per 1,000 person-years (Table 1). For our uncertainty analysis (Appendix Table, <https://wwwnc.cdc.gov/EID/article/25/12/19-1110-App1.pdf>), we used a lower estimate of 11.8 per 1,000, from a longitudinal study of the 2006–2011 influenza seasons in England (6), and a higher estimate of 21.4 per 1,000, from a time-series statistical analysis of the 2000–2008 influenza seasons in England and Wales (4).

We estimated that 726 antibiotic prescriptions are written for every 1,000 influenza-attributable GP consultations (5). For our uncertainty analysis, we used a lower estimate of 313 per 1,000, derived from electronic health records of prescriptions within 30 days of a consultation for influenza-like illness (ILI) or acute cough in England during 2013–2015 (7).

We assumed that LAIV prevents 49% of symptomatic influenza cases on average, using a previously published mathematical model of pediatric LAIV in England and Wales, which assumes 50% uptake and either 70% (matched-year) or 42% (unmatched-year) efficacy among 2–16-year-olds (3). This reduction is consistent with a pilot study comparing consultation rates in treatment with control areas before and after LAIV rollout (8). For our uncertainty analysis, we used lower and higher estimates of 32% and 63% fewer influenza cases from the same model, assuming an uptake of 30% and 70%, respectively.

To predict the healthcare benefits of reducing unnecessary prescribing, we used linear regression with a country's rate of primary-care antibiotic use as the predictor variable and previously published 2015 estimates of adverse health outcomes associated with 16 resistant bacterial strains across European countries (9) as the response variables. We adopted a

Author affiliations: London School of Hygiene and Tropical Medicine, London, UK (C. Chae, N.G. Davies, M. Jit, K.E. Atkins); Public Health England, London (M. Jit); University of Edinburgh, Scotland, UK (K.E. Atkins)

DOI: <https://doi.org/10.3201/eid2601.191110>

¹These authors contributed equally to this article.

Table 1. Projected effect of pediatric LAIV on antibiotic prescription rates, England and Wales*

Age group	Influenza-attributed consultation rate†	Prescriptions per consultation	Direct prescribing rate reduction, unmatched‡	Direct prescribing rate reduction, matched‡	Overall LAIV effectiveness§	Overall prescribing rate reduction¶
0–6 mo	29.7 (23.7–35.9)	0.597 (0.474–0.719)	–	–	0.574 (0.501–0.651)	10.2 (7.03–13.5)
6 m–4 y	29.7 (23.7–35.9)	0.597 (0.474–0.719)	7.46 (5.31–9.64)	12.4 (8.85–16.1)	0.663 (0.618–0.714)	11.8 (8.31–15.4)
5–14 y	22.1 (17.6–26.7)	0.588 (0.466–0.708)	5.46 (3.89–7.06)	9.11 (6.48–11.8)	0.754 (0.709–0.794)	9.81 (6.97–12.8)
15–44 y	12.8 (10.2–15.4)	0.676 (0.536–0.814)	3.64 (2.59–4.70)	6.06 (4.31–7.83)	0.446 (0.394–0.502)	3.86 (2.66–5.09)
45–64 y	12.4 (9.84–14.9)	0.805 (0.639–0.970)	–	–	0.423 (0.374–0.484)	4.22 (2.90–5.58)
≥65 y	12.2 (9.67–14.7)	0.857 (0.680–1.03)	–	–	0.477 (0.397–0.561)	4.97 (3.34–6.68)
Overall	14.7 (11.7–17.7)	0.726 (0.576–0.875)	5.80 (4.13–7.49)	9.86 (7.01–12.9)	0.494 (0.446–0.549)	5.32 (3.74–7.00)

*All estimates reported as mean (95% highest density interval). LAIV, live attenuated influenza vaccine; –, age group not subject to pediatric LAIV.

†Per 1,000 person-years in England and Wales.

‡Reduction in antibiotic prescriptions among vaccinees per 1,000 vaccine recipients, not accounting for herd immunity, presented separately for unmatched and matched seasons.

§Reduction in influenza cases assuming a 50% uptake among children 2–16 years of age, accounting for herd immunity.

¶Per 1,000 person-years in England and Wales, accounting for herd immunity.

published cost estimate of \$1,415 per resistant infection (2016 USD) (10), adjusted for inflation and health-care purchasing power parity to £520 (2015 GBP).

We used Monte Carlo sampling to explore uncertainty across estimates for consultation rate, prescribing rate, and LAIV effectiveness, weighting age

groups by using 2015 demographic data for England and Wales. (Analysis code and data at http://github.com/nicholasdavies/laiv_amr_ew; additional details in the Appendix.)

We found that pediatric LAIV has the potential to reduce antibiotic consumption by 5.3 (95% highest

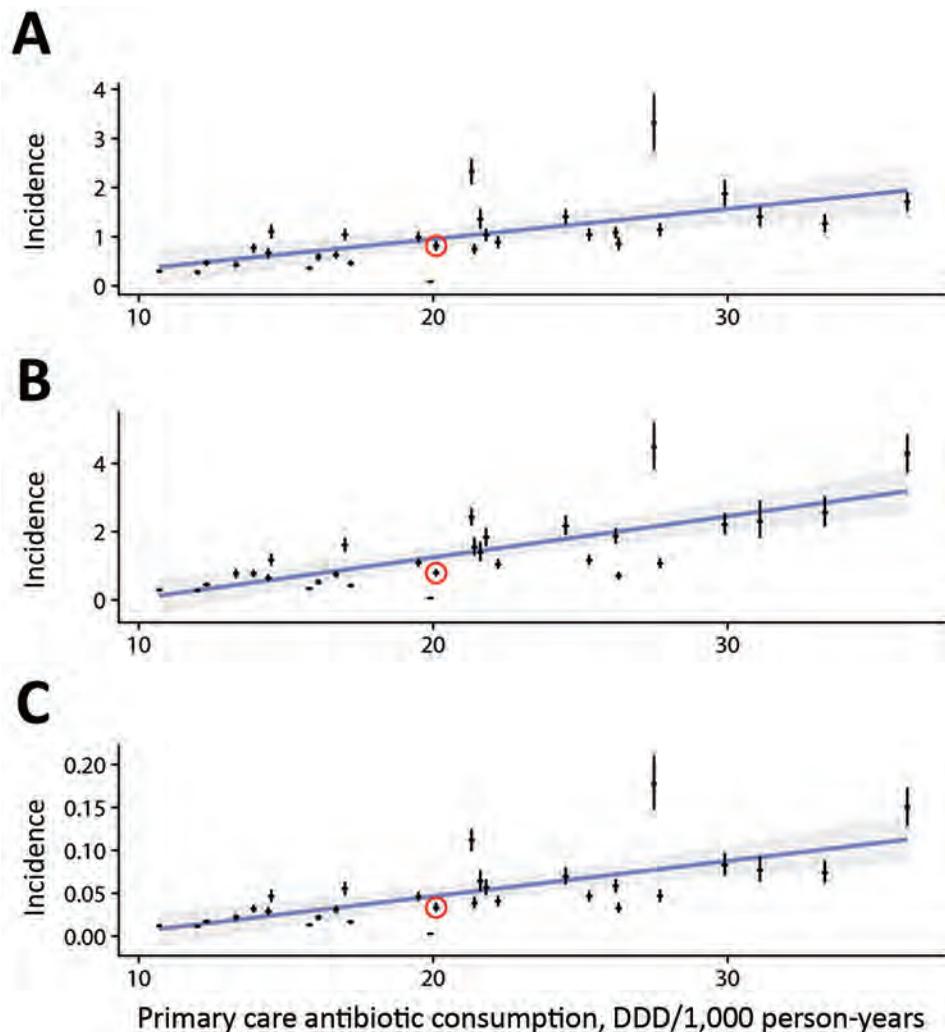


Figure 1. Estimated incidence of adverse health outcomes resulting from antibiotic-resistant infections, plotted against the overall antibiotic consumption in primary care settings in 30 countries in Europe, 2015. A) Antibiotic-resistant cases/1,000-person-years; B) attributable DALYs/1,000 person-years; C) attributable deaths/1,000 person-years. Red circles indicate datapoints for the United Kingdom; error bars indicate 95% CIs. Blue lines indicate linear regressions; gray shading indicates 95% confidence regions for linear regressions. DALYs, disability-adjusted life years; DDD, defined daily dose.

Table 2. Projected effect of pediatric LAIV on adverse health outcomes associated with antibiotic resistance, England and Wales*

Outcome	Estimate for 2015, England and Wales	Projected reduction in outcome resulting from LAIV, mean (95% HDI)
DALYs	46,039	642 (450–842)
Cases	47,080	432 (303–566)
Deaths	1,930	22 (16–29)

*DALYs, disability-adjusted life years; HDI, highest density interval; LAIV, live attenuated influenza vaccine.

density interval [HDI] 3.7–7.0) prescriptions per 1,000 person-years (Table 1) across the population of England and Wales, or 0.8% of the antibiotic dispensation rate for primary care in England and Wales in 2015. For comparison with secular trends, this rate has fallen by 2.5% each year during 2012–2018 in England (Appendix Figure). Focusing on vaccine recipients only, we estimated that the direct effectiveness of LAIV on antibiotic consumption is 5.8 (95% HDI 4.1–7.5) fewer prescriptions per 1,000 person-years in unmatched years and 9.9 (95% HDI 7.0–13) in matched years.

Although 0.8% is a small decrease in antibiotic use, it might appreciably improve the cost-effectiveness of pediatric LAIV if the healthcare costs of resistance are substantial enough (Figure 1). We estimated that LAIV has the potential to reduce resistance-attributable disability-adjusted life years (DALYs) by 642, cases by 432, and deaths by 22 per year in England and Wales (Table 2); averted DALYs were spread relatively evenly across the 7 causative pathogens analyzed (Figure 2, panel A). We estimated a yearly cost saving of £224,000 for averted resistant infections. Compared with the projected incremental cost (program cost minus healthcare saving) of pediatric LAIV at £63.6 million, and its projected effect of saving 27,475 quality-adjusted life years and averting 799 deaths yearly (3), accounting for resistance will not substantially increase the cost-effectiveness of pediatric LAIV in this setting. Our uncertainty analysis (Figure 2, panel B) identified the consultation rate as having the greatest influence over the effect of LAIV on resistance-associated adverse health outcomes.

Conclusions

Our estimates for the foreseeable reduction in antibiotic prescribing from the LAIV program in England and Wales might seem surprisingly low, given that sore throat, cough, and sinusitis together account for 53% of all inappropriate prescribing, which in turn accounts for at least 9%–23% of all prescribing in England (1). However, many viral and bacterial pathogens cause these symptoms. By

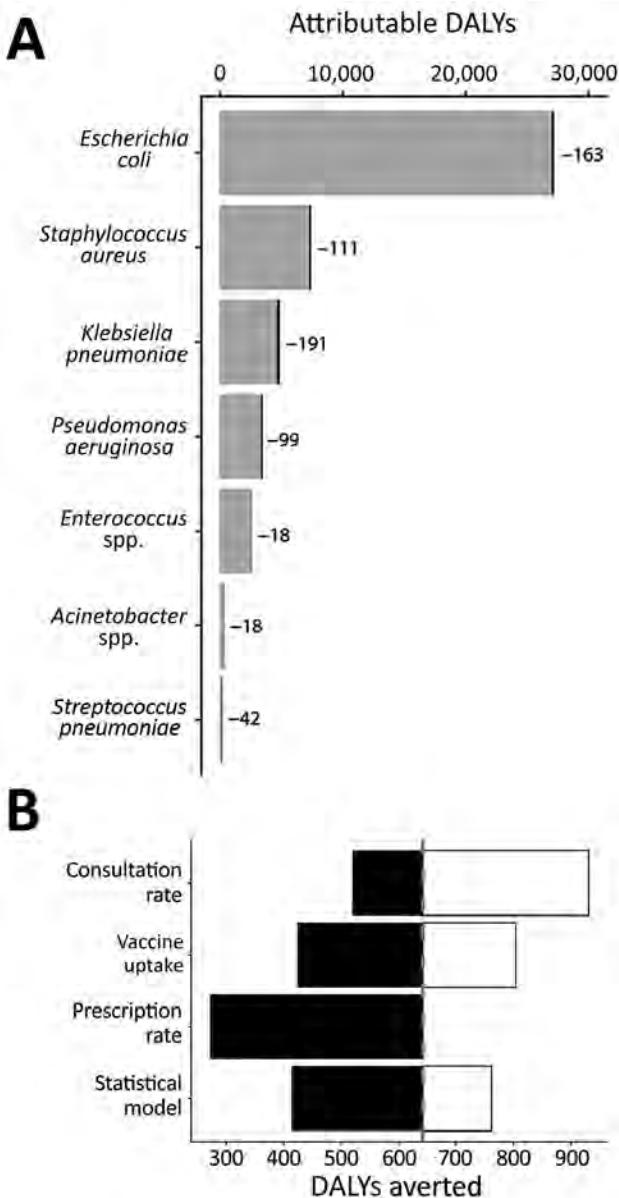


Figure 2. Effect of pediatric LAIV on adverse health outcomes attributable to antibiotic-resistant bacterial strains, England and Wales. A) Estimated DALYs attributable to resistant infections averted by pediatric LAIV, stratified by causative pathogen. The entire width of each bar is the current number of DALYs; potential reductions are highlighted in black and reported next to each bar. B) One-way uncertainty analysis, showing the effect on DALYs averted, of alternative assumptions concerning the rate of influenza-attributable general practitioner consultations, the pediatric uptake of LAIV, the rate of antibiotic prescribing per general practitioner consultation, and how the effect of prescribing on adverse health outcomes associated with resistance is attributed (additional details in Methods and Appendix, <https://wwwnc.cdc.gov/EID/article/25/12/19-1110-App1.pdf>). DALYs, disability-adjusted life years; LAIV, live attenuated influenza vaccine.

one estimate, influenza causes only 11% of GP consultations for acute respiratory illness in England (4), so it might be optimistic to expect influenza vaccination to substantially reduce antibiotic use in this setting.

Our base-case estimate of 726 antibiotic prescriptions per 1,000 influenza-attributable consultations is more than double what electronic health records suggest (7). One explanation is that our estimate, derived from statistical attribution of antibiotic prescriptions to influenza circulation during 1995–2009 (5), feasibly includes prescribing for secondary infections such as otitis media, sinusitis, and pneumonia. Moreover, electronic health records might not reliably reflect antibiotic prescribing rates for influenza: in 1 study, only 8% of consultations for ILI resulted in influenza or ILI being medically recorded (6). Conversely, antibiotic use in England has declined since 1995 (by 22% during 1998–2016) (11). Accordingly, our base-case results should be interpreted as the maximum potential reduction by LAIV of antibiotic use.

In randomized trials, the direct effect of influenza vaccines on vaccinated children has ranged from a 44% reduction (Italy) to a 6% increase (United States) in antibiotic prescriptions over the 4-month period following vaccination, whereas estimates of the effect over entire populations (all ages, vaccinated and unvaccinated) range from 11.3 fewer prescriptions per 1,000 person-years in Ontario, Canada, to 3.9 fewer in South Africa and Senegal (Appendix). This variability might arise from differences in vaccine efficacy and coverage, population risk factors, influenza circulation, or existing patterns of antibiotic use, which make generalizing estimates across settings challenging.

The adverse health outcome estimates that we adopt (9) assume that resistant infections add to, rather than replace, nonresistant infections. Relaxing this assumption would further reduce the projected effect of LAIV, because some prevented resistant infections would be replaced by nonresistant infections (12).

Our framework estimates the effect of influenza vaccination on antibiotic resistance by using the relationship between influenza circulation and antibiotic use in England and Wales, and can be adapted to other settings for which this relationship can be quantified. An alternative approach would be to correlate LAIV uptake, rather than influenza circulation, directly with antibiotic use. Challenges with that approach include appropriately controlling for confounding factors in the relationship between vaccine uptake and antibiotic use and quantifying

herd immunity. However, consistent with our approach, UK-specific empirical estimates have suggested little or no effect of LAIV uptake on prescribing: a self-controlled case-series study found that 2–4-year-old LAIV recipients took 13.5% fewer amoxicillin courses in the 6 months after vaccination (13), whereas an LAIV pilot study detected no difference in prescribing rates for respiratory tract infections between treatment groups (14). No single vaccine is likely to substantially reduce inappropriate antibiotic use in the United Kingdom.

Acknowledgments

We thank Edwin van Leeuwen for providing results from the mathematical model of influenza transmission and vaccination, Diamantis Plachouras for correspondence, and David R.M. Smith, Edwin van Leeuwen, and Marc Baguelin for discussion.

N.G.D., M.J., and K.E.A. were funded by the National Institute for Health Research Health Protection Research Unit in Immunisation at the London School of Hygiene and Tropical Medicine, in partnership with Public Health England. The views expressed are those of the authors and not necessarily those of the NHS, National Institute for Health Research, Department of Health, or Public Health England.

About the Author

Mr. Chae is a health economist and pharmacist working at the Korea International Cooperation Agency who began this work while studying for an MSc in public health at the London School of Hygiene and Tropical Medicine. Dr. Davies is a research fellow at the London School of Hygiene and Tropical Medicine, whose work focuses on the ecology and epidemiology of antibiotic resistance.

References

- Smieszek T, Pouwels KB, Dolk FCK, Smith DRM, Hopkins S, Sharland M, et al. Potential for reducing inappropriate antibiotic prescribing in English primary care. *J Antimicrob Chemother.* 2018;73(Suppl_2):ii36–43. <https://doi.org/10.1093/jac/dkx500>
- Atkins KE, Lafferty EI, Deeny SR, Davies NG, Robotham JV, Jit M. Use of mathematical modelling to assess the impact of vaccines on antibiotic resistance. *Lancet Infect Dis.* 2018; 18:e204–13. [https://doi.org/10.1016/S1473-3099\(17\)30478-4](https://doi.org/10.1016/S1473-3099(17)30478-4)
- Baguelin M, Camacho A, Flasche S, Edmunds WJ. Extending the elderly- and risk-group programme of vaccination against seasonal influenza in England and Wales: a cost-effectiveness study. *BMC Med.* 2015;13:236. <https://doi.org/10.1186/s12916-015-0452-y>
- Cromer D, van Hoek AJ, Jit M, Edmunds WJ, Fleming D, Miller E. The burden of influenza in England by age and clinical risk group: a statistical analysis to inform vaccine

- policy. *J Infect*. 2014;68:363–71. <https://doi.org/10.1016/j.jinf.2013.11.013>
5. Fleming DM, Taylor RJ, Haguinet F, Schuck-Paim C, Logie J, Webb DJ, et al. Influenza-attributable burden in United Kingdom primary care. *Epidemiol Infect*. 2016; 144:537–47. <https://doi.org/10.1017/S0950268815001119>
 6. Hayward AC, Fragaszy EB, Bermingham A, Wang L, Copas A, Edmunds WJ, et al.; Flu Watch Group. Comparative community burden and severity of seasonal and pandemic influenza: results of the Flu Watch cohort study. *Lancet Respir Med*. 2014;2:445–54. [https://doi.org/10.1016/S2213-2600\(14\)70034-7](https://doi.org/10.1016/S2213-2600(14)70034-7)
 7. Pouwels KB, Dolk FCK, Smith DRM, Robotham JV, Smieszek T. Actual versus ‘ideal’ antibiotic prescribing for common conditions in English primary care. *J Antimicrob Chemother*. 2018;73(Suppl_2):19–26. <https://doi.org/10.1093/jac/dkx502>
 8. Pebody RG, Green HK, Andrews N, Boddington NL, Zhao H, Yonova I, et al. Uptake and impact of vaccinating school age children against influenza during a season with circulation of drifted influenza A and B strains, England, 2014/15. *Euro Surveill*. 2015;20:1–11. <https://doi.org/10.2807/1560-7917.ES.2015.20.39.30029>
 9. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al.; Burden of AMR Collaborative Group. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. *Lancet Infect Dis*. 2019;19:56–66. [https://doi.org/10.1016/S1473-3099\(18\)30605-4](https://doi.org/10.1016/S1473-3099(18)30605-4)
 10. Shrestha P, Cooper BS, Coast J, Oppong R, Do Thi Thuy N, Phodha T, et al. Enumerating the economic cost of antimicrobial resistance per antibiotic consumed to inform the evaluation of interventions affecting their use. *Antimicrob Resist Infect Control*. 2018;7:98. <https://doi.org/10.1186/s13756-018-0384-3>
 11. Curtis HJ, Walker AJ, Mahtani KR, Goldacre B. Time trends and geographical variation in prescribing of antibiotics in England 1998–2017. *J Antimicrob Chemother*. 2019;74:242–50.
 12. de Kraker MEA, Jarlier V, Monen JCM, Heuer OE, van de Sande N, Grundmann H. The changing epidemiology of bacteraemias in Europe: trends from the European Antimicrobial Resistance Surveillance System. *Clin Microbiol Infect*. 2013;19:860–8. <https://doi.org/10.1111/1469-0691.12028>
 13. Hardelid P, Ghebremichael-Weldeselassie Y, Whitaker H, Rait G, Gilbert R, Petersen I. Effectiveness of live attenuated influenza vaccine in preventing amoxicillin prescribing in preschool children: a self-controlled case series study. *J Antimicrob Chemother*. 2018;73:779–86. <https://doi.org/10.1093/jac/dkx463>
 14. Muller-Pebody B, Sinnathamby MA, Warburton F, Rooney G, Andrews N, Henderson K, et al. Impact of the new childhood influenza vaccine programme on antibiotic prescribing rates in primary care in England [abstract]. 2019 [cited 2019 Jul 24]. https://www.esmid.org/esmid_publications/esmid_elibrary/material/?mid=66623

Address for correspondence: Nicholas G. Davies, London School of Hygiene and Tropical Medicine, Department of Infectious Disease Epidemiology, Keppel St, London WC1E 7HT, UK; email: nicholas.davies@lshtm.ac.uk

EID SPOTLIGHT TOPIC: Antimicrobial Resistance

Antibiotics and similar drugs, together called antimicrobial agents, have been used for the past 70 years to treat patients who have infectious diseases. Since the 1940s, these drugs have greatly reduced illness and death from infectious diseases. However, these drugs have been used so widely and for so long that the infectious organisms the antibiotics are designed to kill have adapted to them, making the drugs less effective.

Each year in the United States, at least 2 million people become infected with bacteria that are resistant to antibiotics and at least 23,000 people die each year as a direct result of these infections.



EMERGING INFECTIOUS DISEASES <http://wwwnc.cdc.gov/eid/page/resistance-spotlight>

Locally Acquired Human Infection with Swine-Origin Influenza A(H3N2) Variant Virus, Australia, 2018

Yi-Mo Deng, Frank Y.K. Wong, Natalie Spirason, Matthew Kaye, Rebecca Beazley, Miguel L. Grau, Songhua Shan, Vittoria Stevens, Kanta Subbarao, Sheena Sullivan, Ian G. Barr, Vijaykrishna Dhanasekaran

In 2018, a 15-year-old female adolescent in Australia was infected with swine influenza A(H3N2) variant virus. The virus contained hemagglutinin and neuraminidase genes derived from 1990s-like human seasonal viruses and internal protein genes from influenza A(H1N1)pdm09 virus, highlighting the potential risk that swine influenza A virus poses to human health in Australia.

Long-term circulation of influenza A viruses (IAVs) among swine poses a public health threat. The 2009 pandemic was caused by a reassortant swine influenza A(H1N1) virus with genes that originated from human and avian IAVs that had circulated among swine for several years (1,2). Since then, globally enhanced influenza surveillance among swine has indicated continuous introduction of human seasonal influenza viruses into swine, followed by reassortment with influenza A viruses endemic in swine (IAV-S) and persistence of many lineages in swine for several decades (3). Although IAV-S are normally limited to transmission among swine, since 2010, a total of 430 cases of human infection with swine-origin influenza A(H3N2) variant viruses (H3N2v) have been detected in the United States (4), primarily in young persons exposed to swine at agricultural fairs. Most patients had

self-limited influenza-like illness (5). Recent data also suggest that IAV-S have been endemic to Australia for many decades, including viruses that were originally derived from human H3N2 viruses as early as 1968, pre-2009 seasonal H1N1 viruses, and influenza A(H1N1)pdm09 (pH1N1) viruses (6).

The Study

In September 2018, a case of human infection with a swine-origin influenza virus was detected in Australia through routine human influenza virus surveillance by the World Health Organization (WHO) Collaborating Centre for Reference and Research on Influenza (Melbourne, Victoria, Australia), which is part of the WHO Global Influenza Surveillance and Response System. The sample was from a 15-year-old female adolescent living in a semirural area in South Australia, ≈100 km from Adelaide. The patient sought outpatient care for a mild respiratory illness ≈8 days after illness onset. The attending physician collected a nasal swab sample and sent it for testing to a laboratory in Adelaide, where influenza A was detected by real-time reverse transcription PCR (RT-PCR) but not subtyped. The sample was subsequently forwarded to the WHO Collaborating Centre for further characterization. It was later determined that the patient had not been vaccinated against influenza in 2018 and had had contact with animals at an agricultural show in South Australia the day before illness onset.

We isolated an influenza A virus in SIAT-1 MDCK cells (7) and designated it as A/South Australia/85/2018 (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/19-1144-App1.pdf>). Testing of this isolate by real-time RT-PCR with an influenza diagnostic kit from the US Centers for Disease Control and Prevention confirmed that the virus was an

Affiliations: World Health Organization Collaborating Centre for Reference and Research on Influenza, Melbourne, Victoria, Australia (Y.-M. Deng, N. Spirason, M. Kaye, K. Subbarao, S. Sullivan, I.G. Barr, V. Dhanasekaran); CSIRO Australian Animal Health Laboratory, Geelong, Victoria, Australia (F.Y.K. Wong, S. Shan, V. Stevens); South Australian Department of Health and Wellbeing, Adelaide, South Australia, Australia (R. Beazley); Monash University, Melbourne (M.L. Grau, V. Dhanasekaran); University of Melbourne, Melbourne (S. Sullivan, I.G. Barr)

DOI: <https://doi.org/10.3201/eid2601.191144>

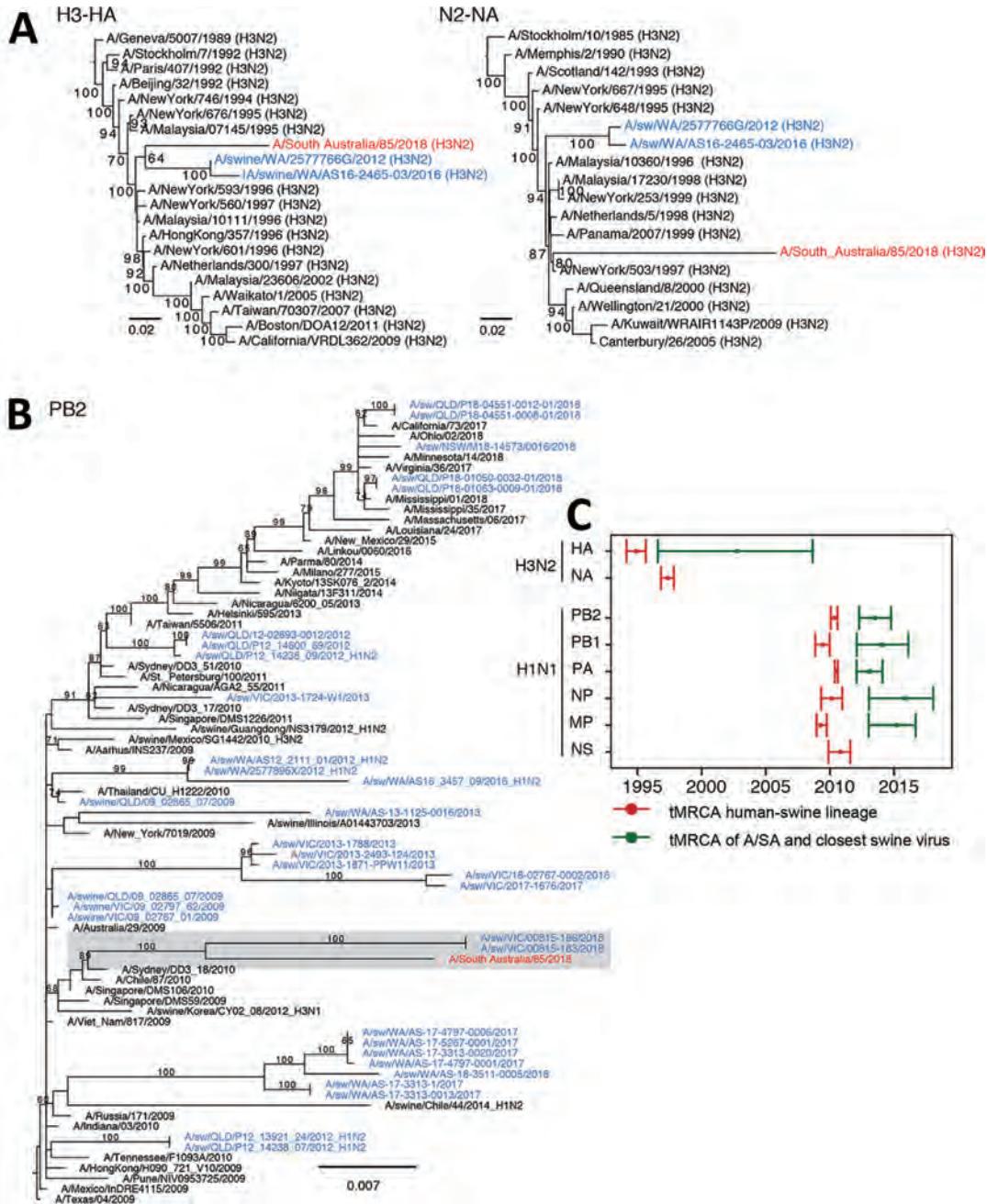


Figure 1. Genetic origin of influenza A/South Australia/85/2018 virus isolated from a human patient in Australia (red) from swine influenza A(H3N2) and H1N1pdm09 viruses. Blue indicates influenza A viruses from swine in Australia. A, B) Maximum-likelihood phylogenies estimated by using RAxML version 8 (10) of the HA and NA genes (A) and PB2 gene (B) showing bootstrap values at branch nodes (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/19-1144-App1.pdf>). The origins of the remaining 5 internal protein genes (PB1, PA, NP, MP, and NS) are provided in Appendix Figure 2, and the GenBank accession numbers and dates of sampling are provided in Appendix Table 4. Scale bars indicate nucleotide substitutions per site. C) Calculation of tMRCAs. Red indicates means and 95% CIs of the time of origin of each of the Australia swine influenza A virus lineages from human seasonal influenza viruses. Numbers denote viruses that shared the same tMRCAs and that formed a similar lineage. Green indicates the time of divergence of A/South Australia/85/2018 from A/swine/WA/2577766G/2012 (H3N2) (for the H3 HA gene) and A/swine/Victoria/18-04095-0003/2018 (H1N1) (for 5 internal protein genes: PB2, PB1, PA, NP, and MP). N2 and NS proteins of A/South Australia/85/2018 are directly derived from human viruses. Divergence times were estimated by using the uncorrelated log-normal relaxed clock model (11) in a Bayesian Markov chain Monte Carlo framework in BEAST version 1.10 (<https://beast.community>). A/SA, A/South Australia/85/2018 virus; HA, hemagglutinin; MP, matrix protein; NA, neuraminidase; NP, nucleoprotein; NS, nonstructural; PA, polymerase acidic; PB, polymerase basic; A(H1N1) pdm09 virus, 2009 pandemic influenza H1N1 virus; tMRCAs, time to most recent common ancestor.

influenza A(H3N2) virus with a pH1N1-like nucleoprotein (NP) gene. Hemagglutination inhibition (HI) assays of the isolate showed that this virus had >5-fold antigenic divergence to ferret antiserum raised against

human H3N2 viruses collected during 1993–2016 and an H3N2v from the United States (A/Minnesota/11/2010) (Appendix Table 1) (6). The A/South Australia/85/2018 virus showed good cross-reactivity

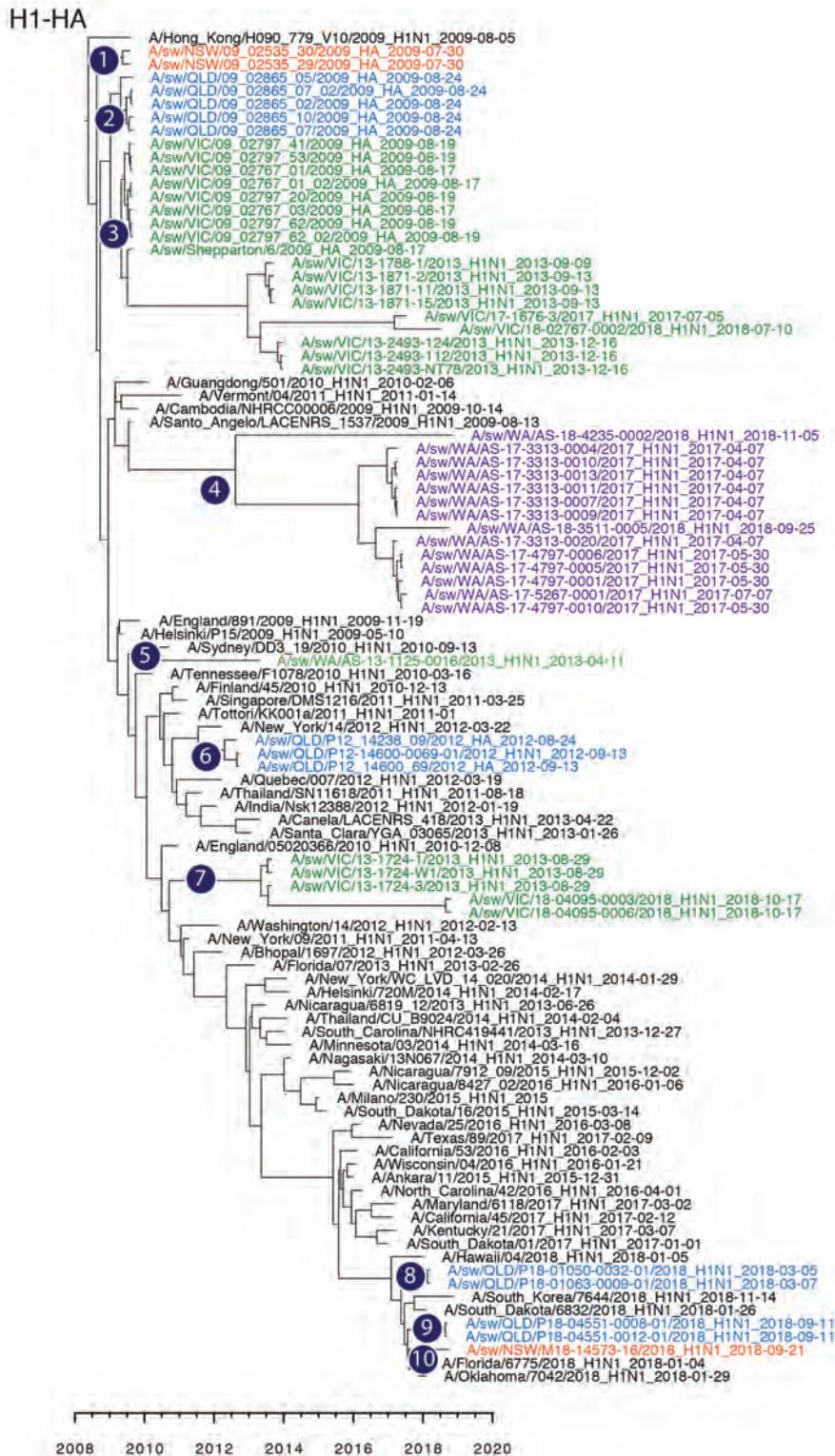


Figure 2. Maximum-clade credibility tree showing the time of emergence from humans and divergence of swine influenza A and influenza A(H1N1)pdm09 virus among swine populations in Australia. Numbers 1–10 denote inferences of individual introductions from humans to swine, and virus names are colored by state of collection: red, New South Wales; blue, Queensland; green, Victoria; purple, Western Australia. Time-scaled phylogenies were estimated by using the uncorrelated log-normal relaxed clock model (11) in a Bayesian Markov chain Monte Carlo framework in BEAST version 1.10 (<https://beast.community>). HA, hemagglutinin.

(within 2-fold of the homologous HI titer) with pig antiserum to a US swine A(H3N2) virus, A/swine/New York/A01104005/2011 (Appendix Table 2). Although both viruses showed high genetic divergence, they contained a common hemagglutinin (HA) Y155H substitution within antigenic site B (Appendix Table 3); its role in antigenicity remains unknown.

Whole-genome sequencing of A/South Australia/85/2018 indicated that the virus was a 2:6 reassortant of 2 human influenza virus lineages introduced to swine in Australia; the H3 HA and N2 NA genes originated independently from seasonal human influenza viruses that circulated during 1995–1999, and the genes for internal proteins originated from pH1N1 viruses from late 2009 through early 2010. Using a fluorescence-based NA enzyme inhibition assay, we found that A/South Australia/85/2018 was sensitive to 1 class of influenza drugs, the NA inhibitors (oseltamivir and zanamivir); however, it would be expected to be resistant to the adamantane class of drugs (amantadine/rimantadine) because it had an S31N substitution in the matrix (M) 2 gene, which is known to confer high-level resistance to adamantane (8).

To identify the evolutionary origins and the epidemiologic links of A/South Australia/85/2018, we further generated the whole and partial genomes of an additional 44 available IAV-S collected in piggeries across several states in Australia during 2013–2018; all were pH1N1-like viruses (Appendix Table 4). These viruses were derived from swine nasal, tracheal, or pooled lung tissue samples submitted on an ad hoc basis for diagnostic investigations during 2012–2018 to the Australian Animal Health Laboratories (Geelong, Victoria, Australia) by commercial piggeries in New South Wales, Queensland, Victoria, and Western Australia.

Phylogenies inferred with all sequences of swine influenza viruses from Australia, as well as with a representative set of those collected from humans and swine globally (6,9) (Appendix), showed that the HA gene of A/South Australia/85/2018 was most closely related to swine H3N2 viruses collected from a commercial piggery in Western Australia during 2012–2016 (6) (Figure 1, panel A). This H3 swine lineage from Australia was poorly supported (64% bootstrap) and originated from the phylogenetic backbone of human seasonal H3N2 virus, clustering with viruses collected during 1995–1996, whereas their NA was most closely related to that of human H3N2 viruses circulating during 1997–1999 (Appendix Figure 1) and not most closely related to that of swine samples from Australia.

Of note, 5 of 6 internal segments of A/South Australia/85/2018 were most closely related to a swine pH1N1 virus collected in Victoria in 2018

(A/swine/Victoria/00815–183/2018) (Figure 1, panel B). Although there were no IAV-S data from South Australia to trace the immediate origins of A/South Australia/85/2018, these phylogenetic relationships confirm that A/South Australia/85/2018 was acquired locally from swine herds endemically infected with influenza A viruses that had circulated since the mid-1990s and the 2009 H1N1 pandemic.

The genomic relationship of A/South Australia/85/2018 to IAV-S collected across at least 2 geographically distinct states, Western Australia for the HA gene and Victoria for 5 internal segments, suggests the possibility of IAV-S movement between states in Australia, although IAV-S data for Australia are missing for at least 2–10 years (Figure 1, panel C). This suggestion is, however, contradictory to the data from IAV-S HA collected across 4 states in this study: 10 distinct monophyletic lineages (Figure 2) derived independently from the human pH1N1 lineage, with each group exclusive to 1 state, suggesting that there are spatial restrictions for farms in Australia.

Conclusions

A comparison of divergence times between the IAV-S segments from Australia showed that reassortment of endemic viruses with introduced human lineages had been continual (Appendix Figure 3), thereby potentially maintaining sustained transmission on individual swine farms. The risk for emergence of A/South Australia/85/2018-like viruses in humans is potentially high because all 6 internal protein genes are derived from human-adapted pH1N1 virus. The human-origin HA and NA genes of A/South Australia/85/2018 were widely circulating in the human population 20–25 years ago. Hence, children probably have little or no immunity to the HA/NA of this virus, making them more susceptible to infection with this virus subtype, as in the case reported here and in children infected with swine H3N2v virus in the United States (12–14).

The genomic and antigenic properties and epidemiologic characteristics of zoonotic IAV-S are useful for identifying the potential risk for emergence and spread into the human population. These data also enable better identification of potential nationally relevant mitigation strategies, including measures such as public awareness programs and influenza vaccination of swine herds to eliminate sustained transmission of influenza virus in swine populations (15). Our study highlights the risk to the general human population in Australia for infection with IAV-S and the need for more vigilant surveillance of swine and persons who are in close contact with swine to enable early detection and characterization of zoonotic influenza infections.

Acknowledgments

We acknowledge the technical support of Rob Shaw, Naomi Komadina, Jeff Butler, Kelly Davies, Debbie Eagles, Patrick Mileto, and Jianning Wang. We thank Amy Vincent for donating reference swine antiserum and virus antigens. We thank the Australian State Animal Health Laboratories at the AgriBio Centre at Agriculture Victoria Research, the Biosecurity Sciences Laboratory of the Department of Agriculture and Fisheries in Queensland, the Elizabeth Macarthur Agricultural Institute of the New South Wales Department of Primary Industries and the Western Australia Department of Primary Industries, and Regional Development Diagnostic Laboratory Services for diagnostic investigations and testing of swine samples. We also thank the Offices of the Chief Veterinary Officer of New South Wales, Queensland, South Australia, and Western Australia, for facilitating the use of virus data from the animal health sector for this study. Finally, we acknowledge the laboratories that generated the influenza virus sequences and listed them on GISAID; for a full list of submitting laboratories and accession numbers, see Appendix Table 5 (<https://wwwnc.cdc.gov/EID/article/26/1/19-1144-App1.pdf>).

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health, and the Australian Animal Health Laboratories facility is supported by the Department of Agriculture and Water Resources and the National Collaborative Research Infrastructure Strategy. V.D. and M.G. are supported by contract HHSN272201400006C from the National Institute of Allergy and Infectious Diseases, National Institutes of Health, US Department of Health and Human Services.

About the Author

Dr. Deng leads the molecular biology group at the WHO Collaborating Centre for Reference and Research on Influenza, Melbourne, Australia. Her primary research interest is the use of novel sequencing and analysis methods to infer the epidemiology and mechanisms of evolution of zoonotic and human influenza viruses.

References

- Smith GJ, Vijaykrishna D, Bahl J, Lycett SJ, Worobey M, Pybus OG, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. *Nature*. 2009;459:1122–5. <https://doi.org/10.1038/nature08182>
- Dawood FS, Jain S, Finelli L, Shaw MW, Lindstrom S, Garten RJ, et al.; Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team. Emergence of a novel swine-origin influenza A (H1N1) virus in humans. *N Engl J Med*. 2009;360:2605–15. <https://doi.org/10.1056/NEJMoa0903810>
- Vincent A, Awada L, Brown I, Chen H, Claes F, Dauphin G, et al. Review of influenza A virus in swine worldwide: a call for increased surveillance and research. *Zoonoses Public Health*. 2014;61:4–17. <https://doi.org/10.1111/zph.12049>
- Bowman AS, Nelson SW, Page SL, Nolting JM, Killian ML, Sreevatsan S, et al. Swine-to-human transmission of influenza A(H3N2) virus at agricultural fairs, Ohio, USA, 2012. *Emerg Infect Dis*. 2014;20:1472–80. <https://doi.org/10.3201/eid2009.131082>
- Centers for Disease Control and Prevention (CDC). Influenza A (H3N2) variant virus-related hospitalizations: Ohio, 2012. *MMWR Morb Mortal Wkly Rep*. 2012;61:764–7.
- Wong FYK, Donato C, Deng YM, Teng D, Komadina N, Baas C, et al. Divergent human-origin influenza viruses detected in Australian swine populations. *J Virol*. 2018;92:e00316-18. <https://doi.org/10.1128/JVI.00316-18>
- Matrosovich M, Matrosovich T, Carr J, Roberts NA, Klenk HD. Overexpression of the alpha-2,6-sialyltransferase in MDCK cells increases influenza virus sensitivity to neuraminidase inhibitors. *J Virol*. 2003;77:8418–25. <https://doi.org/10.1128/JVI.77.15.8418-8425.2003>
- Hayden FG, Hay AJ. Emergence and transmission of influenza A viruses resistant to amantadine and rimantadine. *Curr Top Microbiol Immunol*. 1992;176:119–30. https://doi.org/10.1007/978-3-642-77011-1_8
- Deng YM, Iannello P, Smith I, Watson J, Barr IG, Daniels P, et al. Transmission of influenza A(H1N1) 2009 pandemic viruses in Australian swine. *Influenza Other Respir Viruses*. 2012;6:e42–7. <https://doi.org/10.1111/j.1750-2659.2012.00337.x>
- Stamatakis A. RAXML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*. 2014;30:1312–3. <https://doi.org/10.1093/bioinformatics/btu033>
- Drummond AJ, Ho SY, Phillips MJ, Rambaut A. Relaxed phylogenetics and dating with confidence. *PLoS Biol*. 2006;4:e88. <https://doi.org/10.1371/journal.pbio.0040088>
- Duwell MM, Blythe D, Radebaugh MW, Kough EM, Bachaus B, Crum DA, et al. Influenza A(H3N2) variant virus outbreak at three fairs – Maryland, 2017. *MMWR Morb Mortal Wkly Rep*. 2018;67:1169–73. <https://doi.org/10.15585/mmwr.mm6742a1>
- Epperson S, Jhung M, Richards S, Quinlisk P, Ball L, Moll M, et al.; Influenza A (H3N2)v Virus Investigation Team. Human infections with influenza A(H3N2) variant virus in the United States, 2011–2012. *Clin Infect Dis*. 2013;57(Suppl 1):S4–11. <https://doi.org/10.1093/cid/cit272>
- Jhung MA, Epperson S, Biggerstaff M, Allen D, Balish A, Barnes N, et al. Outbreak of variant influenza A(H3N2) virus in the United States. *Clin Infect Dis*. 2013;57:1703–12. <https://doi.org/10.1093/cid/cit649>
- Animal Health Australia. AUSVETPLAN manuals and documents. Influenza A viruses in swine (Version 4.0) [cited 2019 Jul 5]. <https://www.animalhealthaustralia.com.au/our-publications/ausvetplan-manuals-and-documents>

Address for correspondence: Yi-Mo Deng, WHO Collaborating Centre for Reference and Research on Influenza, The Peter Doherty Institute for Infection and Immunity, 792 Elizabeth St, Melbourne, VIC 3000, Australia; email: yi-mo.deng@influenzacentre.org; Vijaykrishna Dhanasekaran, Department of Microbiology, Biomedicine Discovery Institute, Monash University, 19 Innovation Walk, Clayton, VIC 3800, Australia; email: vijay.dhanasekaran@monash.edu

Use of Ambulance Dispatch Calls for Surveillance of Severe Acute Respiratory Infections

Susana Monge, Janneke Duijster, Geert Jan Kommer, Jan van de Kasstelee, Gé A. Donker, Thomas Krafft, Paul Engelen, Jens P. Valk, Jan de Waard, Jan de Nooij, Wim van der Hoek, Liselotte van Asten

Author affiliations: European Centre for Disease Prevention and Control, Stockholm, Sweden (S. Monge); National Institute for Public Health and the Environment, Bilthoven, the Netherlands (S. Monge, J. Duijster, G.J. Kommer, J. van de Kasstelee, W. van der Hoek, L. van Asten); Nivel Primary Care Database, Utrecht, the Netherlands (G.A. Donker); Maastricht University, Maastricht, the Netherlands (T. Krafft); Meldkamersupport, Hellevoetsluis, the Netherlands (P. Engelen); Dispatch Center Regional Ambulance Services Noord Nederland, Groningen, the Netherlands (J.P. Valk); University Medical Center, Groningen (J.P. Valk); Regional Ambulance Service Hollands Midden, Leiden, the Netherlands (J. de Waard, J. de Nooij)

DOI: <https://doi.org/10.3201/eid2601.181520>

Ambulance dispatches for respiratory syndromes reflect incidence of influenza-like illness in primary care. Associations are highest in children (15%–34% of respiratory calls attributable to influenza), out-of-office hours (9%), and highest urgency-level calls (9%–11%). Ambulance dispatches might be an additional source of data for severe influenza surveillance.

Influenza virus infection is associated with severe illness and death and causes a high burden of disease (1). The World Health Organization recommends that countries improve surveillance of severe influenza infections (2). However, surveillance based on hospital and intensive care unit admissions for severe acute respiratory infections or laboratory-confirmed influenza is still limited in most countries, raising the question whether there are other routinely collected data that could be used to monitor severe influenza infections (2,3).

Ambulance dispatch centers manage patient telephone calls by using a clinical triage system to collect and record information in real time and determine the need for an ambulance and the urgency of the need. This data source is attractive for surveillance because it is recorded continuously, is quickly available, has high coverage within a region, has low running costs,

and is highly standardized (4–6). We aimed to assess whether telephone calls to ambulance dispatch centers were a possible source for surveillance of severe influenza, by showing the association with influenza-like illness (ILI) incidence, the most critical influenza indicator in primary care.

We retrospectively reviewed 289,307 ambulance calls with high urgency (A1 and A2: ambulance required to arrive within 15 or 30 minutes) received in 4 call centers in the Netherlands, covering 4.2 million persons (25% of the population), from January 1, 2014, through December 31, 2016. Therefore, our data covered 2 full respiratory seasons (2014–15 and 2015–16) and 2 half seasons: 2013–14 (weeks 1–26) and 2016–17 (weeks 27–52). Triage diagnostic codes from the Advanced Medical Priority Dispatch System associated with possible respiratory infection were combined as respiratory syndrome calls (RSCs) and aggregated weekly; 6.7% of calls were RSCs (weekly average 123, range 69–165; i.e., 2.9/100,000 inhabitants/wk). RSCs showed a periodic pattern, peaking in winter, with lower interseasonal peaks.

We estimated how many RSCs were attributable to influenza circulation in the community, using weekly ILI incidence from sentinel general-practitioner practices (7). We used a binomial generalized linear model with an identity link function to model weekly number of RSCs (numerator) relative to total number of calls (denominator). Model coefficients estimate risk differences; that is, percentage increase in RSCs per each additional case of ILI per 10,000 inhabitants. We evaluated the presence of a linear time trend and periodic patterns and conceptualized it as the baseline (RSCs not attributed to ILI). We then evaluated the association of ILI incidence either in the current week or lagged up to 4 weeks forward in time (+ lags) or 4 weeks backward in time (– lags). Finally, we evaluated whether this association differed between epidemiologic years (week 27 of one year to week 26 of the following year). We repeated the analysis for different age groups, office hours (8:00 AM–16:59 PM Monday–Friday), out-of-office hours (17:00 PM–7:59 AM Monday–Friday, plus the weekend), and calls with the highest urgency level (A1: immediately life-threatening, ambulance required to arrive within 15 minutes).

All models showed a significant positive association ($p < 0.05$) between ILI and RSCs. Increases in RSCs occurred generally 1–3 weeks later than increases in ILI. For patients <15 years of age, RSC increases occurred 1 week earlier than ILI, possibly reflecting respiratory syncytial virus activity rather than influenza activity. Among highest-urgency calls and children,

the association varied by epidemiologic year and was highest for 2013–14 and 2016–17.

Overall, an increase in ILI weekly incidence of 1 case/10,000 inhabitants was associated with an increase of 0.114% in weekly RSCs. In our study population, this finding translated into an average of 110 RSCs/year, or 2.6 cases/100,000 inhabitants/year.

When applying these effects to observed weekly ILI incidence (4.98 cases/10,000 inhabitants), we attributed 12.9 RSCs/100,000 inhabitants/year to ILI. The highest proportion of ILI-attributed RSCs was in patients <15 years of age (15%–34%, depending on the season), during out-of-office hours (9%), and highest-urgency calls (9%–11%). In all models, the ILI-attributed percentage was higher during the winter peaks,

but non-ILI baseline still explained most RSCs during these winter periods (Figure), showing the expected high background incidence in dispatch data of other respiratory conditions, even during the peak of the influenza season, as also reported by others (4,5,8).

In conclusion, ambulance calls for respiratory syndrome show variability attributable to ILI and probably reflect the severe end of the ILI spectrum, especially when restricted to calls for the young, during out-of-office hours, or with the highest urgency level. Ambulance dispatch center data could be used to monitor the severity of the influenza epidemic, but its true usefulness and added value should be studied prospectively. Such surveillance could potentially inform planning of healthcare services and

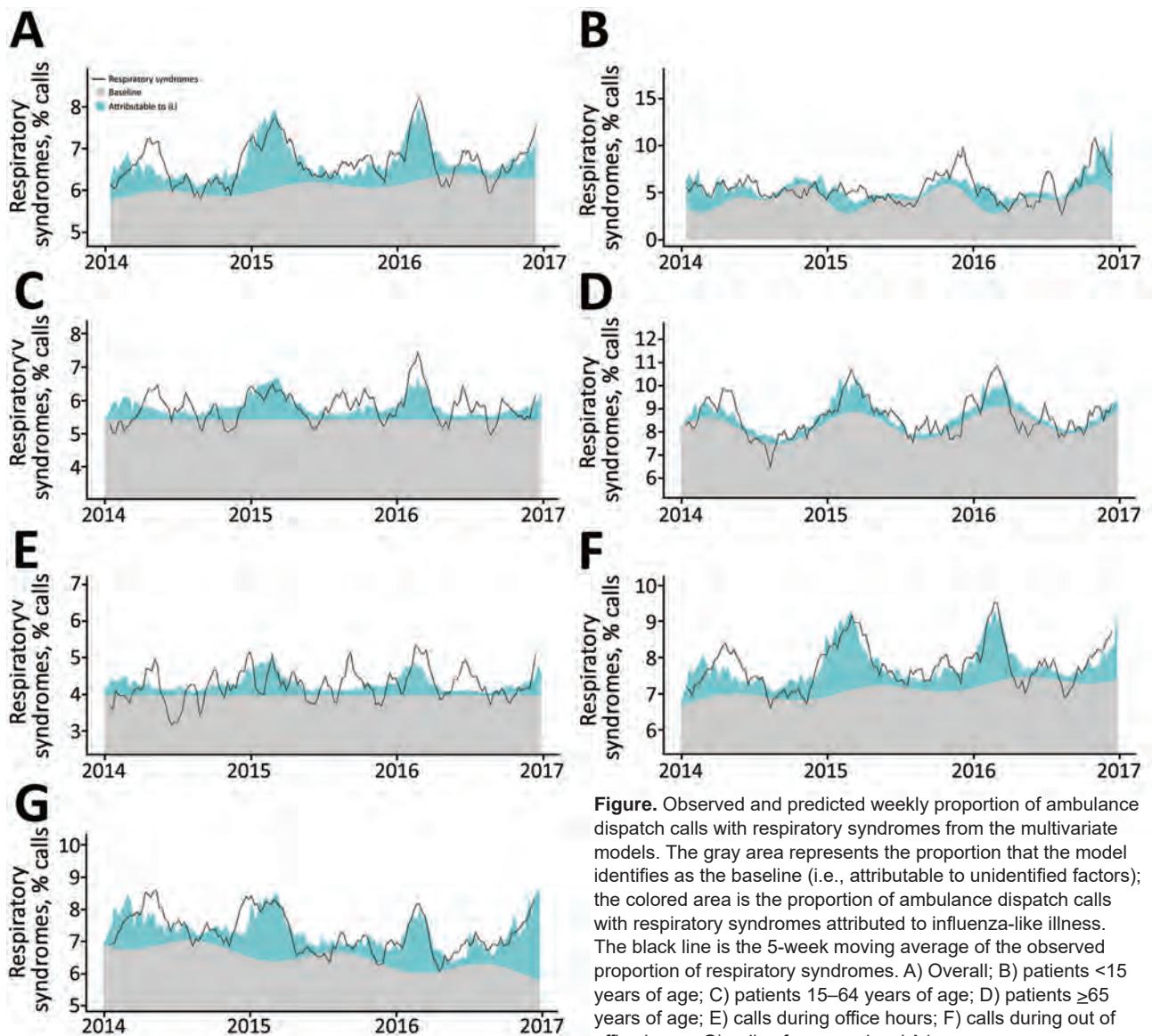


Figure. Observed and predicted weekly proportion of ambulance dispatch calls with respiratory syndromes from the multivariate models. The gray area represents the proportion that the model identifies as the baseline (i.e., attributable to unidentified factors); the colored area is the proportion of ambulance dispatch calls with respiratory syndromes attributed to influenza-like illness. The black line is the 5-week moving average of the observed proportion of respiratory syndromes. A) Overall; B) patients <15 years of age; C) patients 15–64 years of age; D) patients ≥65 years of age; E) calls during office hours; F) calls during out of office hours; G) calls of urgency level A1.

public health actions such as vaccination, especially following the emergence of new influenza strains. Our results also estimate the impact of influenza on ambulance services and can add to the estimations of burden of disease for influenza.

Acknowledgments

We thank the 4 regional ambulance services participating in this project (Hollands Midden, Brabant Midden-West, Brabant Noord, and Noord Nederland). We also thank Jaap Hatenoer, Myrthe Mos, and Susan Hahné.

This work was funded from the regular budget of the Dutch Centre for Infectious Disease Control, made available by the Ministry of Health, Welfare, and Sport, project number V/150207.

Author contributions: L.v.A. and W.v.H. conceived the idea and initiated this project. J.D. and G.J.K. centralized and managed the ambulance dispatch data, and G.A.D. collected and monitored weekly ILI data. S.M. performed the analysis with the help of L.v.A., J.D., and J.v.K., and drafted the first version of the manuscript under the supervision of L.v.A. and W.v.H. All authors participated in the interpretation of results and critically reviewed the manuscript.

About the Author

While developing this work, Dr. Monge was an EPIET fellow at the National Institute for Public Health and the Environment in the Netherlands. She is currently head of section at the Centre for the Coordination of Health Alerts and Emergencies in the Ministry of Health, Consumption and Social Welfare, Spain. Her primary research interest is in infectious diseases epidemiology.

References

- van Lier A, McDonald SA, Bouwknegt M, Kretzschmar ME, Havelaar AH, Mangen MJ, et al. EPI rGoup. Disease burden of 32 infectious diseases in the Netherlands, 2007–2011. *PLoS One*. 2016;11:e0153106. <https://doi.org/10.1371/journal.pone.0153106>
- World Health Organization. Pandemic Influenza Severity Assessment (PISA): a WHO guide to assess the severity of influenza epidemics & pandemics. 2017 [cited 2019 Nov 12]. http://www.who.int/influenza/surveillance_monitoring/pisa/guidance
- World Health Organization. Global epidemiological surveillance standards for influenza. 2013 [cited 2019 Nov 12]. https://www.who.int/influenza/resources/documents/WHO_Epidemiological_Influenza_Surveillance_Standards_2014.pdf
- Coory MD, Kelly H, Tippett V. Assessment of ambulance dispatch data for surveillance of influenza-like illness in Melbourne, Australia. *Public Health*. 2009;123:163–8. <https://doi.org/10.1016/j.puhe.2008.10.027>
- Rosenkötter N, Ziemann A, Riesgo LG, Gillet JB, Vergeiner G, Krafft T, et al. Validity and timeliness of syndromic influenza surveillance during the autumn/winter wave of A(H1N1) influenza 2009: results of emergency medical dispatch, ambulance, and emergency department data from three European regions. *BMC Public Health*. 2013;13:905. <https://doi.org/10.1186/1471-2458-13-905>
- Duijster JD, Pilot S, van der Hoek E, Kommer W, van der Sande GJ, Krafft M, et al. L. Emergency medical service data for syndromic surveillance of infectious diseases: a scoping review. *Eur J Public Health*. In press 2019.
- Donker GA. NIVEL primary care database – sentinel practices 2015. 2016 [cited 2019 Nov 12]. https://www.nivel.nl/sites/default/files/bestanden/Peilstations_2015_Engel.pdf
- Todkill D, Loveridge P, Elliot AJ, Morbey RA, Edgheere O, Rayment-Bishop T, et al. Utility of ambulance data for real-time syndromic surveillance: a pilot in the West Midlands region, United Kingdom. *Prehosp Disaster Med*. 2017; 32:667–72. <https://doi.org/10.1017/S1049023X17006690>

Address for correspondence: Liselotte van Asten, Centre for Infectious Disease Control Netherlands, National Institute for Public Health and the Environment, Antonie van Leeuwenhoeklaan 9, 3721 MA, Bilthoven, the Netherlands; email: liselotte.van.asten@rivm.nl

Hantavirus Pulmonary Syndrome in Traveler Returning from Nepal to Spain

Elena Sulleiro,¹ Maria Luisa Aznar,¹ Núria Serre-Delcor, Fernando Salvador, Adrian Sanchez-Montalvá, Mateu Espasa, Daniel Molina, Fernando de Ory, M. Paz Sanchez-Seco, Israel Molina, Candido Diaz-Lagares, Miguel J. Martinez, Tomàs Pumarola, Inés Oliveira

Author affiliations: Vall d'Hebrón University Hospital, PROSICS Barcelona, Universitat Autònoma de Barcelona, Barcelona, Spain (E. Sulleiro, M.L. Aznar, N. Serre-Delcor, F. Salvador, A. Sanchez-Montalvá, M. Espasa, D. Molina, I. Molina, C. Diaz-Lagares, T. Pumarola, I. Oliveira); Instituto de Salud Carlos III, Majadahonda, Spain (F. de Ory, M.P. Sanchez-Seco); Hospital Clínic, Barcelona (M.J. Martinez)

DOI: <https://doi.org/10.3201/eid2601.181685>

¹These authors contributed equally to this article.

Most human hantavirus infections occur in Asia, but some cases have been described in Europe in travelers returning from Asia. We describe a case of hantavirus pulmonary syndrome in a previously healthy traveler occurring shortly after he returned to Spain from Nepal. Serologic tests suggested a Puumala virus–like infection.

More than 24 pathogenic hantaviruses that are known to be pathogenic to humans have been identified worldwide (1). The diseases these viruses have caused have been traditionally divided into 2 major clinical syndromes: hemorrhagic fever with renal syndrome (HFRS) in Europe, Asia, and Africa, and hantavirus pulmonary syndrome (HPS; sometimes referred to as hantavirus cardiopulmonary syndrome) in the Americas (2). We describe a life-threatening hantavirus infection in a patient with respiratory failure returning to Spain from Nepal.

In October 2017, a 28-year-old man sought care at the outpatient clinic of Drassanes Tropical Medicine Unit at Hospital Vall d'Hebrón, Barcelona, Spain, on day 2 after onset of symptoms. He had recently returned from a 5-week trip to Nepal (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/18-1685-App1.pdf>), during which he stayed at basic hostels, where sounds of rats or mice were audible. The patient reported onset of fever, malaise, weakness, headache, arthromyalgia, and abdominal pain during his flight back to Spain. Medical examination revealed only fever (38.2°C) and a mild diffuse macular rash on his trunk. Laboratory tests showed thrombocytopenia (platelets $125 \times 10^9/L$) and mild liver enzyme elevation (alanine aminotransferase 127 IU/L, aspartate aminotransferase 81 IU/L); results of tests for various pathogens, including hantavirus, were negative (Table).

The patient returned home, but on day 4, he incurred a head wound after a fall caused by dizziness, requiring treatment at the Vall d'Hebrón Hospital emergency department; he was discharged on day 5 in stable condition and with normal chest radiograph results. On day 6, he was readmitted for dyspnea; within 12 hours, respiratory failure developed, requiring admission to the intensive care unit with high-flow nasal cannula oxygen therapy and vasoactive support. Results of a new chest radiograph revealed bilateral pleural effusion and extensive alveolar edema. Electrocardiogram results showed no abnormality, but echocardiogram results showed mild ventricular dysfunction (left ventricular ejection fraction 50%) without evidence of pericardial effusion.

The patient required respiratory and vasoactive support in the intensive care unit for 5 days; and was then transferred to a regular room. At that point, 12

days after symptom onset, repeat testing showed positive results for hantavirus IgM (weak positive) and IgG (Table). The diagnosis was established as HPS, as defined by the US Centers for Disease Control and Prevention (CDC) classification (3). The patient was discharged to his home 2 days later. Repeat test results on day 22 after symptom onset remained positive for hantavirus IgM and IgG. A month after discharge, the patient still reported a mild dyspnea and fatigue. Pulmonary function tests showed no abnormality. Intolerance to exercise lasted for 2 months after discharge. One year later, he is fully recovered without sequelae.

The course of this patient was as classically described for HPS: an initial prodromal phase with influenza-like symptoms, followed by a rapid progression to abrupt onset of respiratory failure (3). Elevated levels of C-reactive protein and lactate dehydrogenase were found soon after symptom onset (Table), as described in the literature (4). Results of coagulation, cardiac enzyme, and renal function tests were normal throughout hospitalization, but proteinuria and microhematuria were not evaluated.

Once the hantavirus diagnosis was established, we contacted the patient's trip partner, who accompanied him during the first 2 weeks in Nepal. He was asymptomatic; results of a serologic test (ELISA) performed 7 weeks after his return was negative for hantavirus IgM and IgG.

Little is known about the incidence of hantavirus infections in Nepal. Thottapalayam virus, a genetically distant virus from other Old World hantaviruses, has been detected in the Asian house shrew (*Suncus murinus*) (5); no human cases of infection with this virus have been reported. However, serologic evidence of hantavirus infection in patients with fever of unknown origin has been reported in Nepal (6). Serum and urine samples from our patient tested negative by an in-house nested reverse transcription PCR (RT-PCR) targeting the small segment of the viral genome for detection of New World and Old World hantaviruses. Viral RNA is rarely found in blood more than a few days after onset of fever, and similar negative results in RT-PCR have been previously reported (7–9). Alternatively, the infection may have been caused by a genetically different hantavirus not detected by the in-house hantavirus RT-PCR. Furthermore, the serum sample was frozen and thawed several times, which may have degraded the RNA.

The pattern of serologic findings able to confirm only a transitory presence of Puumala virus (PUUV) IgG suggest a cross-reaction with an unknown hantavirus, because the known PUUV reservoir, the *Myodes*

Table. Clinical and laboratory findings over time of a patient with hantavirus pulmonary syndrome after travel, Spain*

Findings	Acute phase, day 2	ICU admission, day 6	ICU discharge, day 12	Follow-up, day 22	Follow-up, day 82
Clinical	Fever, myalgia, headache, vomiting, and diarrhea	Acute noncardiac pulmonary edema and hypotension; pleural effusions and respiratory failure	Eupneic, resolution of the pulmonary edema	Mild dyspnea general condition with fatigue	Completely recovered
Hematologic	Leukocytes 4.38 × 10 ⁹ cells/L; platelets 125 × 10 ⁹ /L	Leukocytes 6.17 × 10 ⁹ cells/L; platelets 92 × 10 ⁹ /L	Leukocytes 7.00 × 10 ⁹ cells/L; platelets 306 × 10 ⁹ /L	Leukocytes 4.91 × 10 ⁹ cells/L; platelets 205 × 10 ⁹ /L	Leukocytes 5.82 × 10 ⁹ cells/L; platelets 208 × 10 ⁹ /L
Biochemical	AST 81 IU/L; ALT 127 IU/L; ALP 142 IU/L; GGT 128 IU/L; creatinine 1.02 g/dL; urea 37 mg/dL; Na ⁺ 140 mmol/L; K ⁺ 4.24 mmol/L; cholesterol 113 mg/dL; triglycerides 78 mg/dL; CRP 10.33	AST 448 IU/L; ALT 424 IU/L; ALP 151 IU/L; GGT 150 IU/L; cardiac biomarkers (troponin and natriuretic peptide) normal; creatinine 0.86 mg/dL; urea 53 mg/dL; Na ⁺ 135.6 mmol/L; K ⁺ 4.39 mmol/L; LDH 830 UI/L; CRP 14.58	AST 117 IU/L; ALT 205 IU/L; Creatinine 0.56 mg/dL; Urea 28 mg/dL; Na ⁺ 141.8 mmol/L; K ⁺ 4.38 mmol/L; cholesterol 121 mg/dL; triglycerides 218 mg/dL; CRP 2.81	AST 46 IU/L; ALT 106 IU/L; ALP 176 IU/L; GGT 143 IU/L; creatinine 0.71 mg/dL; Urea 30 mg/dL; Na ⁺ 139.5 mmol/L; K ⁺ 4.58 mmol/L	AST 24 IU/L; ALT 19 IU/L; GGT 12 IU/L; creatinine 0.95 mg/dL; urea 33 mg/dL; Na ⁺ 140.2 mmol/L; K ⁺ 4.78 mmol/L
Arterial blood gas analysis	NA	pH 7.36; PaCO ₂ 32 mm Hg; PaO ₂ 40 mm Hg; SaO ₂ 85.5%; HCO ₃ ⁻ 26.3 mm Hg	NA	NA	NA
Pleural effusion characteristics	NA	Glucose 114 mg/dL; proteins 2.1 g/dL; LDH 423 IU/L; cytology inflammatory process with lymphocyte predominance	NA	NA	NA
Hantavirus diagnosis	IgG (ELISA) negative; IgG (IBT) negative; IgM (ELISA) negative; IgM (IBT) negative; PCR (serum + urine) negative	NA	IgG (ELISA) weak positive; IgG (IBT) positive, PUUV indeterminate, Dobrava Seoul, Hantaan, Sin Nombre and Andes negative; IgM (ELISA) indeterminate; IgM (IBT) negative; PCR (serum) negative; DENV IgG, IgM negative, PCR (serum) negative	IgG (ELISA) positive; IgG (IBT) positive; PUUV indeterminate; Dobrava, Sin Nombre and Andes and negative Seoul and Hantaan; IgM (ELISA) positive; IgM (IBT) negative; PCR (serum) negative	IgG (ELISA) indeterminate; IgG (IBT) indeterminate; PUUV, Dobrava, and Seoul negative; Hantaan, Sin Nombre and Andes; IgM (ELISA) negative; IgM (IBT) negative; PCR (serum) negative

*Days are days after initial symptom onset. An expanded table including additional results is available (<https://wwwnc.cdc.gov/EID/article/26/1/18-1685-T1.htm>). ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate transaminase; CRP, C-reactive protein; DENV, dengue virus; GGT, gamma-glutamyl transpeptidase; IBT, immunoblot; ICU, intensive care unit; LDH, lactate dehydrogenase; NA, not applicable; PUUV, Puumala virus.

glareolus bank vole, is absent from Nepal and India. The PUUV IgG and IgM seroconversion and the classical HPS manifestation (3) are highly reminiscent of 2 fatal HFRS/HPS cases previously described in South India (8,9) that were also PUUV immunoblot positive.

Hantaviruses are emerging zoonotic pathogens and, although recognition of the infection in humans has greatly improved worldwide during the past decade, many cases probably remain undiagnosed. This case highlights the importance of clinical suspicion of hantavirus infection in travelers, even in countries where no cases have been previously reported.

Acknowledgements

We thank the patient, who provided written, informed consent for publication of this case.

About the Author

Dr. Sulleiro is a microbiologist in the Department of Microbiology, Vall d'Hebrón University Hospital, PROSICS Barcelona. Her primary research interests are Chagas disease and arbovirus. Dr. Aznar is a physician working in the Tropical Medicine Unit Drassanes-Vall d'Hebrón Infectious Diseases Department, Vall d'Hebrón University Hospital, PROSICS Barcelona. Her primary research interests are tropical medicine and tuberculosis.

References

- Hofmann J, Weiss S, Kuhns M, Zinke A, Heinsberger H, Kruger DH. Importation of human Seoul virus infection to Germany from Indonesia. *Emerg Infect Dis.* 2018;24:1099-102. <https://doi.org/10.3201/eid2406.172044>

2. Jiang H, Zheng X, Wang L, Du H, Wang P, Bai X. Hantavirus infection: a global zoonotic challenge. *Virology*. 2017;32:32-43. <https://doi.org/10.1007/s12250-016-3899-x>
3. Centers for Disease Control and Prevention. Hantavirus pulmonary syndrome (HPS) 2015 case definition. 2015 [cited 2019 Oct 11]. <https://www.cdc.gov/nndss/conditions/hantavirus-pulmonary-syndrome/case-definition/2015>
4. Clement J, LeDuc JW, McElhinney LM, Reynes J-M, Van Ranst M, Calisher CH. Clinical characteristics of ratborne Seoul hantavirus disease. *Emerg Infect Dis*. 2019;25:387-8. <https://doi.org/10.3201/eid2502.181643>
5. Kang HJ, Kosoy MY, Shrestha SK, Shrestha MP, Pavlin JA, Gibbons RV, et al. Short report: genetic diversity of Thottapalayam virus, a hantavirus harbored by the Asian house shrew (*Suncus murinus*) in Nepal. *Am J Trop Med Hyg*. 2011;85:540-5. <https://doi.org/10.4269/ajtmh.2011.11-0034>
6. Thompson CN, Blacksell SD, Paris DH, Arjyal A, Karkey A, Dongol S, et al. Undifferentiated febrile illness in Kathmandu, Nepal. *Am J Trop Med Hyg*. 2015;92:875-8. <https://doi.org/10.4269/ajtmh.14-0709>
7. Sunil-Chandra NP, Clement J, Maes P, DE Silva HJ, Van Esbroeck M, Van Ranst M. Concomitant leptospirosis-hantavirus co-infection in acute patients hospitalized in Sri Lanka: implications for a potentially worldwide underestimated problem. *Epidemiol Infect*. 2015;143:2094. <https://doi.org/10.1017/S0950268815000412>
8. Clement J, Maes P, Muthusetupathi M, Nainan G, Van Ranst M. Hantavirus in India: a new deadly disease with an as yet unknown hantavirus strain. *Acta Clin Belg*. 2008;64:171.
9. Clement J, Maes P, Muthusetupathi M, Nainan G, Van Ranst M. First evidence of fatal hantavirus nephropathy in India, mimicking leptospirosis. *Nephrol Dial Transplant*. 2006;21:826-7. <https://doi.org/10.1093/ndt/gfi334>

Address for correspondence: Inés Oliveira, Vall d'Hebrón University Hospital, Infectious Diseases Department, PROSICS Barcelona, Barcelona, Spain; email: ioliveira@vhebron.net

Visceral Leishmaniasis, Northern Somalia, 2013–2019

Mikko K. Aalto, Temmy Sunyoto, Mohamed Ahmed Ali Yusuf, Abdiaziz Ahmed Mohamed, Gert Van der Auwera, Jean-Claude Dujardin

Author affiliations: Bosaso General Hospital, Bosaso, Somalia (M.K. Aalto, M.A.A. Yusuf, A.A. Mohamed); Institute of Tropical Medicine, Antwerp, Belgium (T. Sunyoto, G. Van der Auwera, J.-C. Dujardin)

DOI: <https://doi.org/10.3201/eid2601.181851>

We identified visceral leishmaniasis caused by *Leishmania donovani* in a previously unknown focus in northern Somalia. Clinical and epidemiologic characteristics of 118 cases during 2013–2019 in Bosaso, the region's commercial capital, have raised suspicion of visceral leishmaniasis endemicity status there.

Visceral leishmaniasis (VL), the fatal form of a parasitic disease caused by *Leishmania donovani* complex, has been known to exist in southern Somalia since the 1930s, but its presence in the northern part of the country is unclear (1–4). We report VL existence through initial investigation of suspected case-patients in Bosaso General Hospital (Bosaso, Somalia) during December 2013–February 2019. Bosaso is a city in the northeastern Bari Province of Somalia, which serves as the region's commercial capital and is a major seaport on the southern coast of the Gulf of Aden.

Clinicians suspected VL disease in 2013 in several infants with extreme wasting, splenomegaly, pancytopenia, and death. In previous years, leukemia was misdiagnosed in such children. VL was eventually confirmed through microscopic demonstration of *Leishmania* amastigotes in bone marrow and spleen aspirates. Furthermore, these patients responded well when empirically treated with sodium stibogluconate, the mainstay of VL therapy in eastern Africa.

Since then, the hospital's clinicians have maintained a database of patients with suspected and confirmed VL (case definition: fever >2 weeks' duration, splenomegaly, wasting, and pancytopenia). A total of 118 patients were confirmed to have VL during 2013–2019 by microscopy, in vitro culture (introduced in 2016 [Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/18-1851-App1.pdf>]), or serology. After Bosaso General Hospital reported the first cases to the World Health Organization (WHO) in 2014, WHO provided rK39 rapid diagnostic tests (Kalazar Detect; Inbios, <https://inbios.com/kalazar-detecttm-rapid-test-for-visceral-leishmaniasis-intl>) for use in accordance with Somalia's national leishmaniasis guidelines of 2012 (5).

Among the 118 identified patients, nearly all (107 [91%]) were children. Patients' ages ranged from 6 months to 60 years; 78 (66%) were male. The most frequent symptoms were wasting, splenomegaly, and severe or moderate pancytopenia, along with persistent fever. Lymphadenopathy was absent, as was post-kala-azar dermal leishmaniasis, although this condition might have been missed because no follow-up system was in place. Whenever possible, patients with confirmed VL were treated with sodium

stibogluconate (20 mg/kg) combined with paromomycin (20 mg/kg), both intramuscularly. However, because of the low availability of paromomycin, sodium stibogluconate monotherapy was administered when paromomycin was unavailable. Outcome records were available for 103 (87%) patients, 85 (85%) of whom were clinically cured. Eighteen (17%) patients died. As part of routine data collection, the patients' origins were documented: all patients came from Sanaag and Bari regions in northern Somalia and had no history of traveling outside this area.

For 3 patients, we attempted to identify *Leishmania* species by extracting DNA from microscopy slides and sequencing partial fragments of the PCR-amplified HSP70 gene (6). In 2 patients, *L. donovani* was identified (European Nucleotide Archive study PRJEB34786, accession nos. LR723650 and LR723651 [Appendix, section 1]).

Suspected VL patients whose illnesses fit the case definition first underwent rapid diagnostic testing to exclude malaria (5). During 2013–2019, only 2 were found positive for malaria, which was confirmed by microscopy. Both also had confirmed VL and subsequently received antimalarial and antileishmanial therapy. In accordance with national guidelines (5), patients also were tested for HIV; no VL patient was positive. Screening for other underlying conditions was undertaken when feasible; for example, with chest radiograph. One VL case-patient with concomitant pulmonary tuberculosis was referred to the tuberculosis center after finishing antileishmanial treatment.

We describe a previously unreported focus of VL in northern Somalia. The age distribution of the case-patients indicates that VL seems to be endemic in this region; it is unlikely that all cases were imported or present as an "outbreak" such as that described in Huddur (Bakool region) in 2001 (7). From the perspectives of families of the patients and the local health workers, the disease has been known in this area for years. Despite ongoing war and unrest in southern Somalia and the prevalence of displacement in the country, it appears implausible that the VL patients in Bosaso came from there. The nearest known focus is across the Gulf of Aden, in southern Yemen (8,9), where Somali diaspora is present. Because sea travel with small wooden boats is common across the gulf and has been for centuries, this focus might play a role in the cases described here and merits further exploration. Surveillance for VL should be strengthened in northern Somalia, and access to adequate diagnosis

and treatment must be provided to reduce transmission, illness, and death. Support and collaboration across stakeholders, including WHO and national health actors, must be continued to tackle the disease in a comprehensive manner. Further investigation (e.g., a cross-sectional survey) might be considered to define the infection rate in this newly identified focus and determine its level of endemicity.

About the Author

Dr. Aalto is a surgeon in the surgical department of Bosaso General Hospital. His primary research interests include malaria, leishmaniasis, and the control of their vectors.

References

1. World Health Organization. Somalia leishmaniasis country profile [cited 2019 Aug 20]. https://www.who.int/leishmaniasis/burden/Leishmaniasis_Somalia
2. World Health Organization. Global leishmaniasis surveillance update, 1998–2016 [cited 2019 Oct 28]. <https://apps.who.int/iris/bitstream/handle/10665/275337/WER9340-530-540.pdf>
3. Sunyoto T, Potet J, Boelaert M. Visceral leishmaniasis in Somalia: a review of epidemiology and access to care. *PLoS Negl Trop Dis*. 2017;11:e0005231. <http://dx.doi.org/10.1371/journal.pntd.0005231>
4. World Health Organization. Control of visceral leishmaniasis in Somalia: achievements in a challenging scenario, 2013–2015. *Wkly Epidemiol Rec*. 2017;92:566–72.
5. Somali Federal Government Ministry of Health. Guidelines for diagnosis, treatment and prevention of visceral leishmaniasis in Somalia [cited 2019 Jul 1]. https://www.who.int/leishmaniasis/burden/Guidelines_for_diagnosis_treatment_and_prevention_of_VL_in_Somalia.pdf
6. Van der Auwera G, Maes I, De Doncker S, Ravel C, Cnops L, Van Esbroeck M, et al. Heat-shock protein 70 gene sequencing for *Leishmania* species typing in European tropical infectious disease clinics. *Euro Surveill*. 2013;18:20543. <http://dx.doi.org/10.2807/1560-7917.ES2013.18.30.20543>
7. Marlet MVL, Sang DK, Ritmeijer K, Muga RO, Onsongo J, Davidson RN. Emergence or re-emergence of visceral leishmaniasis in areas of Somalia, north-eastern Kenya, and south-eastern Ethiopia in 2000–01. *Trans R Soc Trop Med Hyg*. 2003;97:515–8. [http://dx.doi.org/10.1016/S0035-9203\(03\)80012-3](http://dx.doi.org/10.1016/S0035-9203(03)80012-3)
8. Postigo JAR. Leishmaniasis in the World Health Organization Eastern Mediterranean Region. *Int J Antimicrob Agents*. 2010;36 (Suppl 1):S62–5. <http://dx.doi.org/10.1016/j.ijantimicag.2010.06.023>
9. Pigott DM, Bhatt S, Golding N, Duda KA, Battle KE, Brady OJ, et al. Global distribution maps of the leishmaniases. *eLife*. 2014;3:1–21. <http://dx.doi.org/10.7554/eLife.02851>

Address for correspondence: Mikko K. Aalto, Bosaso General Hospital—Surgery, Bosaso, Bari, Somalia; email: mikko_aalto@yahoo.com

Autochthonous Human Fascioliasis, Belgium

Sandrine Milas,¹ Camelia Rossi, Ivan Philippart, Pierre Dorny, Emmanuel Bottieau

Author affiliations: University Hospital Center Tivoli, La Louvière, Belgium (S. Milas); University Hospital Center Ambroise Paré, Mons, Belgium (C. Rossi); Regional Hospital Mons-Hainaut, Mons (I. Philippart); Institute of Tropical Medicine, Antwerp, Belgium (P. Dorny, E. Bottieau)

DOI: <https://doi.org/10.3201/eid2601.190190>

We report 2 cases of human fascioliasis (HF) in Belgium, likely caused by consumption of vegetables from a garden that was flooded by pasture runoff. Because autochthonous HF is rare and the route of transmission was unusual, HF was not diagnosed until 6 months after symptom onset in both cases.

Human fascioliasis (HF) is a plantborne and waterborne infection caused by the trematodes *Fasciola hepatica* in temperate areas and *F. gigantica* in tropical areas (1,2). *Fasciola* spp. trematodes infect herbivorous mammals and humans. The *Fasciola* life cycle requires 2 hosts; ruminants carry adult worms and excrete eggs into the environment in feces; lymnaeid snails are invaded and release cercariae, which encyst as metacercariae on aquatic vegetation. Humans become infected by ingesting raw aquatic vegetables or consuming plants or water containing metacercariae (3). Symptoms of fascioliasis are stage-specific and related to hepatic migration by larva or obstruction of the biliary ducts by adult worms (1,2).

In Belgium, only 6 cases of HF have been published since 1960 (4–6). We describe 2 autochthonous cases of HF. The cases were seen in different hospitals and initially were not linked epidemiologically.

Case 1 was in a 72-year-old man with no underlying medical conditions and no history of travel outside the country who was referred to the Regional Hospital of Mons in November 2008. He had fever, abdominal pain, rash, and hypereosinophilia that had lasted for 8 weeks (Table). Fecal egg detection was negative. Several serologic tests targeting parasitic infections were performed (Table); results for *Trichinella spiralis* were positive, but this diagnosis was discarded in the absence of myalgia and elevated creatine kinase. Eventually, a diagnosis of idiopathic hypereosinophilic syndrome was made. The patient

received high doses of corticosteroids, but his condition did not improve. He was reevaluated in March 2009, and HF was considered on the basis of combined clinical, laboratory, and radiologic findings (Table). An indirect hemagglutination test for *Fasciola* spp. was performed by using ELIHA Distoma (ELITech Group, <https://www.elitechgroup.com>), and results were positive. The patient received triclabendazole (10 mg/kg/d) for 2 consecutive days. His symptoms abated, and his eosinophil count was nearly normal 1 month later (Table).

Case 2 was in the index case's neighbor, who experienced similar symptoms that lasted for 3 months before she was seen at the University Hospital Center Ambroise Paré in Mons in December 2008. She also was misdiagnosed initially (Table). In February 2009, 2 stool examinations were negative for parasite eggs. In March, an indirect hemagglutination test for *Fasciola* was performed and was highly positive. The patient received a single dose of triclabendazole (15 mg/kg) and recovered fully within 5 months (Table).

A detailed anamnesis revealed that both patients consumed unwashed raw vegetables from case-patient 2's garden, which was flooded with runoff from a neighboring cattle pasture in August 2008. We hypothesize the vegetables were contaminated by metacercariae, either by *Fasciola*-infested amphibious snails washed into the garden or directly by runoff. We did not perform sampling of the garden. Cases related to garden vegetables contaminated by flooding have been reported previously, such as in Corsica (3).

HF is not a notifiable disease in Belgium. Among 6 published cases, 3 occurred in a cluster related to consumption of homegrown watercress, and 3 non-clustered cases had a questionable autochthonous nature (4–6). Consumption of watercress and dandelions is uncommon in Belgium, but common in France, where ≈300 HF cases occur annually (7). However, *Fasciola* infection in cattle is common in Belgium; herd prevalence is 37.3% in Flemish dairy cattle (8). In addition, a 2008 survey of snails showed 1.31% of *Galba truncatula* and 0.16% of *Radix* spp. were infected by *F. hepatica* trematodes (9).

Both cases in this study experienced an acute invasive stage and a considerable delay in HF diagnosis. Clinicians should be aware of key elements of HF, including potential diet exposure, clinical signs and symptoms, and imaging and laboratory findings. Contrast-enhanced computed tomography scans of the liver sometimes show tortuous subscapular tracts associated with hypodense nodules and hepatomegaly during the acute phase (2). In industrialized countries, human cases occur singly or in small clusters,

¹Current affiliation: University Center of Charleroi, Charleroi, Belgium.

Table. Clinical, laboratory, and radiologic features of 2 autochthonous cases of human fascioliasis, Mons, Belgium, 2008–2009*

Features	Case 1	Case 2
Patient age, y/sex	72/M	59/F
Date of initial examination	Nov 2008	Dec 2008
Laboratory tests, date		
Eosinophil cell count/mL (% leukocytes)†		
Nov 2008	9,688 (56)	ND
Dec 2008	7,504 (56)	6,750 (43)
Mar 2009	8,830 (56.6)	8,525 (55)
1 mo after triclabendazole	627 (10.8)	3,526 (41)
5 mo after triclabendazole	512 (8.4)	812 (14.5)
Aspartate aminotransferases, IU/L‡		
Nov 2008	53	ND
Dec 2008	30	21
Mar 2009	25	23
1 mo after triclabendazole	26	32
5 mo after triclabendazole	17	ND
Serologic test (method), date	Dec 2008	Jan 2009
<i>Ascaris</i> spp. (immunodiffusion)	–	–
<i>Echinococcus granulosus</i> (ELISA)	–	–
<i>Echinococcus multilocularis</i> (ELISA)	–	ND
<i>Toxocara</i> spp. (ELISA)	–	–
<i>Taenia solium</i>	ND	–
<i>Trichinella spiralis</i> (ELISA)	+	–
<i>Fasciola</i> (indirect hemagglutination), Mar 2009§	1/640	1/2,560
1 mo. after triclabendazole	1/640	ND
5 mo. after triclabendazole	1/320	1/320
Abdominal CT, date	Nov 2008, multiple hypodense liver nodules	Mar 2009, multiple hypodense liver nodules
Liver biopsy, date	Dec 2008, chronic active hepatitis with acute necrosis, presence of eosinophils in portal spaces	ND

*Both case-patients had an early acute phase of disease during Sep–Dec 2008 with high fever, abdominal pain, generalized itching, and urticarial skin rash. Both cases then had a later disease phase during Nov 2008–Mar 2009 with persistent fatigue and weight loss. CT, computed tomography; IU, International Units; ND, not done; –, negative; +, positive.
†Normal values, 0–400/mL ($\leq 7\%$).
‡Normal values 10–40 IU/L.
§Normal value $< 1/320$.

and diagnosis usually is made during the invasive phase by combined clinical, laboratory, and imaging findings. Serologic tests can detect antibodies within 2 weeks after infection but might have low specificity. *Fasciola* eggs can appear in stool 3–4 months postinfection, depending on the infection burden and the ability of the flukes to produce eggs. Intermittent shedding can occur (1,2). Co-proantigens are detectable 8 weeks after infection and have a high sensitivity, but 1 negative result despite high egg shedding has been reported (2).

Triclabendazole, licensed in Europe only by Novartis (<https://www.novartis.com>), at 10–15 mg/kg/day in 1 dose or on 2 consecutive days, is the preferred treatment, and patients usually recover rapidly. Resistance increasingly is described in ruminants and treatment failures have been reported in humans (2,10).

Although overlooked in countries in northern Europe, HF should be considered in cases of unexplained eosinophilia associated with liver symptoms, even in the absence of ingestion of freshwater plants. This zoonotic condition highlights the need for good

epidemiologic communication between human and animal health workers.

Acknowledgment

We thank Quentin Groom from the Botanic Garden Meise, Meise, Belgium, for his contribution to this article.

About the Author

At the time of this study, Dr. Milas was an infectious disease specialist at University Hospital Center Tivoli, La Louvière, Belgium. She is currently an infectious disease specialist at University Hospital Center of Charleroi, Charleroi, Belgium. Her research interests include general infectious diseases, travel medicine, and infection control.

References

- Mas-Coma S, Bargues MD, Valero MA. Fascioliasis and other plant-borne trematode zoonoses. *Int J Parasitol.* 2005;35:1255–78. <https://doi.org/10.1016/j.ijpara.2005.07.010>
- Mas-Coma S, Bargues MD, Valero MA. Diagnosis of human fascioliasis by stool and blood techniques: update for the present global scenario. *Parasitology.* 2014;141:1918–46. <https://doi.org/10.1017/S0031182014000869>

3. Mas-Coma S, Bargues MD, Valero MA. Human fascioliasis infection sources, their diversity, incidence factors, analytical methods and prevention measures. *Parasitology*. 2018; 145:1665–99. <https://doi.org/10.1017/S0031182018000914>
4. Jeanty C. A new focus of hepatic distomatosis due to *Fasciola hepatica* in Belgium. Extraction of a cutaneous fluke. *Acta Gastroenterol Belg*. 1960;23:210–27.
5. Orlent H, Selleslag D, Vandecasteele S, Jalal PK, Bank S, Hines J. Clinical challenges and images in GI. *Fasciola hepatica* infection and Von Hippel-Lindau disease type 1 with pancreatic and renal involvement. *Gastroenterology*. 2007;132:15–6, 467–8. <https://doi.org/10.1053/j.gastro.2006.11.050>
6. Pilet B, Deckers F, Pouillon M, Parizel P. *Fasciola hepatica* infection in a 65-year-old woman. *J Radiol Case Rep*. 2010;4:13–9. <https://doi.org/10.3941/jrcr.v4i4.384>
7. Mailles A, Capek I, Ajana F, Schepens C, Ilief D, Vaillant V. Commercial watercress as an emerging source of fascioliasis in Northern France in 2002: results from an outbreak investigation. *Epidemiol Infect*. 2006;134:942–5. <https://doi.org/10.1017/S095026880600611X>
8. Bennema S, Vercruysse J, Claerebout E, Schnieder T, Strube C, Ducheyne E, et al. The use of bulk-tank milk ELISAs to assess the spatial distribution of *Fasciola hepatica*, *Ostertagia ostertagi* and *Dictyocaulus viviparus* in dairy cattle in Flanders (Belgium). *Vet Parasitol*. 2009;165:51–7. <https://doi.org/10.1016/j.vetpar.2009.07.006>
9. Caron Y, Martens K, Lempereur L, Saegerman C, Losson B. New insight in lymnaeid snails (Mollusca, Gastropoda) as intermediate hosts of *Fasciola hepatica* (Trematoda, Digenea) in Belgium and Luxembourg. *Parasit Vectors*. 2014;7:66. <https://doi.org/10.1186/1756-3305-7-66>
10. Cabada MM, Lopez M, Cruz M, Delgado JR, Hill V, White AC Jr. Treatment failure after multiple courses of triclabendazole among patients with fascioliasis in Cusco, Peru: a case series. *PLoS Negl Trop Dis*. 2016;10:e0004361. <https://doi.org/10.1371/journal.pntd.0004361>

Address for correspondence: Sandrine Milas, CHU Charleroi, Infectious Diseases and Infection Control, 140 Chaussée de Bruxelles, 6042 Lodelinsart, Belgium; email: milassandrine@yahoo.fr

Recombinant Nontypeable Genotype II Human Noroviruses in the Americas

Kentaro Tohma, Cara J. Lepore, Juan I. Degiuseppe, Juan A. Stupka, Mayuko Saito, Holger Mayta, Mirko Zimic, Lauren A. Ford-Siltz, Robert H. Gilman, Gabriel I. Parra

Author affiliations: US Food and Drug Administration, Silver Spring, Maryland, USA (K. Tohma, C.J. Lepore, L.A. Ford-Siltz, G.I. Parra); INEI-ANLIS “Dr. Carlos G. Malbrán,” Buenos Aires, Argentina (J.I. Degiuseppe, J.A. Stupka); Tohoku University Graduate School of Medicine, Sendai, Japan (M. Saito); Universidad Peruana Cayetano Heredia, Lima, Peru (H. Mayta, M. Zimic); Johns Hopkins University Bloomberg School of Public Health, Baltimore, Maryland, USA (R.H. Gilman)

DOI: <https://doi.org/10.3201/eid2601.190626>

We report multiple nontypeable genotype II noroviruses circulating in South America; nucleotides differed by $\geq 25\%$ from those of other genotypes. These viruses have been circulating in the Americas for ≈ 20 years and show recombination with other genotypes. Clues to norovirus natural history can guide development of treatment and prevention plans.

Norovirus is a leading cause of acute gastroenteritis (1). The norovirus RNA genome is organized into 3 open reading frames (ORFs). ORF1 encodes for 6 nonstructural proteins, including the RNA-dependent RNA polymerase (RdRp). ORF2 and ORF3 encode for the major capsid protein (VP1) and minor capsid protein (VP2). Norovirus classification was recently updated, and these viruses are now classified into 10 genogroups (GI–GX) and ≈ 40 genotypes (2). This classification is based on the genetic diversity presented by VP1 and RdRp (3). Human noroviruses are mostly represented by the GI and GII strains.

Advances in genome sequencing approaches enabled us to detect a novel (nontypeable) GII norovirus circulating in Peru in 2008 (4). As part of a larger study to sequence the genomes from noroviruses circulating on different continents and over different decades, we found 7 additional nontypeable GII norovirus strains: 2 in fecal samples (PNV024019 and PNV027026) from children in Peru with diarrhea (5) and 5 in fecal samples collected in Buenos Aires, Argentina (6) (Appendix 1 Table, <https://wwwnc.cdc.gov/EID/article/26/1/19-0626-App1.pdf>). These strains were detected by routine PCR screening and were either incorrectly assigned as GII.22 or could not be assigned to any genotypes in the

Noronet Typing Tool (5,6). To investigate the genetic structures and their evolutionary relationship with other norovirus strains, we performed norovirus GII-specific amplicon-based next-generation sequencing as described previously (4). We obtained nearly complete genomes for all 7 strains; average depth coverage (sequenced nucleotides/genome position) was $\geq 5,253\times$ (GenBank accession nos. MK733201–MK733207).

The maximum-likelihood phylogenetic tree of the RdRp-encoding nucleotide sequences showed that the newly obtained strains from Argentina and Peru were closely related to each other and clustered with a group of nontypeable strains (recently classified as GII.P23–GII.P27) (2) (Figure). This genetic group diverged from GII.P22, GII.P25, GII.P38 (previously known as GII.Pn), and GII.P40 (previously known as GII.P22). One of the strains, Arg1382, was classified as GII.P26 under the updated classification system; however, this strain did not distinctly cluster with other GIIP26 strains on the tree. The other 6 strains remain unclassified.

Similar to the RdRp phylogenetic tree, the analysis of the VP1-encoding nucleotide sequences showed a distinct group of nontypeable strains (recently

classified as GII.23–GII.27) (Figure). This genetic group branched out from the GII.22 and GII.25 strains (mean nucleotide difference in VP1 25.9% and mean amino acid difference 19.2%) and further diverged into different genotypes. All strains we report were assigned as GII.27 except Arg1382. Of note, Arg1382, which was the oldest virus among the strains with a nontypeable RdRp, displayed VP1 from the GII.12 genotype.

The analysis of the VP2-encoding nucleotide sequences also showed a distinct group of those GII.23–27 genotypes; however, clustering of genotypes (particularly GII.27) seen in VP1 was disrupted in the VP2 tree (Figure). These genotypes were clustered with the VP2 sequences from GII.3 strains, suggesting possible recombination of the VP1 and VP2 among those strains. Although recombination at the ORF2/ORF3 junction region has been reported for different GII.4 variants (7), we showed possible recombination between different genotypes. Simplot analyses provided further support that these nontypeable strains have recombined with multiple genotypes at the ORF1/ORF2 and ORF2/ORF3 junction regions (Appendix 1 Figure 1) during their evolutionary history.

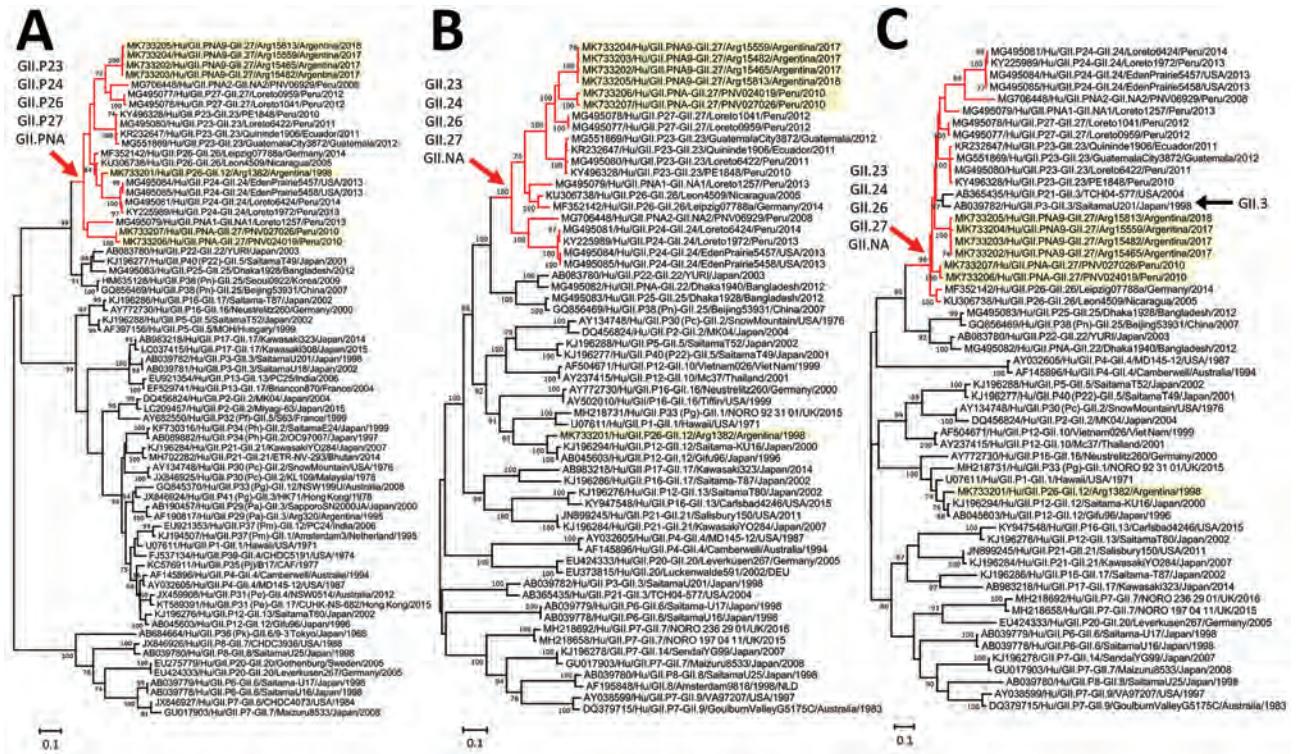


Figure. Phylogenetic analyses of nontypeable norovirus GII strains locally distributed in the Americas. Maximum-likelihood phylogenetic trees of the RNA-dependent RNA polymerase—encoding nucleotide sequences (≥ 771 nt) (A), major capsid protein—encoding nucleotide sequences ($\geq 1,605$ nt) (B), and the minor capsid protein—encoding nucleotide sequences (≥ 536 nt) (C) from human GII norovirus strains were created by using the Tamura-Nei model. Yellow highlighting indicates strains detected in this study; red branches and arrows indicate the divergence of a group of nontypeable GII strains reported in the Americas; and black arrow indicates GII.3 strains that diverged in the same branches with nontypeable GII strains. Bootstrap values (≥ 70) from 100 replicates are shown on the nodes. GenBank accession numbers are shown. Scale bars indicate genetic distance (nucleotide substitutions/site).

All strains in this group were detected in the Americas (United States, Nicaragua, Guatemala, Ecuador, Peru, and Argentina) (Appendix 1 Figure 2). One previous study reported strains associated with this group outside the Americas; an immunocompromised patient in Germany was infected with a similar virus for ≈ 3 years since 2014 (the first strain detected during the prolonged shedding, Leipzig07788a, is shown in the Figure) (8). Detection of these viruses as early as 1998 (Appendix 1 Table) suggests long-term circulation of these nontypeable strains. Their long-term geographically limited detection could result from multiple factors including, but not limited to, low transmissibility, restrictions on the mutability of the virus, host-related susceptibility (9), or under-detection because of limited norovirus surveillance or nucleotide mismatches in the PCR primers and probes for detection. In that regard, we found a substitution (C5047T) on a common probes binding site used for quantitative PCR detection in 6 nontypeable strains (Appendix 2, <https://wwwnc.cdc.gov/EID/article/26/1/19-0626-App2.xlsx>). Further studies would provide insight into the apparent limited geographic distribution of these viruses.

Although only a few genotypes (e.g., GII.4, GII.3, GII.2, GII.6) are most prevalent among humans, those with limited circulation can emerge, causing large outbreaks (e.g., GII.17) (10,11). Monitoring of minor genotypes is helpful for preparing for the emergence of novel viruses and possible future outbreaks. Information about the circulation of each of the norovirus genotypes will provide clues to the natural history of norovirus disease and guide the development of specific treatment and prevention plans.

Acknowledgments

We thank the Facility for Biotechnology Resources at the US Food and Drug Administration (FDA) for their support with next-generation sequencing, Karina Rivero for her technical support, the community of San Juan de Miraflores for collaboration, and Asociación Benéfica PRISMA for the fieldwork in Peru.

Financial support for this work was provided by FDA intramural funds (program no. Z01 BK 04012-01 LHV to G.I.P.). K.T. and C.J.L. are recipients of an FDA Center for Biologics Evaluation and Research–sponsored Oak Ridge Institute for Science and Education fellowship. The funders had no role in the study design, data collection, data interpretation, or the decision to submit the work for publication.

About the Author

Dr. Tohma is a postdoctoral fellow at the Division of Viral Products, Food and Drug Administration. His research interests include genetic and antigenic evolution, epidemiology, and norovirus vaccine development.

References

1. Pires SM, Fischer-Walker CL, Lanata CF, Devleeschauwer B, Hall AJ, Kirk MD, et al. Aetiology-specific estimates of the global and regional incidence and mortality of diarrhoeal diseases commonly transmitted through food. *PLoS One*. 2015;10:e0142927. <https://doi.org/10.1371/journal.pone.0142927>
2. Chhabra P, de Graaf M, Parra GI, Chan MC, Green K, Martella V, et al. Updated classification of norovirus genogroups and genotypes. *J Gen Virol*. 2019. <https://doi.org/10.1099/jgv.0.001318>
3. Kroneman A, Vega E, Vennema H, Vinjé J, White PA, Hansman G, et al. Proposal for a unified norovirus nomenclature and genotyping. *Arch Virol*. 2013;158:2059–68. <https://doi.org/10.1007/s00705-013-1708-5>
4. Tohma K, Saito M, Mayta H, Zimic M, Lepore CJ, Ford-Siltz LA, et al. Complete genome sequence of a nontypeable GII norovirus detected in Peru. *Genome Announc*. 2018; 6:e00095-18. <https://doi.org/10.1128/genomeA.00095-18>
5. Saito M, Goel-Apaza S, Espetia S, Velasquez D, Cabrera L, Loli S, et al.; Norovirus Working Group in Peru. Multiple norovirus infections in a birth cohort in a Peruvian periurban community. *Clin Infect Dis*. 2014;58:483–91. <https://doi.org/10.1093/cid/cit763>
6. Bereciartu A, Bok K, Gómez J. Identification of viral agents causing gastroenteritis among children in Buenos Aires, Argentina. *J Clin Virol*. 2002;25:197–203. [https://doi.org/10.1016/S1386-6532\(02\)00010-0](https://doi.org/10.1016/S1386-6532(02)00010-0)
7. Eden JS, Tanaka MM, Boni MF, Rawlinson WD, White PA. Recombination within the pandemic norovirus GII.4 lineage. *J Virol*. 2013;87:6270–82. <https://doi.org/10.1128/JVI.03464-12>
8. Pietsch C, Ennuschat N, Härtel S, Liebert UG. Within-host evolution of virus variants during chronic infection with novel GII.P26–GII.26 norovirus. *J Clin Virol*. 2018;108:96–102. <https://doi.org/10.1016/j.jcv.2018.09.013>
9. Nordgren J, Svensson L. Genetic susceptibility to human norovirus infection: an update. *Viruses*. 2019;11:E226. <https://doi.org/10.3390/v11030226>
10. Chan MC, Lee N, Hung TN, Kwok K, Cheung K, Tin EK, et al. Rapid emergence and predominance of a broadly recognizing and fast-evolving norovirus GII.17 variant in late 2014. *Nat Commun*. 2015;6:10061. <https://doi.org/10.1038/ncomms10061>
11. Matsushima Y, Ishikawa M, Shimizu T, Komane A, Kasuo S, Shinohara M, et al. Genetic analyses of GII.17 norovirus strains in diarrheal disease outbreaks from December 2014 to March 2015 in Japan reveal a novel polymerase sequence and amino acid substitutions in the capsid region. *Euro Surveill*. 2015;20:21173. <https://doi.org/10.2807/1560-7917.ES2015.20.26.21173>

Address for correspondence: Kentaro Tohma, US Food and Drug Administration, 10903 New Hampshire Ave, Rm 1376, Bldg 52/72, Silver Spring, MD 20993, USA; email: kentaro.tohma@fda.hhs.gov.; Gabriel I. Parra, US Food and Drug Administration, 10903 New Hampshire Ave, Rm 1308, Bldg 52/72, Silver Spring, MD 20993, USA; email: gabriel.parra@fda.hhs.gov

***Legionella pneumophila* as Cause of Severe Community-Acquired Pneumonia, China**

Huahua Yi, Jie Fang, Jingwen Huang, Bing Liu, Jieming Qu, Min Zhou

Author affiliation: Ruijin Hospital, Shanghai, China

DOI: <https://doi.org/10.3201/eid2601.190655>

We report a case of community-acquired pneumonia in a patient in China. We verified *Legionella pneumophila* infection through next-generation sequencing of blood, sputum, and pleural effusion samples. Our results show the usefulness of next-generation sequencing and of testing different samples early in the course of illness to identify this bacterium.

Community-acquired pneumonia (CAP) can lead to high mortality rates worldwide (1). Possible causes are *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Mycoplasma chlamydia*, and *Legionella* spp. bacteria, as well as various respiratory viruses; although identifying pathogens in a timely manner is critical, it is not done in most cases. Next-generation sequencing (NGS) has emerged as a high-throughput method of pathogen identification and is superior to current microbiologic diagnostic measures for identifying hard-to-culture pathogens. We report a case of CAP in China caused by *Legionella pneumophila*, determined through NGS of sputum, blood, and pleural effusion samples.

The patient was a 65-year-old woman with a history of breast cancer who sought care for fever on December 1, 2018, in a hospital in her community (Appendix 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-0655-App1.pdf>); her fever was accompanied by vomiting. Emergency room staff diagnosed gastroenteritis and prescribed ceftriaxone. The next day, she experienced frequent urination and urinary pain followed by diarrhea and returned to the hospital. A computed tomography (CT) scan of her abdomen showed gallbladder calculi and a low-density node in the right lobe of the liver. Treatment did not resolve fever and diarrhea. On the fifth day, the patient experienced respiratory symptoms including cough and purulent sputum and was admitted to the gastroenterology department in the same hospital. Over the next 2 days, respiratory symptoms worsened, and she was transferred to the infectious disease department of Ruijin Hospital in Shanghai.

At admission, PCR and serologic testing were performed as well as bacterial culture from blood and

sputum samples. Chest CT results showed patchy consolidation in the lower lobe of the left lung (Figure, panels A and B). The patient was transferred to respiratory intensive care. A respiratory clinician, suspecting *L. pneumophila* as the cause, prescribed the quinolone moxifloxacin. We repeated serologic tests for *L. pneumophila* and cultured respiratory samples again. We sent samples of blood, sputum, and pleural effusion to BGI Group (Shenzhen, China) for NGS, and urine to Shanghai East Hospital for *L. pneumophila* antigen testing. Serum antibody and urine antigen tests were negative, but NGS indicated *L. pneumophila* in blood, sputum, and pleural effusion. For blood, the coverage rate of *L. pneumophila* was 3.2% with 1,136 raw reads; it was 3.8% with 1,353 raw reads for sputum and 8% with 2,867 raw reads for pleural effusion (Appendix 2 Tables 1–3, <https://wwwnc.cdc.gov/EID/article/26/1/19-0655-App2.xlsx>). Under moxifloxacin treatment, the patient's symptoms disappeared in 1 week. We conducted another NGS for blood without any evidence for *L. pneumophila*. Patchy consolidation in the left lower lung lobe was almost completely absorbed (Figure 1, panel C).

We confirmed the presence of *L. pneumophila* bacteria by PCR for the remaining pleural infusion and sputum of this patient. We did not investigate the source of the *L. pneumophila* bacteria.

L. pneumophila bacteria is 1 of the 3 most common pathogens that cause severe CAP and is isolated in 1%–40% of hospital-acquired pneumonia cases (2). Extrapulmonary symptoms are always present in *L. pneumophila* infection, which may increase the risk for misdiagnosis early in the infection. Furthermore, culturing *L. pneumophila* requires a specific medium, which makes it difficult to obtain a diagnosis without positive microbiology test results. *L. pneumophila* infections have been reported previously (3,4) but were confirmed by PCR with target microorganisms and not by NGS.

BGI Group conducted NGS as described previously (5), collecting 3–4 mL blood in ethylenediaminetetraacetic acid tubes, staining in RT for 3–5 minutes, and centrifuging at $1,600 \times g$ for 10 min at 4°C. They then collected 0.5–3 mL sputum or pleural effusion sample following standard procedures and, using a 1.5-mL microcentrifuge tube, agitated the sample at 2,800–3,200 rpm for 30 min. They separated 0.3 mL of the sample into a new tube and extracted DNA using the TIANamp Micro DNA Kit (DP316; Tiangen Biotech, <http://tiangen.com>) according to the manufacturer's recommendation. DNA libraries were constructed through DNA fragmentation, end repair, adapter ligation, and PCR amplification.

BGI Group sequenced the qualified libraries by using the BGISEQ-100 platform (6). They generated

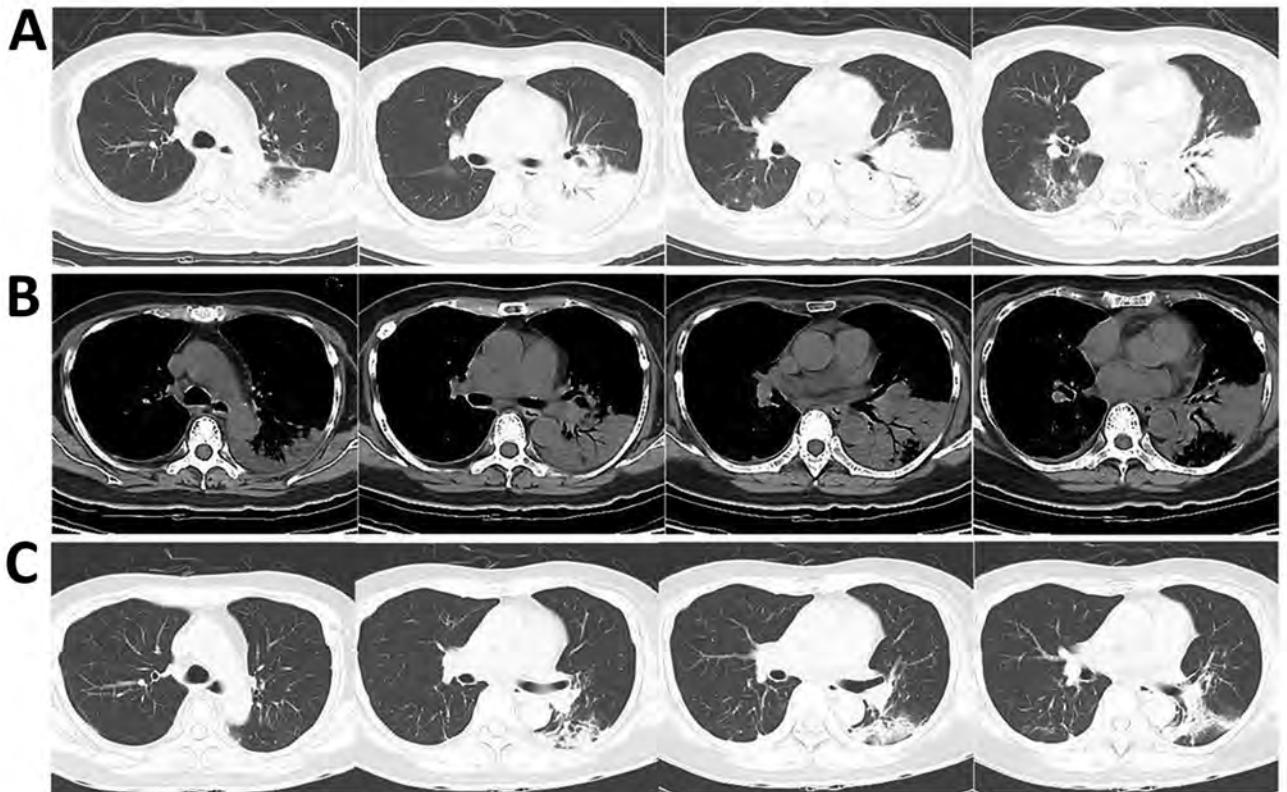


Figure. Computed tomography images of the lungs of a 65-year-old woman with community-acquired pneumonia caused by *Legionella pneumophila* bacteria, China, showing absorption of infusion in left lobes after effective treatment. Lung window (A) and bone window (B) at the beginning of treatment showed consolidation in the left lobes; after 1 week of treatment (C), infusion is mostly absorbed in the left lobes.

high-quality sequencing data by removing low-quality and short (length <35 bp) reads, then performed computational subtraction of human host sequences mapped to the human reference genome (hg19) using Burrows-Wheeler alignment (7). They classified the remaining data by removing low-complexity reads and simultaneously aligning the sequences to microbial genome databases for bacteria, viruses, fungi, and parasites downloaded from the US National Center for Biotechnology Information (<ftp://ftp.ncbi.nlm.nih.gov/genomes>). Experts in respiratory illness, microbiology, and radiology evaluated patients' status and interpreted results of NGS testing together, thus identifying possible etiologic agents.

Because turnaround time is short and nonspecific primers can be used, NGS is useful for detecting unculturable pathogens, especially in severely ill patients (8). NGS is highly sensitive and expensive, so clinicians must assess the value of NGS for identifying etiologic pathogens. In this case, we diagnosed *L. pneumophila* infection on the basis of 3 kinds of samples that had not been reported previously, which shows the importance of testing multiple samples

early in the course of illness to identify the etiologic agent and begin appropriate treatment.

Acknowledgments

We thank BGI-Shenzhen for conducting next-generation sequencing and the clinicians of Shanghai East Hospital for urine antigen.

The study was supported by National Key R&D Program of China (2017YFC1309700 and 2017YFC1309701); National Natural Science Foundation of China (81570029); Shanghai Key Discipline for Respiratory Diseases (2017ZZ02014); and the Innovative Research team of high-level local universities in Shanghai.

About the Author

Dr. Yi is a PhD candidate at Shanghai Jiao Tong University School of Medicine under the supervision of Dr. Min Zhou, specializing in respiratory infections and chronic obstructive pulmonary diseases. Her primary research interest is the diagnosis and treatment of respiratory infectious diseases, especially among immunocompromised patients.

References

1. Prina E, Ranzani OT, Torres A. Community-acquired pneumonia. *Lancet*. 2015;386:1097–108. [https://doi.org/10.1016/S0140-6736\(15\)60733-4](https://doi.org/10.1016/S0140-6736(15)60733-4)
2. Diederer BM. *Legionella* spp. and Legionnaires' disease. *J Infect*. 2008;56:1–12. <https://doi.org/10.1016/j.jinf.2007.09.010>
3. Zhang Q, Zhou H, Chen R, Qin T, Ren H, Liu B, et al. Legionnaires' disease caused by *Legionella pneumophila* serogroups 5 and 10, China. *Emerg Infect Dis*. 2014;20:1242–3. <https://doi.org/10.3201/eid2007.131343>
4. Buchholz U, Reber F, Lehfeld AS, Brodhun B, Haas W, Schaefer B, et al. Probable reinfection with *Legionella pneumophila* – a case report. *Int J Hyg Environ Health*. 2019;222:315–8. <https://doi.org/10.1016/j.ijheh.2018.11.001>
5. Miao Q, Ma Y, Wang Q, Pan J, Zhang Y, Jin W, et al. Microbiological diagnostic performance of metagenomic next-generation sequencing when applied to clinical practice. *Clin Infect Dis*. 2018;67:S231–S240.
6. Jeon YJ, Zhou Y, Li Y, Guo Q, Chen J, Quan S, et al. The feasibility study of non-invasive fetal trisomy 18 and 21 detection with semiconductor sequencing platform. *PLoS One*. 2014;9:e110240. <https://doi.org/10.1371/journal.pone.0110240>
7. Freitas TAK, Li P-E, Scholz MB, Chain PSG. Accurate read-based metagenome characterization using a hierarchical suite of unique signatures. *Nucleic Acids Res*. 2015;43:e69.
8. Naccache SN, Federman S, Veerarahavan N, Zaharia M, Lee D, Samayoa E, et al. A cloud-compatible bioinformatics pipeline for ultrarapid pathogen identification from next-generation sequencing of clinical samples. *Genome Res*. 2014;24:1180–92. <https://doi.org/10.1101/gr.171934.113>

Address for correspondence: Min Zhou, Ruijin Hospital, Shanghai Jiao Tong University Medical School Affiliated, Department of Respiratory and Critical Care Medicine, No. 197 Ruijin Er Rd, Shanghai, 200025, China; email: doctor_zhou_99@163.com

Training for Foodborne Outbreak Investigations by Using Structured Learning Experience

Florian Burckhardt, Esther Kissling

Author affiliations: European Programme for Intervention Epidemiology Training Alumni Network, Heidelberg, Germany (F. Burckhardt); Epiconcept, Paris, France (E. Kissling)

DOI: <https://doi.org/10.3201/eid2601.190755>

We created a free and interactive training activity based on playing cards (disease detective cards) that introduces foodborne outbreak investigations to public health professionals and students. Competencies taught covered selected descriptive and analytic epidemiologic topics, such as case definition, epidemic curve, 2-by-2 tables, relative risks, attack rates, stratification, and confounding.

Training programs, such as the Epidemic Intelligence Service of the Centers for Disease Control and Prevention (Atlanta, GA, USA) (1) and the European Programme for Intervention Epidemiology Training of the European Centre for Disease Prevention and Control (Stockholm, Sweden) (2), teach future epidemiologists outbreak investigation skills. Their case studies for foodborne outbreaks are often based on historic investigations whose real-world complexity can be overwhelming for beginners.

An introductory study (3) used 14 exposures to explain the concept of risk factors and their association with disease. We designed a scripted foodborne outbreak investigation as a structured learning experience with illustrated playing cards that depict exposures (food eaten) and outcome (symptoms, time to first symptoms). With the cards and a flipchart as props, facilitators teach core skills and competencies of outbreak investigations in an interactive group exercise.

We modeled a hypothetical *Salmonella* species outbreak investigation with a cohort design in Excel 2010 (Microsoft, <https://www.microsoft.com>). We took symptoms and incubation periods from our own unpublished outbreak investigations and literature research (4,5). We reverse-engineered relative risks for univariable and stratified analysis with their 2-by-2 tables into a line list with the desired properties and transcribed onto playing cards in which each card represents 1 person attending the exposure event.

Dynamic illustrations of gastroenteric symptoms and food items were hand drawn with pen and ink on Bristol board, scanned, and colored by using the GNU Image Manipulation Program (<https://www.gimp.org>). The illustrations depict characters from our earlier work, the disease detectives (6). We used OpenOffice software (<https://www.openoffice.org>) for layout of the cards. The teaching script is a restructured and simplified adaptation of outbreak case studies used during European Programme for Intervention Epidemiology Training introductory courses (2,3). The cards and other resources are available (<https://www.disease-detectives.org>) under a Creative Commons open-culture license in multiple formats for home printing or as templates for high-quality printing services.

The exercise is for 16–26 students who play roles of participants in the outbreak event and investigate their own outbreak. Each participant draws 1 card that illustrates their fictional health status, food eaten, symptoms if ill, and time of onset (Figure). Facilitators subsequently collect the information from the cards and summarize them on a board during the 1–2-hour teaching activity as the outbreak investigation unfolds. The facilitator should have professional experience in investigating foodborne outbreaks, accompanying statistical methods, and adult learning. Their role is to tap into experiences of the participants and weave them into the narrative of the fictional outbreak investigation. Depending on the learner audience and their skills, the facilitator can use a lecture style or an activity-centered style with small group discussions and collaborative summaries (7). The exercise is designed to work without electricity and only a board or flip-chart. It covers the skills of developing a case definition, collecting data for preparing an epidemic curve, and calculating relative risks as a measure of strength of association between exposure and outcome.

In addition, exposure-specific attack rates and cases explained by exposure, stratification, and confounding can be introduced at the facilitators' discretion. The teaching script suggests the sequence of introducing epidemiologic concepts and collection of relevant data from the cards of individual

participants. The exercise is limited to the epidemiologic descriptive and analytic part of an outbreak investigation and does not cover environmental investigations such as trace back or cover laboratory methods, results, or interpretation. The card model can be extended to train other outbreak patterns (e.g., norovirus) with more vomiting as a symptom and a 2-peak epidemic curve caused by secondary human-to-human exposure.

Since 2014, we have used the teaching activity in 4 district health authority trainings and 1 training of hospital hygiene doctors in Rhineland-Palatinate, Germany. The German Federal Office of Consumer Protection and Food Safety in Berlin used the activity in 2 training sessions on outbreak investigations during 2018, and the Robert-Koch Institute in Berlin used the cards in their outbreak investigation module of an annual training for public health professionals in infectious disease surveillance and during Kids Day (8).

Source material can be modified to adapt the exercise to different cultural contexts. During training workshops in Sudan and Tunisia, under the German Biosecurity Programme of the Federal Foreign Office during 2016 and 2018 (9), hamburger and potato salad were substituted with shawarma and salade niçoise as more culturally appropriate food for northern Africa. The interactive card training tool had been positively reviewed in the training evaluation

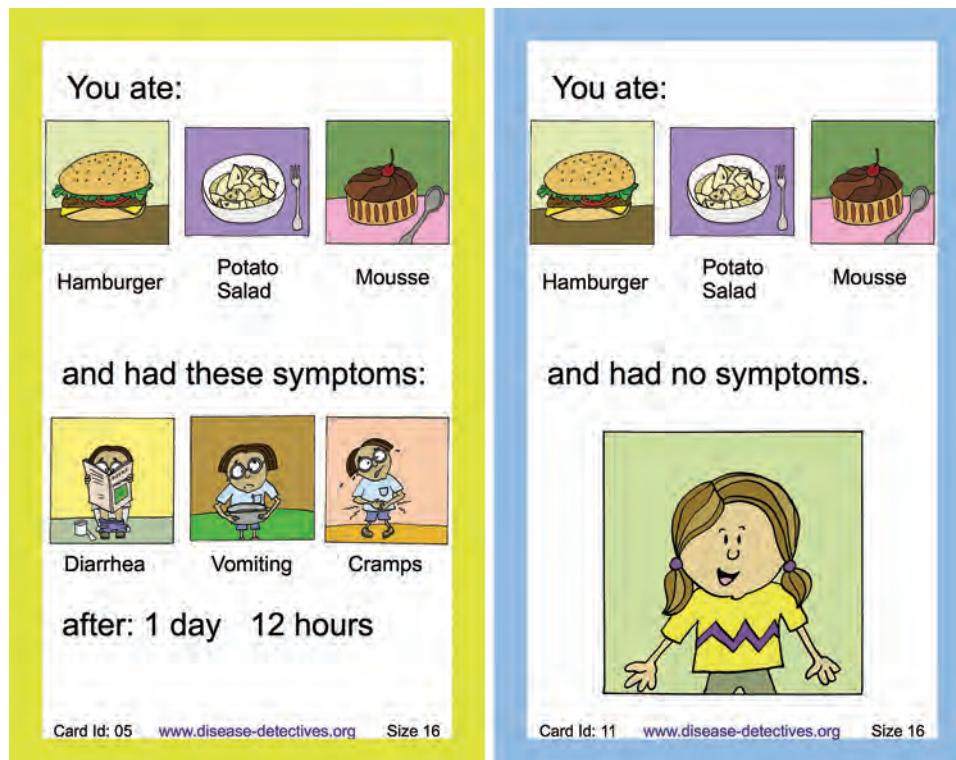


Figure. Playing cards used to describe symptoms, their timing, and food eaten as exposure for training for foodborne outbreak investigations by using structured learning experience.

comments, and participants remained engaged throughout the session. We are planning a formal evaluation of the training tool with the field epidemiology training programs for Germany and Europe.

The exercise is a tool for building outbreak response capacities and teaches the topic in an engaging way. It can be used on its own or embedded as an ice breaker into a field epidemiology curriculum for health professionals or for school classes looking for health-related project work. Supported languages are German, English, Russian, and French. Further translations and adaptations are encouraged and will be referenced on our web page (<https://www.disease-detectives.org>).

Acknowledgments

We thank Nadine Zeitlmann, Michaela Diercke, and Hannah Lewis Winter for thoroughly reading the draft version of the manuscript and adapting the German script into an English 10-steps facilitator guide; Ariane Halm and Juliane Wunderlich for performing the same activity for the French version; and Yanina Lenz for supporting the Russian version.

About the Author

Mr. Burckhardt is a fellow supervisor and trainer at the European Programme for Intervention Epidemiology Training Alumni Network, Heidelberg, Germany. His research interests are outbreak investigations and disease surveillance. Ms. Kissling is an epidemiologist and trainer at Epiconcept, Paris, France. Her research interests are vaccine effectiveness and influenza.

References

1. Jones DS, Dicker RC, Fontaine RE, Boore AL, Omolo JO, Ashgar RJ, et al. Building global epidemiology and response capacity with field epidemiology training programs. *Emerg Infect Dis*. 2017;23. <https://doi.org/10.3201/eid2313.170509>
2. Bremer V, Bosman A, Coulombier D. New perspectives after the transition of EPIET to ECDC: the future of the programme. *Euro Surveill*. 2009;14:19374.
3. Centers for Disease Control and Prevention. Oswego: an outbreak of gastrointestinal illness following a church supper; 2003 [cited 2019 Oct 21]. <https://www.cdc.gov/eis/casestudies/xoswego.401-303.student.pdf>
4. Centers for Disease Control and Prevention. Guide to confirming an etiology in foodborne disease outbreak; 2017 [cited 2019 Oct 21]. https://www.cdc.gov/foodsafety/outbreaks/investigating-outbreaks/confirming_diagnosis.html
5. Mertens E, Kreher H, Rabsch W, Bornhofen B, Alpers K, Burckhardt F. Severe infections caused by *Salmonella* Enteritidis PT8/7 linked to a private barbecue. *Epidemiol Infect*. 2013;141:277–83. <https://doi.org/10.1017/S0950268812000726>
6. Burckhardt F, Kissling E. 'The disease detectives': a comic perspective on outbreak investigations. *Euro Surveill*. 2007;12:E071122.4.
7. World Health Organization. Immunization hand-book and facilitators' guide. South-East Asia Regional Office [cited 2019 Jan 17]. http://www.searo.who.int/india/topics/routine_immunization/Facilitators_Guide_Medical_Officers_Handbook_2010.pdf
8. Robert Koch Institute. Investigate outbreaks module [in German]. *Epidemiol Bull*. 2019; (17):143.
9. Foreign Office. The German biosecurity programme; 2017 [cited 2019 Oct 21]. <https://www.auswaertiges-amt.de/en/ausenpolitik/themen/abruestung/uebersicht-bcwaffen-node/-/239362>

Address for correspondence: Florian Burckhardt, European Programme for Intervention Epidemiology Training Alumni Network, Römerstrasse 59, 69115 Heidelberg, Germany; email: florian@burckhardt.de

Emergence of *Vibrio cholerae* O1 Sequence Type 75 in Taiwan

Yueh-Hua Tu, Bo-Han Chen, Yu-Ping Hong, Ying-Shu Liao, Yi-Syong Chen, Yen-Yi Liu, Ru-Hsiou Teng, You-Wun Wang, Chien-Shun Chiou

Author affiliations: Centers for Disease Control, Taichung, Taiwan

DOI: <https://doi.org/10.3201/eid2601.190934>

We investigated the epidemiology of cholera in Taiwan during 2002–2018. *Vibrio cholerae* sequence type (ST) 75 clone emerged in 2009 and has since become more prevalent than the ST69 clone from a previous pandemic. Closely related ST75 strains have emerged in 4 countries and may now be widespread in Asia.

Cholera, an acute diarrheal disease caused by the toxigenic *Vibrio cholerae* serogroup O1 and its derivative serogroup O139, remains a severe public health threat in some regions of the world (1). Seven cholera pandemics have occurred in the past 200 years; the most recent, caused primarily by a sequence type (ST) 69 *V. cholerae* clone, originated in Indonesia in 1961 and remains ongoing (2,3). In Taiwan, cholera appeared in 1962 and resulted in 383 cases and 24 deaths during a 3-month outbreak (4). Cholera has been rare in Taiwan since the 1962

Table. Distribution of cholera and sequence types of *Vibrio cholerae* isolates, Taiwan, 1962 and 2002–2018

Category	1962	2002	2003	2004	2005	2006	2007	2008	2009	2010	2011	2012	2013	2014	2015	2016	2017	2018	Total
No. cases	383	2	1	1	2	1	0	1	3	5	3	5	7	4	10	9	2	7	63
No. isolates	4	2	1	1	2	1	0	1	3	4	3	3	6	3	8	9	2	7	56
Sequence type																			
69	4		1	1	2	1		1		1	1		2			3	1		14
75 and variants									3	3	2	3	4	3	8	6	1	7	40
723		2																	2

outbreak; however, incidence has increased in recent years. The average number of cholera cases increased from 1.5 cases/year in 1991–2008 to 5.5 cases/year in 2009–2018 (5).

For this study, we investigated the epidemiology of cholera in Taiwan for 2002–2018 and the source of *V. cholerae* strains in those cases. During 2002–2018, Taiwan reported 63 total cholera cases, ranging from 0 to 10 cases per year (Table). Among the patients, 62 were from Taiwan and 1 from Japan; 35 (55.6%) were male. Three (4.8%) patients were in the <14 year age range, 37 (58.7%) in the 15–64 age range, and 23 (36.5%) in the ≥65 age range. Nearly all cases, 61, were sporadic; 2 were part of a family cluster. Seven patients had traveled within the incubation period (5 days) before onset of symptoms: 2 to Indonesia, 1 to Malaysia, 1 to Thailand, 2 to the Philippines, and 1 to Vietnam.

Using pulsed-field gel electrophoresis (PFGE) and whole-genome sequencing analysis, we characterized 60 *V. cholerae* isolates: 4 recovered from patients of the 1962 cholera outbreak and 56 recovered from patients during 2002–2018. Using PFGE, the 2 most prevalent isolates we identified were VcN09.014 (n = 25) and VcN09.012 (n = 7) (Appendix Figure 1, <https://wwwnc.cdc.gov/EID/article/26/1/19-0934-App1.pdf>). We compared those PFGE patterns with those in the *Vibrio cholerae* database maintained by the US Centers for Disease Control and Prevention in 2016 and found no match for VcN09.014, but we did find 11 isolates from Guam and the Philippines that matched with VcN09.012 (identification no. KZGN11.0102).

We identified 7 ST types for the 60 isolates: ST69 (18 isolates), ST723 (2 isolates), ST75 (35 isolates), ST725 (1 isolate), ST726 (2 isolates), ST727 (1 isolates), and ST728 (1 isolate). ST725, ST726, and ST728 are single-locus variants and ST727 a double-locus variant of ST75. ST75 and its derivatives first appeared in 2009 and subsequently become the most prevalent types (Table). Among the 7 patients who had traveled abroad, we found ST69 in those returning from Thailand, the Philippines, and Malaysia; ST75 in the person returning from Vietnam; and ST723 in those

returning from Indonesia. We performed cluster analyses on whole-genome single nucleotide polymorphism profiles for the 60 isolates, which revealed 4 distinct clades (Appendix Table, Figure 2). The fact that a total of 5 isolates in clades 2 and 3 did not harbor the quinolone-resistant gene *qnrVC4* suggests that the resistance gene was introduced after the ST75 strains had emerged.

We compared core genome multilocus sequence typing profiles of the 60 isolates with 5,048 genomes in the National Center for Biotechnology Information databases as of January 19, 2019. We found that the 38 isolates in clade 2 were closely related to 10 strains from China (6), strain MS6 that was identified in Thailand in 2008 (7), and a UK strain recovered from a traveler who returned from Thailand in 2017 (8) (Appendix Figure 3). Strains from the 2 isolates in clade 3 were distantly removed from strains in clade 2 and those found near the US Gulf Coast (9,10) but more closely related to a strain recovered in 2018 and another strain from Russia. The ST75 strains from China were recovered from well water, carriers, and patients during 2005–2014 (6). One ST75 strain in clade 2 was obtained from a Taiwanese person who returned from Vietnam in 2015.

In summary, for most of recent history, cholera has been rare and primarily sporadic in Taiwan. However, the per-year rate of cholera cases has increased since 2009, concurrent with the emergence of strains of the ST75 clone. Over this time, ST75 strains have replaced ST69 as the most prevalent causative agent of cholera in Taiwan. Because closely related ST75 strains had been identified earlier in China and 2 other Southeast Asia countries, we believe our findings indicate that the ST75 clone is spreading more widely in Asia.

Acknowledgments

We sincerely thank Peter Gerner-Smidt and his colleagues at the US Centers for Disease Control and Prevention for conducting PFGE pattern similarity searching in the US PFGE National Database; Marie Anne Chattaway of Public Health England in the United Kingdom and Kazuhisa Okada of Osaka University in Japan for providing the

historical information of the *V. cholerae* ST75 strains they studied; and our colleagues in the Biobank Section of Taiwan Centers for Disease Control for providing *V. cholerae* isolates.

This study was funded by the Ministry of Health and Welfare, Taiwan (grant no. MOHW108-CDC-C-315-122129).

About the Author

Mr. Tu is a research associate in the Centers for Disease Control, Ministry of Health and Welfare, Taiwan. His expertise includes systems biology, computational biology, and machine learning, including developing *Vibrio cholerae* core genome multilocus sequence typing profiling pipelines for the organization.

References

1. World Health Organization. Cholera Annual Report 2017. *Wkly Epidemiol Rec.* 2018;93:489–500.
2. Mutreja A, Kim DW, Thomson NR, Connor TR, Lee JH, Kariuki S, et al. Evidence for several waves of global transmission in the seventh cholera pandemic. *Nature.* 2011;477:462–5. <https://doi.org/10.1038/nature10392>
3. Lippi D, Gotuzzo E, Caini S. Cholera. *Microbiol Spectr.* 2016;4.
4. Yen CH. A recent study of cholera with reference to an outbreak in Taiwan in 1962. *Bull World Health Organ.* 1964;30:811–25. [10.1128/microbiolspec.PoH-0012-2015](https://doi.org/10.1128/microbiolspec.PoH-0012-2015)
5. Taiwan National Infectious Disease Statistics System [cited 2019 Feb 1]. <https://nidss.cdc.gov.tw>
6. Luo Y, Octavia S, Jin D, Ye J, Miao Z, Jiang T, et al. US Gulf-like toxigenic O1 *Vibrio cholerae* causing sporadic cholera outbreaks in China. *J Infect.* 2016;72:564–72. <https://doi.org/10.1016/j.jinf.2016.02.005>
7. Okada K, Na-Ubol M, Natakathung W, Roobthaisong A, Maruyama F, Nakagawa I, et al. Comparative genomic characterization of a Thailand-Myanmar isolate, MS6, of *Vibrio cholerae* O1 El Tor, which is phylogenetically related to a “US Gulf Coast” clone. *PLoS One.* 2014;9:e98120. <https://doi.org/10.1371/journal.pone.0098120>
8. Greig DR, Schaefer U, Octavia S, Hunter E, Chattaway MA, Dallman TJ, et al. Evaluation of whole-genome sequencing for identification and typing of *Vibrio cholerae*. *J Clin Microbiol.* 2018;56:e00831–18. <https://doi.org/10.1128/JCM.00831-18>
9. Watve SS, Chande AT, Rishishwar L, Mariño-Ramírez L, Jordan IK, Hammer BK. Whole-genome sequences of 26 *Vibrio cholerae* isolates. *Genome Announc.* 2016;4:e01396–16. <https://doi.org/10.1128/genomeA.01396-16>
10. Kaper JB, Bradford HB, Roberts NC, Falkow S. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. *J Clin Microbiol.* 1982;16:129–34.

Address for correspondence: Chien-Shun Chiou, Centers for Disease Control, Central Region Laboratory, 5F, 20 Wen-Sin South 3rd Rd, Taichung City 40855, Taiwan; email: nipmcs@cdc.gov.tw

Diabetes Mellitus, Hypertension, and Death among 32 Patients with MERS-CoV Infection, Saudi Arabia

Khalid H. Alanazi, Glen R. Abedi, Claire M. Midgley, Abdulrahim Alkhamis, Taghreed Alsaqer, Abdullah Almoaddi, Abdullah Algwizani, Sameeh S. Ghazal, Abdullah M. Assiri, Hani Jokhdar, Susan I. Gerber, Hail Alabdely, John T. Watson

Author affiliations: Ministry of Health, Riyadh, Saudi Arabia (K.H. Alanazi, A. Alkhamis, T. Alsaqer, A. Almoaddi, A. Algwizani, S.S. Ghazal, A.M. Assiri, H. Jokhdar, H. Alabdely); Centers for Disease Control and Prevention, Atlanta, Georgia, USA (G.R. Abedi, C.M. Midgley, S.I. Gerber, J.T. Watson)

DOI: <https://doi.org/10.3201/eid2601.190952>

Diabetes mellitus and hypertension are recognized risk factors for severe clinical outcomes, including death, associated with Middle East respiratory syndrome coronavirus infection. Among 32 virus-infected patients in Saudi Arabia, severity of illness and frequency of death corresponded closely with presence of multiple and more severe underlying conditions.

First described in 2012, infection with Middle East respiratory syndrome coronavirus (MERS-CoV) has been reported worldwide. More than 2,200 cases have been reported to the World Health Organization, and more than one third have resulted in death (1).

Certain underlying conditions, including diabetes mellitus (DM), hypertension, chronic cardiac disease, and chronic renal disease, are recognized risk factors for illness and death caused by infection with MERS-CoV (2,3). We further explored this relationship among MERS patients admitted to a referral hospital in Riyadh, Saudi Arabia, during August 1, 2015–August 31, 2016. Enrollment criteria and data collection methods have been described (4).

We considered persons with a medical history of DM as having documented DM and persons with multiple recorded periods of hyperglycemia during hospitalization as having possible DM (4). We similarly identified patients with hypertension or chronic kidney disease (CKD) by using documentation in the medical chart. We defined cardiovascular disease as having documentation of coronary artery disease or a history of heart failure or stroke. We considered patients with cardiovascular disease or CKD to have chronic organ dam-

age (COD). We performed statistical analysis by using SAS version 9.4 (<https://www.sas.com>) and Microsoft Excel (<https://www.microsoft.com>).

Of 33 enrolled patients, medical history was available for 32 through medical charts. Underlying disease status among the 32 patients were no DM, hypertension, or COD ($n = 11$); DM without hypertension or COD ($n = 5$); DM and hypertension without COD ($n = 5$); and DM or hypertension with COD ($n = 11$). Of the 21 patients who had DM, 19 had DM documented in the medical chart, and 2 had possible DM, with random glucose readings >350 mg/dL during hospitalization. All 15 patients with hypertension had concomitant DM (Figure; Appendix Table, <https://wwwnc.cdc.gov/EID/article/26/1/19-0952-App1.pdf>). Of the 11 patients who had COD, 8 had cardiovascular disease, 1 had CKD, and 2 had cardiovascular disease and CKD. Of the 10 patients who had cardiovascular disease, 5 had a history of coronary disease, 3 had a history of heart failure, and 4 had a history of stroke; of the 3 patients with a history of heart failure, 1 also had coronary disease and 1 had a history of stroke.

Age was associated with presence of DM (mean 59 years vs. 30 years; $p < 0.0001$, by t -test), hypertension (mean 64 years vs. 36 years; $p < 0.0001$ by t -test), and cardiovascular disease (median 66 years vs. 41 years; $p < 0.0001$ by t -test). Sex was not significantly associated with DM (68% of patients with DM were male vs. 45% of patients without DM; $p = 0.2159$ by χ^2 test), hypertension (67% of patients with hypertension

were male vs. 59% of patients without hypertension; $p = 0.6474$ by χ^2 test, or cardiovascular disease (60% of patients with cardiovascular disease were male vs. 64% of patients without cardiovascular disease; $p = 0.8439$ by χ^2 test).

Of the 32 patients, 21 survived until discharge, including 8 who required supplemental oxygen during hospitalization. Eleven died in the hospital, all of whom required ventilatory support. Case-patients with multiple and more severe underlying conditions generally had worse clinical course and outcomes than those without these conditions (Figure; Appendix Table). Of the 11 case-patients with no DM or hypertension, 100% survived, as did all patients with DM but without hypertension or COD. In comparison, 2 (40%) of 5 with DM and hypertension but without COD died, and 9 (82%) of 11 with DM or hypertension and with COD died (Appendix Table).

In a previous study (5), DM, chronic lung disease, heart disease, and smoking were identified as underlying health conditions and behaviors associated with primary infection with MERS-CoV. Alqatani et al. reported that DM, hypertension, cardiac disease, renal disease, and bronchial asthma were frequent underlying conditions associated with death of MERS-CoV-infected patients and also found that the risk for death increased for patients with multiple comorbidities (3). We found that DM, hypertension, and COD co-occurred frequently in MERS-CoV-infected patients, and severity of illness and frequency

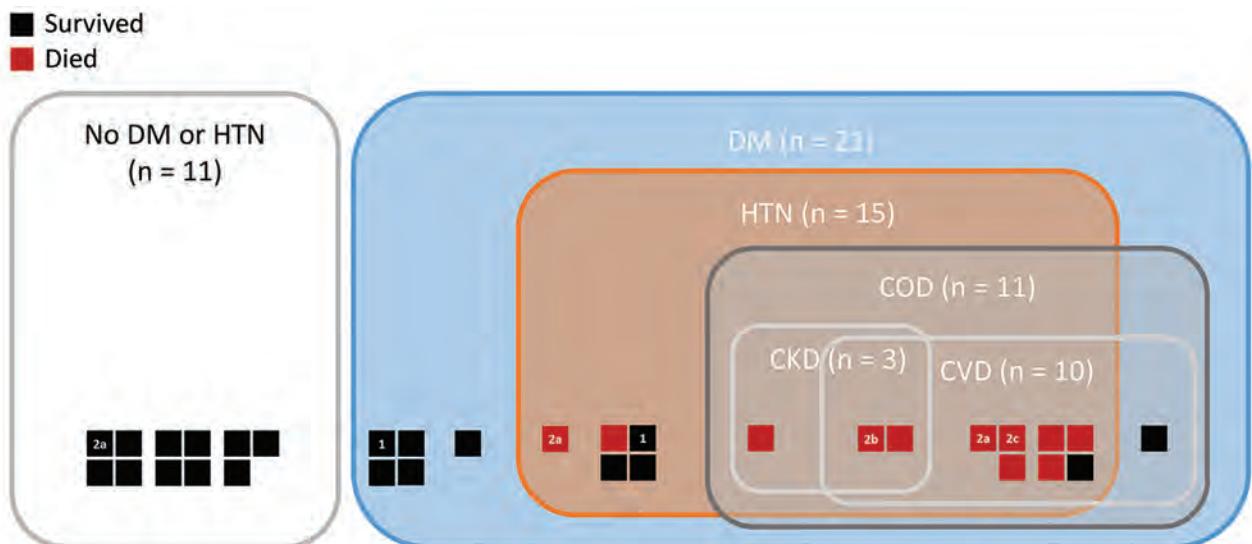


Figure. Characteristics of 32 case-patients infected with Middle East respiratory syndrome coronavirus, by underlying condition and survival status, Saudi Arabia. 1, DM defined as hyperglycemia recorded during hospitalization ($n = 2$). 2a, chronic lung disease in 1 patient with no DM or HTN who survived, 1 patient with DM and HTN but no COD who died, and 1 patient with CVD and not CKD who died. 2b, epilepsy in 1 patient with CVD and CKD who died. 2c, uterine cancer in 1 patient with CVD and not CKD who died. COD, chronic organ damage; CKD, chronic kidney disease; CVD, cardiovascular disease; DM, diabetes mellitus; HTN, hypertension.

of death were higher for patients with multiple and more severe underlying conditions. Further studies are necessary to better clarify the mechanisms that lead to severe outcomes among these patients.

For case-patients infected with MERS-CoV, the presence and compounding of underlying conditions, including DM, hypertension, and, ultimately, COD, corresponded with an increasingly complicated clinical course and death. These findings indicate that increased clinical vigilance is warranted for patients with multiple and severe underlying conditions who are suspected of being infected with MERS-CoV.

About the Author

Dr. Alanazi is director general of infection prevention and control at the Ministry of Health, Riyadh, Saudi Arabia. His research interests include prevention and control of infectious diseases in the healthcare setting.

References

1. World Health Organization. Regional office for the Eastern Mediterranean. MERS situation update; October 2018 [cited 2019 Oct 30]. <http://www.emro.who.int/pandemic-epidemic-diseases/mers-cov/mers-situation-update-october-2018.html>
2. Assiri A, Al-Tawfiq JA, Al-Rabeeh AA, Al-Rabiah FA, Al-Hajjar S, Al-Barrak A, et al. Epidemiological, demographic, and clinical characteristics of 47 cases of Middle East respiratory syndrome coronavirus disease from Saudi Arabia: a descriptive study. *Lancet Infect Dis*. 2013;13:752–61. [https://doi.org/10.1016/S1473-3099\(13\)70204-4](https://doi.org/10.1016/S1473-3099(13)70204-4)
3. Alqahtani FY, Aleanizy FS, Ali El Hadi Mohamed R, Alanazi MS, Mohamed N, Alrasheed MM, et al. Prevalence of comorbidities in cases of Middle East respiratory syndrome coronavirus: a retrospective study. *Epidemiol Infect*. 2018;Nov 5:1–5.
4. Al-Abdely HM, Midgley CM, Alkhamis AM, Abedi GR, Lu X, Binder AM, et al. Middle East respiratory syndrome coronavirus infection dynamics and antibody responses among clinically diverse patients, Saudi Arabia. *Emerg Infect Dis*. 2019;25:753–66. <https://doi.org/10.3201/eid2504.181595>
5. Alraddadi BM, Watson JT, Almarashi A, Abedi GR, Turkistani A, Sadran M, et al. Risk factors for primary Middle East respiratory syndrome coronavirus illness in humans, Saudi Arabia, 2014. *Emerg Infect Dis*. 2016;22:49–55. <https://doi.org/10.3201/eid2201.151340>

Address for correspondence: Glen R. Abedi, Centers for Disease Control and Prevention, 1600 Clifton RD NE, Mailstop A34, Atlanta, GA 30329-4027, USA; email: gabedi@cdc.gov

Influenza D Virus of New Phylogenetic Lineage, Japan

Shin Murakami, Ryota Sato, Hiroho Ishida, Misa Katayama, Akiko Takenaka-Uema, Taisuke Horimoto

Author affiliations: University of Tokyo, Tokyo, Japan (S. Murakami, H. Ishida, M. Katayama, A. Takenaka-Uema, T. Horimoto); Yamagata Livestock Hygiene Service Center, Yamagata, Japan (R. Sato)

DOI: <https://doi.org/10.3201/eid2601.191092>

Influenza D virus (IDV) can potentially cause respiratory diseases in livestock. We isolated a new IDV strain from diseased cattle in Japan; this strain is phylogenetically and antigenically distinguished from the previously described IDVs.

Influenza D virus (IDV; family *Orthomyxoviridae*) is one of the possible bovine respiratory disease complex (BRDC) causative agents. IDVs are detected in and isolated from cattle in many countries in North America, Asia, Europe, and Africa (1–4). In addition, both IDV RNAs and specific antibodies have been detected in many animal species (1,5,6). To date, IDVs have been phylogenetically classified into 3 lineages: D/OK, D/660, and Japanese lineages. We isolated a new IDV strain from cattle in Japan with respiratory disease, whose hemagglutinin-esterase-fusion (HEF) gene did not belong to the known phylogenetical lineages.

At a herd in Yamagata Prefecture in northern Japan, 15 Holsteins (37.5% of bred cattle in the herd) had respiratory signs develop during January 6–10, 2019 (Appendix Table 1, <http://wwwnc.cdc.gov/EID/article/26/1/19-1092-App1.pdf>). We collected nasal swab samples from 9 of 15 cows on January 7 and 10. All samples subjected to IDV-specific real-time reverse transcription PCR (RT-PCR) (7) were found positive. We also subjected these samples to RT-PCR analyses specific for bovine viral diarrhoea virus 1 and 2, infectious bovine rhinotracheitis virus, bovine parainfluenza virus 3, bovine respiratory syncytial virus, bovine coronavirus, bovine rhinitis A virus, *Mycobacterium bovis*, *Mycoplasma bovis*, *Mycoplasma dispar*, *Ureaplasma diversum*, and *Mycoplasma bovirhinis*. All samples were found negative for these pathogens except for the sample from cow no. 5, which was positive for *M. dispar*. Despite these cows receiving antimicrobial drug treatment, we isolated *Mannheimia haemolytica* from 7 of them (nos. 1–5, 7, 8); cow no. 5 died on January 7. Taken together, these

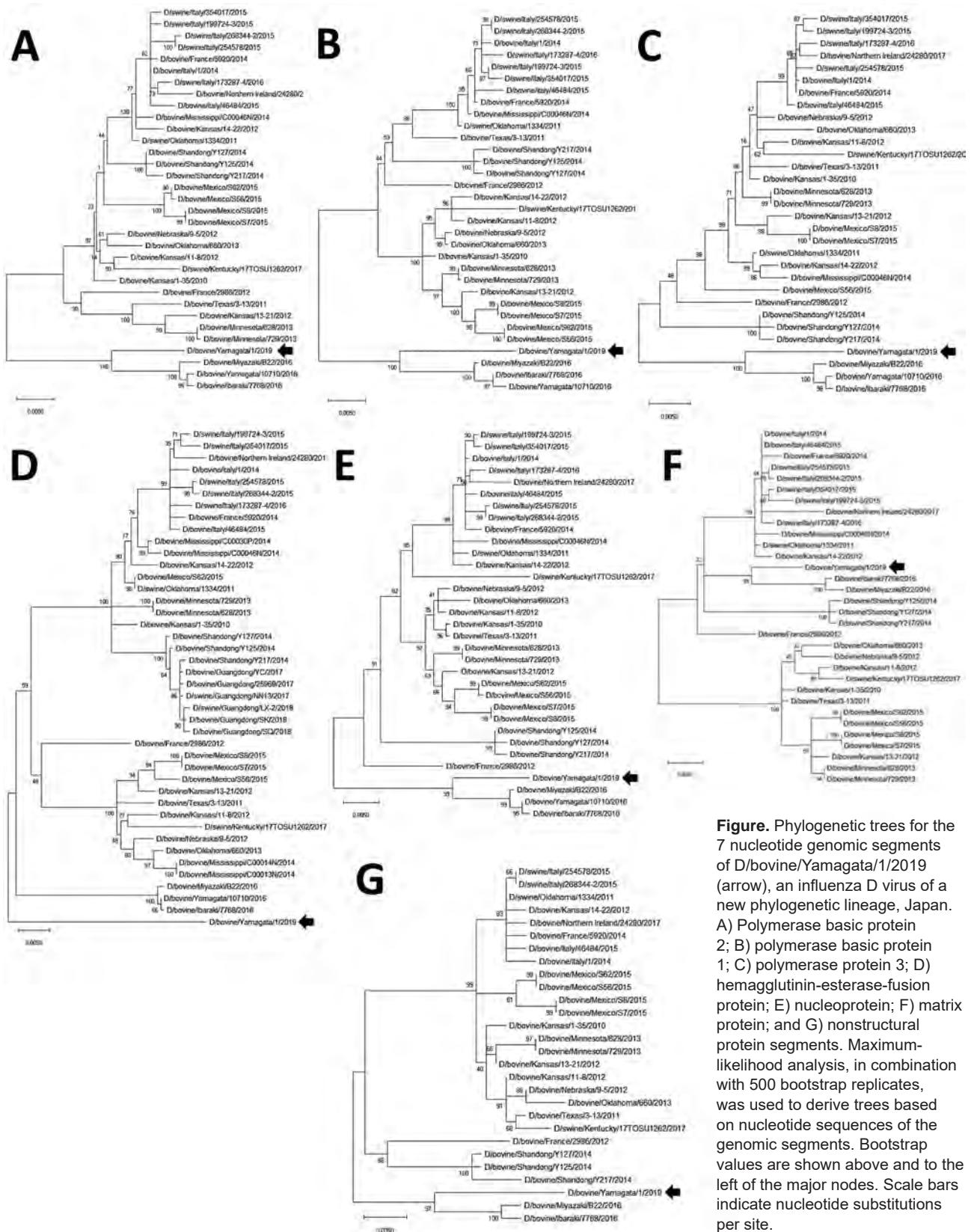


Figure. Phylogenetic trees for the 7 nucleotide genomic segments of D/bovine/Yamagata/1/2019 (arrow), an influenza D virus of a new phylogenetic lineage, Japan. A) Polymerase basic protein 2; B) polymerase basic protein 1; C) polymerase protein 3; D) hemagglutinin-esterase-fusion protein; E) nucleoprotein; F) matrix protein; and G) nonstructural protein segments. Maximum-likelihood analysis, in combination with 500 bootstrap replicates, was used to derive trees based on nucleotide sequences of the genomic segments. Bootstrap values are shown above and to the left of the major nodes. Scale bars indicate nucleotide substitutions per site.

factors suggested that IDV infection was involved in the onset of BRDC in this herd.

To isolate IDV, we inoculated the collected samples into human rectal tumor HRT-18G cells (American Type Culture Collection [ATCC] no. CRL-11663), incubated the cells for 4 days at 37°C, and blindly passaged the supernatants in swine testis ST cells (ATCC no. CRL-1746) in the presence of tosyl phenylalanyl chloromethyl ketone-treated trypsin. We observed a cytopathic effect at the second passage. For further analysis, we used supernatants that tested positive for hemagglutination (HA) and for presence of IDV RNA detected by real-time RT-PCR, indicating successful isolation of IDV (designated as D/bovine/Yamagata/1/2019 [D/Yama2019]). We determined the entire genomic sequence of D/Yama2019 by performing RT-PCR amplification of each viral segment with specific primers, revealing an identical sequence with that detected in swab sample no. 1. We deposited our sequence data in GenBank (accession nos. LC494105–11).

We phylogenetically analyzed the sequences of D/Yama2019 by performing maximum-likelihood analysis using MEGA version X (8). The HEF sequence of D/Yama2019 acquired an independent position different from that exhibited in any other lineage, whereas those of the other segments were positioned relatively close to the preexisting Japanese strains, in which polymerase basic protein 1 and 2, polymerase protein 3, nucleoprotein, and nonstructural protein sequences were of the Japanese lineage, whereas the matrix sequence was of the D/OK lineage (Figure).

We also collected serum samples from 8 cows on January 10 (acute phase of the disease) and February 4 (recovery phase) and examined them for antibodies against D/Yama2019 by using the HA inhibition (HI) test (Appendix Table 1). We treated serum samples with receptor-destroying enzyme (RDEII; Denka Seiken, Tokyo, Japan, <https://www.denka.co.jp>) before the HI test, which was done with the D/Yama2019 antigen and 0.6% turkey erythrocyte suspension. We considered samples with HI titer >1:40 to be positive (4). All serum samples collected on January 7 were HI-negative except for the sample from cow no. 1 (a titer of 1:40), whereas all samples collected on February 4 were HI-positive (a titer range of 1:40–1:160), confirming seroconversion to IDV antibodies in diseased cows. Taken together, these results indicate that cattle were infected with IDV, leading to BRDC in this herd.

We next compared HEF antigenicities among D/Yama2019 and 3 reference IDVs, D/swine/

Oklahoma/1334/2011 (D/OK) in the D/OK-lineage (1), D/bovine/Nebraska/9–5/2012 (D/NE) in the D/660-lineage (9), and D/bovine/Yamagata/10710/2016 (D/Yama2016) in the Japanese lineage (10), by performing HI tests using anti-IDV mouse serum and a panel of anti-HEF monoclonal antibodies (Appendix Table 2). We observed 1-way cross-reactivity between D/Yama2019 and D/OK or D/Yama2016 with antiserum. Monoclonal antibody clones B4 and R36 cross-reacted to D/Yama2019 and other tested viruses with different titers, whereas the clones G22, G27, and G74, which reacted strongly to D/Yama2016, did not react to D/Yama2019. These data indicated HEF antigenic heterogeneity between D/Yama2019 and viruses of the 3 known lineages. Monoclonal antibodies revealed the presence of common epitopes among IDVs, as suggested by previous reports (9,10). Amino acid differences were located on the head region of HEF among the IDVs, possibly causing antigenic heterogeneity.

In summary, we isolated a newly identified IDV from diseased cattle that was phylogenetically and antigenically distinguished from known IDVs. Further epidemiologic studies are needed to clarify invasion and epidemic status of these heterologous IDVs, but our results indicated that heterologous IDVs are circulating in close vicinity within the prefecture.

Acknowledgments

We thank Ben M. Hause for the viruses used in the study.

S.M. is supported by a Grant-in-Aid for the Encouragement of Young Scientists (A) (grant no. 17H05042). T.H. is supported by Livestock Promotion Funds from the Japan Racing Association.

About the Author

Dr. Murakami is an associate professor at the Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan. His research interests include epidemiologic and molecular biological studies of animal viruses, including influenza viruses.

References

- Hause BM, Ducatez M, Collin EA, Ran Z, Liu R, Sheng Z, et al. Isolation of a novel swine influenza virus from Oklahoma in 2011 which is distantly related to human influenza C viruses. *PLoS Pathog*. 2013;9:e1003176. <https://doi.org/10.1371/journal.ppat.1003176>
- Jiang WM, Wang SC, Peng C, Yu JM, Zhuang QY, Hou GY, et al. Identification of a potential novel type of influenza virus in bovine in China. *Virus Genes*. 2014;49:493–6. <https://doi.org/10.1007/s11262-014-1107-3>

3. Dane H, Duffy C, Guelbenzu M, Hause B, Fee S, Forster F, et al. Detection of influenza D virus in bovine respiratory disease samples, UK. *Transbound Emerg Dis*. 2019;66:2184–7. <https://doi.org/10.1111/tbed.13273>
4. Murakami S, Endoh M, Kobayashi T, Takenaka-Uema A, Chambers JK, Uchida K, et al. Influenza D virus infection in herd of cattle, Japan. *Emerg Infect Dis*. 2016;22:1517–9. <https://doi.org/10.3201/eid2208.160362>
5. Salem E, Cook EAJ, Lbacha HA, Oliva J, Awoume F, Aplogon GL, et al. Serologic evidence for influenza C and D virus among ruminants and camelids, Africa, 1991–2015. *Emerg Infect Dis*. 2017;23:1556–9. <https://doi.org/10.3201/eid2309.170342>
6. Murakami S, Odagiri T, Melaku SK, Bazartseren B, Ishida H, Takenaka-Uema A, et al. Influenza D virus infection in dromedary camels, Ethiopia. *Emerg Infect Dis*. 2019;25:1224–6. <https://doi.org/10.3201/eid2506.181158>
7. Faccini S, De Mattia A, Chiapponi C, Barbieri I, Boniotti MB, Rosignoli C, et al. Development and evaluation of a new real-time RT-PCR assay for detection of proposed influenza D virus. *J Virol Methods*. 2017;243:31–4. <https://doi.org/10.1016/j.jviromet.2017.01.019>
8. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol Biol Evol*. 2018;35:1547–9. <https://doi.org/10.1093/molbev/msy096>
9. Collin EA, Sheng Z, Lang Y, Ma W, Hause BM, Li F. Cocirculation of two distinct genetic and antigenic lineages of proposed influenza D virus in cattle. *J Virol*. 2015;89:1036–42. <https://doi.org/10.1128/JVI.02718-14>
10. Odagiri T, Ishida H, Li JY, Endo M, Kobayashi T, Kamiki H, et al. Antigenic heterogeneity among phylogenetic clusters of influenza D viruses. *J Vet Med Sci*. 2018;80:1241–4. <https://doi.org/10.1292/jvms.18-0157>

Address for correspondence: Taisuke Horimoto, University of Tokyo, Graduate School of Agricultural and Life Sciences, Department of Veterinary Microbiology, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan; email: ahorimo@mail.ecc.u-tokyo.ac.jp

Diagnosis of Syphilitic Bilateral Papillitis Mimicking Papilloedema

Alicia Gonzalez-Martinez, Sonia Quintas, Diego Celdrán Vivancos, José Cebrián, José Vivancos

Author affiliation: Hospital Universitario de la Princesa, Madrid, Spain

DOI: <https://doi.org/10.3201/eid2601.191122>

Syphilis produces myriad nonspecific signs and symptoms. For example, optic disk swelling might be seen in patients with syphilis as a result of cranial hypertension (papilloedema), inflammatory optic neuritis with papillitis, or optic perineuritis. We report a case involving differential diagnosis of syphilitic bilateral papillitis mimicking papilloedema.

Syphilis, caused by infection with the bacterium *Treponema pallidum*, is a sexually transmitted infection for which incidence has been increasing since 2002, especially among adult men >55 years of age who engage in risky sex (1). Syphilis has earned its nickname, the “great masquerader,” because it produces myriad nonspecific signs and symptoms that make it difficult to distinguish from certain other diseases. Eye impairment occurs in ≥3% of cases (2,3) and can be the first manifestation (4). Optic nerve involvement, either unilateral or bilateral, in the form of papilloedema, perineuritis, or optic neuritis, is the second most common type of syphilitic ocular impairment (5). Each of these conditions shares findings from funduscopy testing with unilateral or bilateral optic disk swelling (Table), but the etiology and, therefore, the diagnostic algorithm are different. Semiology and ophthalmological findings are the key to achieving a correct syndromic diagnosis.

We describe the case of a 62-year-old man who was admitted to the neurology department at Hospital Universitario de la Princesa in Madrid, Spain, with a 4-day history of bilateral decreased visual acuity. He was obese, an active smoker, and dyslipidemic. He reported neither ocular pain nor dyschromatopsia suggestive of optic neuritis, nor headache or diplopia usually associated with intracranial hypertension. He had no known history of syphilis. Visual acuity was 20/32 in the right eye and 20/63 in the left. Pupils were equal and reactive to light, with no relative afferent pupillary defect, which is typical of unilateral optic neuritis. Slit lamp examination results were normal, showing no inflamed cells in the anterior chambers or vitreous. Neurologic examination was normal. Opening pressure of the cerebrospinal fluid (CSF) on lumbar puncture was 27 cm H₂O, above the reference range of 5–20 cm H₂O. The CSF white cell count was 0, with normal glucose and protein levels. A fundus examination revealed bilateral optic disk swelling and peripapillary retinitis; visual field testing revealed bilateral central scotoma and an enlarged blind spot (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/19-1122-App1.pdf>).

Doctors initiated acetazolamide for suspected idiopathic intracranial hypertension (IIH), but

Table. Differential diagnosis of syphilitic optic disk swelling*

Differential diagnosis	Clinical presentation	Visual acuity	Visual fields	Optic disk appearance	Other ocular abnormalities	Lumbar CSF opening pressure†	Orbital MRI
Papilloedema	Headache, nausea, tinnitus, diplopia, neck stiffness, photophobia	Normal to slow reduction (months)	Enlarged blind spot	Swollen	Flame hemorrhages	High	Normal/flattening of the posterior sclera, dilation of the ONS, and protrusion of the optic disk head
Perineuritis	Asymptomatic	Normal	Enlarged blind spot, constricted peripheral visual field	Slightly swollen	None	Normal	ONS and orbital fat expansion and enhancement
Anterior optic neuritis (papillitis)	Ocular pain, dyschromatopsia	Reduced (hours-days)	Enlarged blind spot, central scotomas, and other field abnormalities	Swollen	None/cellular activity in the posterior vitreous, patchy diffuse retinitis	Normal	Optic nerve gadolinium enhancement

*CSF cerebrospinal fluid; MRI, magnetic resonance imaging; ONS optic nerve sheath.

†Normal lumbar CSF pressure: <25 cm H₂O, <28 cm H₂O in obese patients.

visual acuity decreased to 20/40 in the right eye and 20/200 in the left. The rapid decrease in visual acuity and the lack of response to acetazolamide suggested optic nerve involvement, which seemed atypical for IIH in the absence of other cranial nerve impairment (being the sixth cranial nerve, which is most likely to be affected IIH in the first place).

A cerebral magnetic resonance imaging scan with gadolinium did not reveal any structural lesion or indirect findings of IIH. For this reason, the diagnostic study was expanded. Optical coherence tomography of the nerve fiber layer showed an increase in average thickness in both eyes, reflecting optic nerve edema. Results from laboratory tests for complete blood count, urea, electrolytes, enzymes, hormones, antinuclear antibodies, and protein electrophoresis were within normal ranges. Test results were negative for HIV. A treponemal test reacted negatively to a nontreponemal Venereal Disease Research Laboratory (VDRL) test in serum, but a VDRL test in CSF was reactive without dilution. Treatment with intravenous penicillin G (4×10^6 U, every 8 h for 14 d) was initiated. Fundoscopy results were normal, visual acuity remained stable but unimproved (0/40 in the right eye and 20/200 in the left eye, which was not unexpected), and visual fields remained stable 3 months after penicillin treatment was begun (Appendix). Lumbar puncture at that time revealed normal opening pressure and negative results for VDRL test scores.

Papilloedema refers to optic disk edema caused by increased intracranial pressure. The initial pressure of 27 cm H₂O for this patient, despite being high, was lower than the 28 cm H₂O in obese patients required to diagnose IIH, according to the latest International

Classification of Headache Disorders standards (6). Syphilitic optic perineuritis is usually asymptomatic (7) because the inflammation is restricted to the optic nerve sheath, rather than the nerve itself (8). Clinical suspicion of that condition arises in the presence of optic disk swelling with normal CSF pressure and visual acuity (9). Optic neuritis, unlike these other conditions, is usually accompanied by rapid visual failure, as seen in this patient. Ocular pain is a common finding but not necessary for diagnosis.

Because the optic nerve and retina are considered extensions of the central nervous system, patients with ocular syphilis should undergo lumbar puncture and CSF analysis to confirm neurologic involvement. Diagnosing neurosyphilis relies on a combination of symptoms and signs verified by laboratory studies of blood and CSF (10), ocular symptoms due to bilateral papillitis, and positive treponemal test in serum and VDRL in CSF. This patient met these criteria.

Our findings underline that IIH can only be diagnosed through systematic exclusion of alternative diagnoses and the diagnosis cannot be reached by funduscopy findings alone. Clinical findings are key points for differentiating papilloedema and ocular perineuritis from papillitis. A complete diagnostic work up is required to rule out all other etiologic causes of optic neuropathy and, if the diagnosis of ocular syphilis is reached, a lumbar puncture should be performed to rule out neurosyphilis.

About the Author

Dr. Gonzalez-Martinez is currently completing her residency in neurology at Hospital Universitario de la Princesa, Madrid. She has previously worked at

Karolinska Institutet and Massachusetts General Hospital (Harvard Medical School). Her areas of interest are clinical neurology and translational research.

References

1. STI Study Group. Syphilis and gonorrhoea diagnoses in a network of STI centres, 2005–2008. Madrid:National Centre of Epidemiology; 2010.
2. Doris JP, Saha K, Jones NP, Sukthankar A. Ocular syphilis: the new epidemic. *Eye (Lond)*. 2006;20:703–5. <https://doi.org/10.1038/sj.eye.6701954>
3. Apinyawasisuk S, Poonyathalang A, Preechawat P, Vanikietti K. Syphilitic optic neuropathy: re-emerging cases over a 2-year period. *Neuroophthalmology*. 2016;40:69–73. <https://doi.org/10.3109/01658107.2015.1134586>
4. Bandettini di Poggio M, Primavera A, Capello E, Bandini F, Mazzarello G, Viscoli C, et al. A case of secondary syphilis presenting as optic neuritis. *Neurol Sci*. 2010;31:365–7. <https://doi.org/10.1007/s10072-010-0222-8>
5. Lapere S, Mustak H, Steffen J. Clinical manifestations and cerebrospinal fluid status in ocular syphilis. *Ocul Immunol Inflamm*. 2019;27:126–30. <https://doi.org/10.1080/09273948.2018.1521436>
6. Headache Classification Committee of the International Headache Society (IHS). The International Classification of Headache Disorders, 3rd edition. Cephalgia. 2018;38:1–211. <https://doi.org/10.1177/0333102417738202>
7. O'Connell K, Marnane M, McGuigan C. Bilateral ocular perineuritis as the presenting feature of acute syphilis infection. *J Neurol*. 2012;259:191–2. <https://doi.org/10.1007/s00415-011-6148-0>
8. Meehan K, Rodman J. Ocular perineuritis secondary to neurosyphilis. *Optom Vis Sci*. 2010;87:E790–6. <https://doi.org/10.1097/OPX.0b013e3181f361b0>
9. Gartaganis S, Georgiou S, Monastirli A, Katsimpris J, Pasmazi E, Tsambaos D. Asymptomatic bilateral optic perineuritis in secondary syphilis. *Acta Derm Venereol*. 2000;80:75–6. <https://doi.org/10.1080/000155500750012720>
10. Workowski KA, Berman SM. CDC sexually transmitted diseases treatment guidelines. *Clin Infect Dis*. 2002;35(Suppl 2):S135–7. <https://doi.org/10.1086/342100>

Address for correspondence: Sonia Quintas, Hospital Universitario de la Princesa, Calle Diego de León, 62, Secretaría de Neurología, 28006 Madrid, Spain; email: sonia.qg@gmail.com

Influenza A Virus Infections in Dromedary Camels, Nigeria and Ethiopia, 2015–2017

Daniel K.W. Chu,¹ Ranawaka A.P.M. Perera,¹ Abraham Ali,¹ Jamiu O. Oladipo, Gezahegne Mamo, Ray T.Y. So, Ziqi Zhou, Yen Yeen Chor, Chak Kai Chan, Desalegn Belay, Adamu Tayachew, Mesfin Mengesha, Feyesa Regassa, Nga Ting Lam, Leo L.M. Poon, Malik Peiris

Author affiliations: The University of Hong Kong, Hong Kong, China (D.K.W. Chu, R.A.P.M. Perera, J.O. Oladipo, R.T.Y. So, Z. Zhou, Y.Y. Chor, C.K. Chan, N.T. Lam, L.L.M. Poon, M. Peiris); Ethiopian Public Health Institute, Addis Ababa, Ethiopia (A. Ali, D. Belay, A. Tayachew, M. Mengesha, F. Regassa); Addis Ababa University, Bishoftu, Ethiopia (G. Mamo)

DOI: <https://doi.org/10.3201/eid2601.191165>

We examined nasal swabs and serum samples acquired from dromedary camels in Nigeria and Ethiopia during 2015–2017 for evidence of influenza virus infection. We detected antibodies against influenza A(H1N1) and A(H3N2) viruses and isolated an influenza A(H1N1) pdm09–like virus from a camel in Nigeria. Influenza surveillance in dromedary camels is needed.

Aquatic wild birds are the natural reservoir of influenza A virus, which comprises 16 hemagglutinin and 9 neuraminidase subtypes. Influenza A virus subtypes H1N1, H2N2, and H3N2 have caused pandemics in humans, and subtypes H1N1 and H3N2 circulate in humans as seasonal influenza. Pandemic influenza arises when an animal influenza virus evolves through the reassortment of animal and human virus gene segments (antigenic shift) to sustainably transmit in humans. Avian and swine influenza viruses have caused zoonotic infections, some resulting in fatal disease. Thus, influenza virus surveillance in animals is needed for pandemic preparedness (1).

Dromedary camel populations, estimated to be 30 million globally, can be found in parts of Africa, the Middle East, and Central Asia, often in close proximity to humans. An equine influenza A(H3N8) virus (2) and human influenza A/USSR/90/77(H1N1)–like viruses (which were associated with fatal disease in 1980–1983) (3) have been isolated from Bactrian camels in Mongolia. However, little is known of influenza A virus infections in dromedary populations.

¹These authors contributed equally to this article.

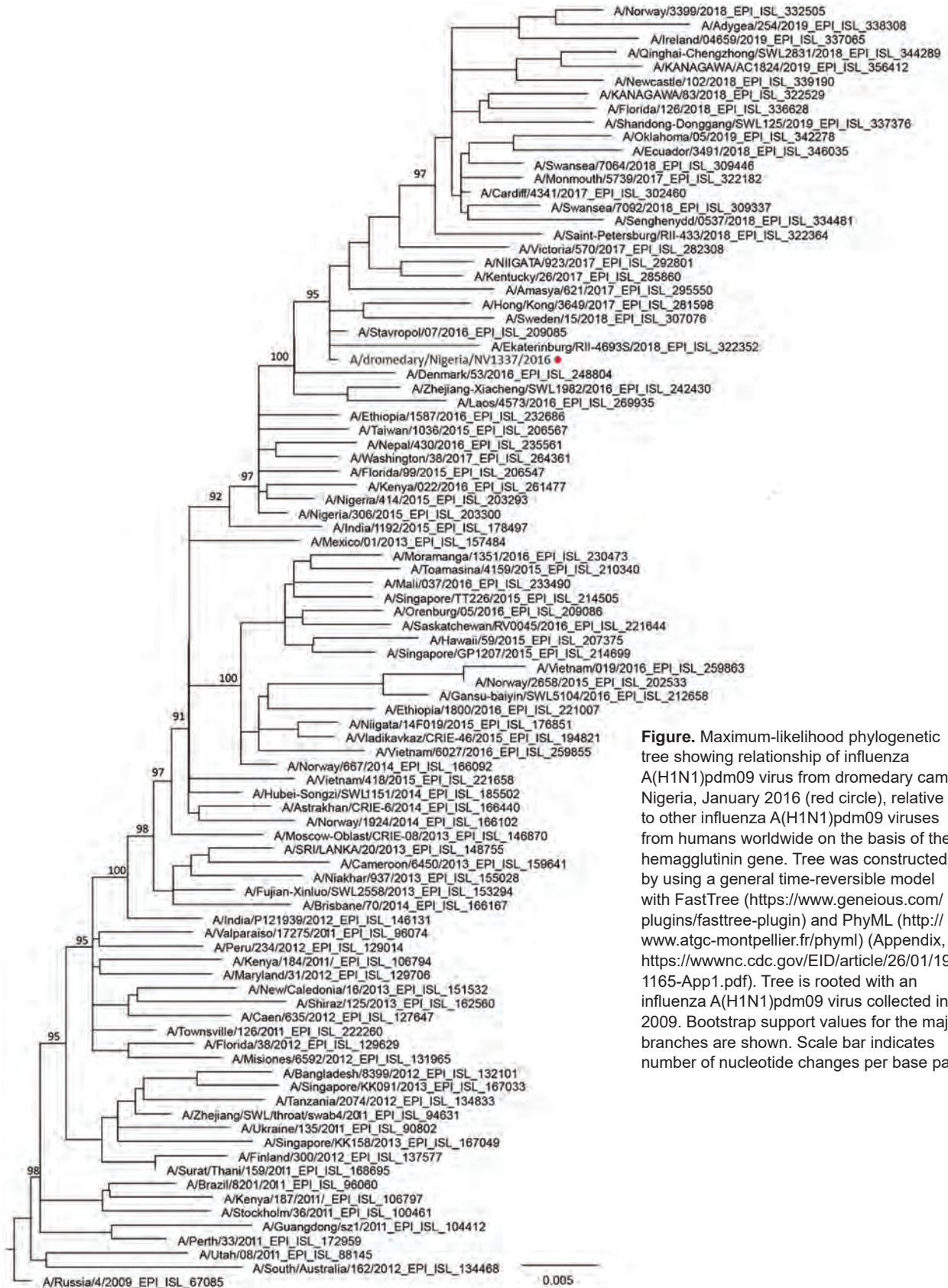


Figure. Maximum-likelihood phylogenetic tree showing relationship of influenza A(H1N1)pdm09 virus from dromedary camel, Nigeria, January 2016 (red circle), relative to other influenza A(H1N1)pdm09 viruses from humans worldwide on the basis of the hemagglutinin gene. Tree was constructed by using a general time-reversible model with FastTree (<https://www.geneious.com/plugins/fasttree-plugin>) and PhyML (<http://www.atgc-montpellier.fr/phyml>) (Appendix, <https://wwwnc.cdc.gov/EID/article/26/01/19-1165-App1.pdf>). Tree is rooted with an influenza A(H1N1)pdm09 virus collected in 2009. Bootstrap support values for the major branches are shown. Scale bar indicates number of nucleotide changes per base pair.

Therefore, we carried out a study to determine the prevalence of influenza A virus infection in dromedary camels.

As part of an investigation of Middle East respiratory syndrome coronavirus conducted during October 2015–February 2016 (4), we collected 2,166 nasal swabs and 150 serum samples from dromedaries at an abattoir in Kano, Nigeria. We also collected 102 nasal swabs and 100 serum samples from nomadic dromedary herds in Amibara, Ethiopia (in January 2017); 109 nasal swabs from herds in Asayita, Ethiopia (in July 2017); and 83 nasal swabs from herds in Dubti, Ethiopia (in July 2017). We put nasal swabs in virus transport medium, stored samples frozen at -80°C , and shipped them to Hong Kong, China, on dry ice for laboratory tests. We performed RNA extraction and detected the influenza A virus matrix gene using molecular methods (Appendix, <https://wwwnc.cdc.gov/EID/article/26/1/19-1165-App1.pdf>) (5).

Twelve nasal swabs from camels in Nigeria collected at different times (October [$n = 5$] and November [$n = 5$] 2015 and January [$n = 1$] and February [$n = 1$] 2016) were positive for the influenza A virus matrix gene; cycle thresholds for these samples were 33.7–38.9 (Appendix Table). One of the nasal swabs collected in Amibara was also positive (cycle threshold 37.8). We inoculated quantitative reverse transcription PCR–positive samples onto Madin Darby canine kidney cells grown in 24-well plates in minimum essential medium with tosylsulfonyl phenylalanyl chloromethyl ketone–treated trypsin (2 $\mu\text{g}/\text{mL}$) (Appendix) (6). We examined inoculated cells for cytopathic effect and passaged each culture twice. We defined virus detection as the appearance of cytopathic effect or the hemagglutination of turkey erythrocytes. We were able to isolate 1 virus, which we designated A/dromedary/NV1337/2016 (H1N1), from a swab collected in Nigeria on January 22, 2016 (Appendix Table).

We performed full-genome sequencing of A/dromedary/NV1337/2016 (Appendix) as previously described (6). We achieved sequencing read coverages of ≥ 100 for each nucleotide and were able to deduce the full virus genome (GenBank accession nos. MN453859–66). All 8 gene segments showed their highest identity (≥ 99.8) to contemporary influenza A(H1N1)pdm09 viruses (data not shown).

In a phylogenetic analysis, we compared the hemagglutinin gene of A/dromedary/NV1337/2016 with that of other influenza A(H1N1)pdm09 viruses available from GenBank and GISAID (<https://platform.gisaid.org>) (Figure). The dromedary influenza A virus

isolated in Nigeria in January 2016 was similar to other influenza viruses circulating in humans at the same time. The sampling dates for the influenza viruses detected in camels in Nigeria overlapped with the human influenza virus season, which typically occurs during October–March (Appendix Table) (7). This finding suggests reverse zoonosis of influenza viruses from humans to dromedaries. Whether these viruses were subsequently maintained in dromedary populations via camel-to-camel transmission is not clear. Further studies are needed to address this question. Transmission of influenza A virus also occurs from humans to swine, and these viruses can be maintained in swine populations for variable periods, sometimes decades (8).

We tested serum samples from dromedary camels for hemagglutination inhibition (HI) antibody against A/dromedary/NV1337/2016(H1N1) using standard methods (Appendix) (5); 4 serum samples from camels in Amibara had HI antibody (titers 1:40, 1:80, 1:160, and 1:160). The dromedaries that had influenza A virus RNA–positive nasal swabs were negative for HI antibody. Dromedaries recently infected with a virus are expected to be seronegative for that virus because antibody responses against viruses take around a week to develop (9), by which time nasal swab specimens are often negative for that virus’s genomic material. Microneutralization tests are more appropriate for testing antibody to contemporary H3N2 viruses (10); hence, we also tested serum samples for antibody to A/Hong Kong/4801/2014(H3N2) virus using the microneutralization test (Appendix). In total, 1 serum sample from a camel in Nigeria was positive at a titer of 1:80.

In conclusion, we provide evidence of influenza A virus infection in dromedaries. Our findings indicate that influenza virus surveillance in dromedary camel populations is needed.

This research was funded by the Theme Based Research Scheme (reference no. T11-705/14N) from the Research Grants Council of the Hong Kong Special Administrative Region, China, and from the US National Institute of Allergy and Infectious Diseases under Centers of Excellence for Influenza Research and Surveillance contract no. HHSN272201400006C.

About the Author

Dr. Chu is a research assistant professor at The University of Hong Kong, Hong Kong. His research focuses on influenza and coronaviruses of humans and animals and his interests focus on diagnostic virology, molecular diagnostics, and virus evolution.

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. PubMed
2. Yondon M, Zayat B, Nelson MI, Heil GL, Anderson BD, Lin X, et al. Equine influenza A(H3N8) virus isolated from Bactrian camel, Mongolia. *Emerg Infect Dis.* 2014;20:2144–7. PubMed <https://doi.org/10.3201/eid2012.140435>
3. Yamnikova SS, Mandler J, Bekh-Ochir ZH, Dachtzeren P, Ludwig S, Lvov DK, et al. A reassortant H1N1 influenza A virus caused fatal epizootics among camels in Mongolia. *Virology.* 1993;197:558–63. <https://doi.org/10.1006/viro.1993.1629>
4. So RT, Perera RA, Oladipo JO, Chu DK, Kuranga SA, Chan KH, et al. Lack of serological evidence of Middle East respiratory syndrome coronavirus infection in virus exposed camel abattoir workers in Nigeria, 2016. *Euro Surveill.* 2018;23. <https://doi.org/10.2807/1560-7917.ES.2018.23.32.1800175>
5. Duchamp MB, Casalegno JS, Gillet Y, Frobert E, Bernard E, Escuret V, et al. Pandemic A(H1N1)2009 influenza virus detection by real time RT-PCR: is viral quantification useful? *Clin Microbiol Infect.* 2010;16:317–21. <https://doi.org/10.1111/j.1469-0691.2010.03169.x>
6. Baudon E, Chu DKW, Tung DD, Thi Nga P, Vu Mai Phuong H, Le Khanh Hang N, et al. Swine influenza viruses in Northern Vietnam in 2013–2014. *Emerg Microbes Infect.* 2018;7:1–16. <https://doi.org/10.1038/s41426-018-0109-y>
7. Newman LP, Bhat N, Fleming JA, Neuzil KM. Global influenza seasonality to inform country-level vaccine programs: an analysis of WHO FluNet influenza surveillance data between 2011 and 2016. *PLoS One.* 2018;13:e0193263. <https://doi.org/10.1371/journal.pone.0193263>
8. Shortridge KF, Webster RG, Butterfield WK, Campbell CH. Persistence of Hong Kong influenza virus variants in pigs. *Science.* 1977;196:1454–5. <https://doi.org/10.1126/science.867041>
9. Abdellatif MM, Ibrahim AA, Khalafalla AI. Development and evaluation of a live attenuated camelpox vaccine from a local field isolate of the virus. *Rev Sci Tech.* 2014;33:831–8. <https://doi.org/10.20506/rst.33.3.2321>
10. Laurie KL, Engelhardt OG, Wood J, Heath A, Katz JM, Peiris M, et al.; CONSISE Laboratory Working Group participants. International laboratory comparison of influenza microneutralization assays for A(H1N1)pdm09, A(H3N2), and A(H5N1) influenza viruses by CONSISE. *Clin Vaccine Immunol.* 2015;22:957–64. <https://doi.org/10.1128/VI.00278-15>

Address for correspondence: Malik Peiris, The University of Hong Kong School of Public Health, No. 7 Sassoon Rd, Pokfulam, Hong Kong; email: malik@hku.hk

Bovine Kobuvirus in Calves with Diarrhea, United States

Leyi Wang, Richard Fredrickson, Michelle Duncan, Jonathan Samuelson, Shih-Hsuan Hsiao

Author affiliations: University of Illinois, Urbana, Illinois, USA (L. Wang, R. Fredrickson, J. Samuelson, S.-H. Hsiao); Western Illinois Veterinary Clinic, Quincy, Illinois, USA (M. Duncan)

DOI: <https://doi.org/10.3201/eid2601.191227>

We detected bovine kobuvirus (BKV) in calves with diarrhea in the United States. The strain identified is related genetically to BKVs detected in other countries. Histopathologic findings also confirmed viral infection in 2 BKV cases. Our data show BKV is a potential causative agent for diarrhea in calves.

Bovine kobuvirus (BKV; species *Aichivirus B*, genus *Kobuvirus*, family *Picornaviridae*) was identified initially as a cytopathic contaminant in a culture medium of HeLa cells in Japan in 2003 (1). Since then, BKV has been reported in Thailand, Hungary, the Netherlands, Korea, Italy, Brazil, China, and Egypt (2–9). However, circulation of BKV in North America remains unclear. We report detection of BKV in calves in the United States.

In April 2019, a fecal sample from a 10–14-day-old calf was submitted to University of Illinois Veterinary Diagnostic Laboratory (Urbana, IL, USA) for testing for enteric pathogens. Results of tests for rotavirus, coronavirus, cryptosporidium, and *Escherichia coli* were positive; results for *Salmonella* were negative.

We extracted nucleic acid from the fecal sample and conducted a sequence-independent single-primer amplification and library preparation by using Nextera XT DNA Library Preparation Kit (Illumina, <https://www.illumina.com>). We conducted sequencing on a MiSeq (Illumina) using MiSeq Reagent Kit V2 (Illumina) at 500 cycles, as previously described (10). We conducted a taxonomic analysis of raw FASTQ files using Kraken version 1 and MiniKraken DB (<https://ccb.jhu.edu/software/kraken>), which showed 15,582 kobuvirus sequence reads in addition to sequences for *E. coli*, coronavirus, and rotavirus. We assembled the complete genome of BKV IL35164 (GenBank accession no. MN336260) with a genome size of 8,337 nt. We used BLAST (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) to search the IL35164 genome and found it is closely related to and shares 89%–91% identities with 4 BKV strains, U-1, EGY-1, SC1, and CHZ. It shares only 77%–82% identity with sheep and ferret kobuviruses.

Sequence analysis showed that IL35164 has a similar genome organization to other BKVs (Figure, panel A) and a 7,392-nt open reading frame encoding 2,463 amino acids, the same length as U-1, EGY-1, and SC1 strains. IL35164 shares polyprotein identities with 4 other BKV strains, 88.5%–90.9% identity in the nucleotide level and 94.9%–96.7% identity in the amino acid level (Appendix Table 1, <http://wwwnc.cdc.gov/EID/article/26/1/19-1227-App1.pdf>). Comparing individual proteins from 4 other BKVs, IL35164 shared only 80.9%–86.8% nucleotide identity with the leader protein and shares its highest identity, 95.5%–98.8%, with the 3B nucleotide in those strains (Appendix Table 1). In addition to 3B, IL35164 shows higher nucleotide identities, 92.6%–

95.4%, to other BKVs in 3D, encoding a viral RNA-dependent RNA polymerase.

Phylogenetic analysis of the complete genome confirmed that IL35164 correlates with 4 BKVs in the *Aichivirus B* species cluster (Figure, panel B). On the phylogenetic tree of complete VP1 nucleotide sequences, IL35164 clusters with 2 BKV strains from Brazil, BRA1991 and BRA2016, rather than BKVs U-1, EGY-1, SC1, and CHZ (Appendix Figure 1). The relatedness of IL35164 to BRA1991 and BRA2016 in other parts of the genome is unclear because complete genomes of the strains from Brazil are unavailable. IL35164 is related distinctly to U-1, EGY-1, SC1, and CHZ on the phylogenetic tree of partial 3D (Appendix Figure 2).

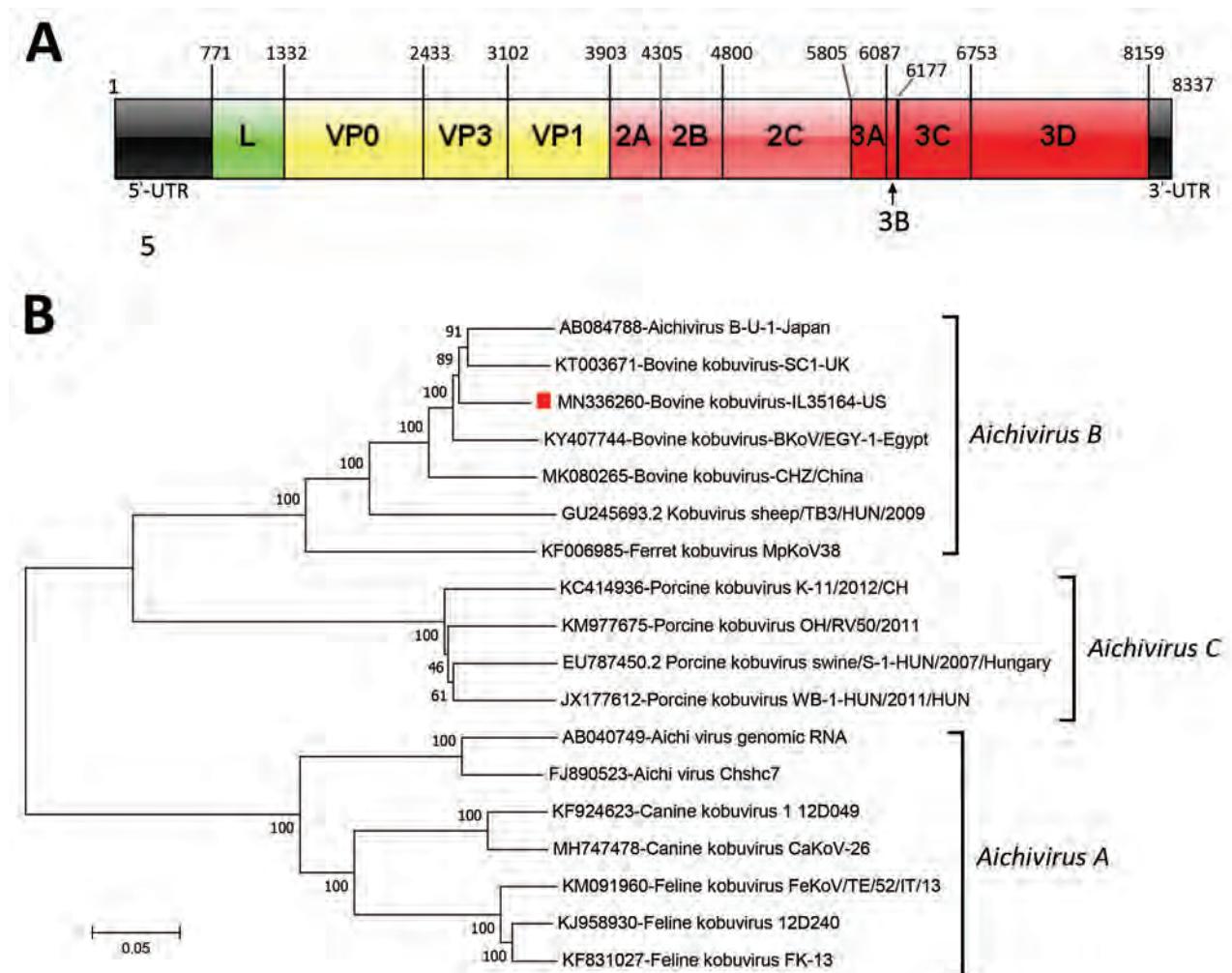


Figure. Genome organization and phylogenetic tree of bovine kobuvirus IL35164 isolated from cattle, United States. A) Genome organization with each gene's initial nucleotide position labeled. The 5' UTR is located in positions 1–770 and the 3' UTR is located in positions 8160–8337. B) Phylogenetic tree of complete genomes of 3 *Aichivirus* species, A, B, and C. The dendrogram was constructed by using the neighbor-joining method in MEGA version 7.0.26 (<http://www.megasoftware.net>). Bootstrap resampling of 1,000 replications was performed and bootstrap values are indicated for each node. Red square indicates bovine kobuvirus IL35164 identified in this study. Scale bar indicates nucleotide substitutions per site. UTR, untranslated region; VP, viral protein.

To further screen BKV in bovine samples, we designed primers and probes (sequences available upon request) targeting 3D to test 9 additional intestinal samples from necropsied calves. Real-time reverse transcription PCR showed 4/9 samples were positive for BKV by cycle thresholds of 23.0 (case IL35146), 29.97 (case IL37122), 32.84 (case IL50179), and 33.61 (case IL34890) but were negative for coronavirus, rotavirus, and bovine viral diarrhea virus (Appendix Table 2). Histopathologic observation of small intestines revealed that 2 cases with diarrhea, IL35146 and IL50179, had necrotizing enteritis with villus atrophy and fusion, suggesting a primary viral infection (Appendix Figure 3). Two other calves without clinically evident diarrhea died, case IL37122 from jejuno-ileal volvulus and case IL34890 from abomasal rupture; both also were positive for BKV.

Among 3 BKV-positive calves with diarrhea, 2 were <1 month of age and 1 was ≈5 months of age. Previous studies reported high prevalence of BKV infection in young calves with diarrhea; 20.9% (38/182) in calves ≤2 months of age in Brazil and 26.7% (23/86) in calves ≤1 month of age in South Korea (5,9). Our study further supports the hypothesis that BKV causes neonatal diarrhea in calves. In addition, BKV also can be detected from cattle without diarrhea or clinical signs of the virus (1,8).

Since initial identification in 2003 (1), BKV has been detected in cattle from several countries, but only from fecal samples; no natural or experimental studies have reported its pathogenesis. Our histologic examination of necropsied cases clearly indicated viral infection, and only BKV was detected, suggesting BKV was the causative agent for diarrhea. Future studies, including virus isolation and virus challenge to calves, are needed to determine whether BKV fulfills the Koch's postulates as a causative agent for diarrhea in calves.

The prevalence of BKV in the United States remains unknown. Continued surveillance is urgently needed to determine rates and distribution of BKV in North America. Although many partial sequences of 3D and viral protein 1 are available at GenBank, only 4 complete sequences are available, limiting evaluation of BKV. Whole-genome sequencing of both previously and newly discovered BKV isolates is needed to analyze genetic diversity and evolution.

About the Author

Dr. Wang is a clinical assistant professor in the College of Veterinary Medicine at the University of Illinois. His research interests focus on diagnosis of viral infectious diseases and novel pathogen discovery.

References

1. Yamashita T, Ito M, Kabashima Y, Tsuzuki H, Fujiura A, Sakae K. Isolation and characterization of a new species of kobuvirus associated with cattle. *J Gen Virol*. 2003;84:3069–77. <https://doi.org/10.1099/vir.0.19266-0>
2. Barry AF, Ribeiro J, Alfieri AF, van der Poel WH, Alfieri AA. First detection of kobuvirus in farm animals in Brazil and the Netherlands. *Infect Genet Evol*. 2011;11:1811–4. <https://doi.org/10.1016/j.meegid.2011.06.020>
3. Chang J, Wang Q, Wang F, Jiang Z, Liu Y, Yu L. Prevalence and genetic diversity of bovine kobuvirus in China. *Arch Virol*. 2014;159:1505–10. <https://doi.org/10.1007/s00705-013-1961-7>
4. Di Martino B, Di Profio F, Di Felice E, Ceci C, Pistilli MG, Marsilio F. Molecular detection of bovine kobuviruses in Italy. *Arch Virol*. 2012;157:2393–6. <https://doi.org/10.1007/s00705-012-1439-z>
5. Jeoung HY, Lim JA, Jeong W, Oem JK, An DJ. Three clusters of bovine kobuvirus isolated in Korea, 2008–2010. *Virus Genes*. 2011;42:402–6. <https://doi.org/10.1007/s11262-011-0593-9>
6. Khamrin P, Maneekarn N, Peerakome S, Okitsu S, Mizuguchi M, Ushijima H. Bovine kobuviruses from cattle with diarrhea. *Emerg Infect Dis*. 2008;14:985–6. <https://doi.org/10.3201/eid1406.070784>
7. Mohamed FF, Mansour SMG, Orabi A, El-Araby IE, Ng TFF, Mor SK, et al. Detection and genetic characterization of bovine kobuvirus from calves in Egypt. *Arch Virol*. 2018;163:1439–47. <https://doi.org/10.1007/s00705-018-3758-1>
8. Reuter G, Egyed L. Bovine kobuvirus in Europe. *Emerg Infect Dis*. 2009;15:822–3. <https://doi.org/10.3201/eid1505.081427>
9. Ribeiro J, Lorenzetti E, Alfieri AF, Alfieri AA. Kobuvirus (*Aichivirus B*) infection in Brazilian cattle herds. *Vet Res Commun*. 2014;38:177–82. <https://doi.org/10.1007/s11259-014-9600-7>
10. Wang L, Stuber T, Camp P, Robbe-Austerman S, Zhang Y. Whole-genome sequencing of porcine epidemic diarrhea virus by Illumina MiSeq Platform. In: Wang L, editor. *Animal coronaviruses*. Totowa (NJ): Humana Press; 2016. p. 201–8.

Address for correspondence: Leyi Wang, University of Illinois College of Veterinary Medicine, Department of Veterinary Clinical Medicine and the Veterinary Diagnostic Laboratory, 2001 S Lincoln Ave, Urbana, IL 61802, USA; email: leyiwang@illinois.edu

Imported Schistosomiasis, China, 2010–2018

Si-Min Dai, Zhou Guan, Li-Juan Zhang, Shan Lv, Chun-Li Cao, Shi-Zhu Li, Jing Xu

Author affiliations: National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, China; World Health Organization Collaborating Centre for Tropical Diseases, Shanghai; National Tropical Disease Research Center, Shanghai

DOI: <https://doi.org/10.3201/eid2601.191250>

China has made remarkable progress in reducing schistosomiasis caused by *Schistosoma japonicum* over the past 7 decades but now faces a severe threat from imported schistosomiasis. Results from national surveillance during 2010–2018 indicate integrating active surveillance into current surveillance models for imported cases is urgently needed to achieve schistosomiasis elimination in China.

Six species of *Schistosoma* trematodes are known to infect humans, but only *S. japonicum* is prevalent in China (1,2). However, because of enhanced international cooperation and communication, 2 *Schistosoma* species were identified from schistosomiasis cases imported to China: *S. haematobium* and *S. mansoni*. In addition, the *Biomphalaria straminea* snail, an intermediate host of *S. mansoni* trematodes, has been introduced into Shenzhen, Guangdong Province (3,4), so the possibility exists that imported *S. mansoni*

trematodes could be transmitted to these snails and result in locally acquired human infections. Therefore, we analyzed imported schistosomiasis status in China through data collected from the National Notifiable Disease Report System (NNDRS) during 2010–2018 to provide information for national policy making on schistosomiasis elimination.

We defined imported schistosomiasis as schistosomiasis in an endemic focus outside mainland China. A confirmed imported case had to meet both of the following diagnostic criteria: the patient was given a diagnosis of schistosomiasis, meaning that schistosome eggs or miracidia were detected in the patient's biological samples; the patient once traveled to and/or worked in schistosomiasis-endemic areas in other countries during transmission seasons (Table).

Twenty-two cases were reported during the study period. All imported cases occurred in men; their ages ranged from 30 to 68 years (mean 48 ± SD 9.98 years). Nineteen (86%) were 30–58 years of age, and 3 (14%) were >58 years of age. Half of them were workers who returned to China from Africa; 4 (18%) were businessmen. No case was reported in Guangdong Province; 4 cases were reported in the neighboring provinces of Guangxi, Jiangxi, and Fujian.

The 22 cases acquired *Schistosoma* infection in 8 countries in Africa. Of the 20 case-patients for whom detailed data were available, most were infected in central Africa (Angola, 6 [30%] cases) and Cameroon and Congo (1 [5%] case each). In West Africa, Nigeria accounted for 6 (30%) cases and Mali for 1 (5%) case; in southern Africa, Zambia accounted for 4 (20%) cases.

Table. Imported schistosomiasis, mainland China, 2010–2018*

Diagnosis year	Reported province	Patient age, y	Source country	Activity/occupation	Parasite
2018	Jiangsu	45	Sudan	Businessman	<i>Schistosoma haematobium</i>
2018	Jiangsu	60	Sudan	Worker	<i>S. haematobium</i>
2018	Jiangsu	55	Angola	Worker	<i>S. haematobium</i>
2018	Jiangsu	49	Angola	Worker	<i>S. haematobium</i>
2016	Beijing	34	Uganda	Civil servant	<i>S. mansoni</i>
2016	Zhejiang	58	Nigeria	Farmer	<i>S. haematobium</i>
2016	Zhejiang	56	Nigeria	Farmer	<i>S. haematobium</i>
2016	Fujian	35	Angola	Housework	<i>S. haematobium</i>
2016	Jiangxi	51	Zambia	Worker	<i>S. haematobium</i>
2016	Sichuan	40	Unknown	Businessman	<i>S. haematobium</i>
2015	Beijing	68	Cameroon	Retiree	Unknown
2015	Beijing	35	Nigeria	Businessman	<i>S. mansoni</i>
2015	Zhejiang	57	Nigeria	Worker	<i>S. haematobium</i>
2015	Guangxi	45	Angola	Seafarer	<i>S. haematobium</i>
2014	Shandong	43	Angola	Farmer	<i>S. haematobium</i>
2012	Fujian	30	Unknown	Businessman	<i>S. haematobium</i>
2012	Shanxi	46	Mali	Worker	Unknown
2012	Shanxi	60	Angola	Worker	Unknown
2010	Shanxi	56	Zambia	Worker	Unknown
2010	Shanxi	55	Zambia	Worker	Unknown
2010	Shanxi	39	Zambia	Worker	Unknown
2010	Shanxi	42	Congo	Worker	Unknown

More than 1 million persons from China are estimated to work in Africa (5). However, during the study period, only 22 confirmed cases were recognized. Considering the high risk for infection in Africa and the existing passive surveillance system, the number of imported schistosomiasis cases might be substantially underestimated.

As a national schistosomiasis surveillance system in China, NNDRS mirrors the local and imported case status passively reported on a national scale: when patients exposed in Africa had symptoms after returning to China, they sought treatment at hospitals on their own. If schistosomiasis was confirmed, the physician reported it to NNDRS, the only case report source in the existing surveillance system. However, this surveillance model has limitations. For example, cases might be misdiagnosed and missed because medical staff have insufficient clinical awareness of African schistosomiasis or because some patients may be asymptomatic or have mild symptoms.

Another challenge is that global integration has contributed to an increasing number of persons from China who travel abroad, and most persons who travel to Africa are not equipped with essential knowledge about schistosomiasis prevention and control. In addition, introduction of the *B. straminea* snail into southern China increases the risk that persons in China might acquire *S. mansoni* infection.

Because imported schistosomiasis may increase the disease burden in China and hinder the process of schistosomiasis elimination, several measures are urgently needed. First, a well-rounded active surveillance system needs to be integrated into the current monitoring model to ensure an accurate and quick response. When performing active surveillance through screening, sensitive and rapid immunologic tests should be widely conducted among the most susceptible group before etiologic examinations are conducted. Our results suggest that male workers returning from Africa are the most susceptible population. Meanwhile, because the intermediate host of African schistosomiasis, the *B. straminea* snail, has been introduced to Guangdong Province in southern China, persons returning from *S. mansoni*-endemic countries or regions. Guangdong Province and adjacent provinces should be a primary target population for surveillance. Identifying migrant workers as the highest priority group for active surveillance will be

of great value. Second, China should collaborate with the World Health Organization and other relevant international institutions to evaluate the feasibility of existing serologic detection kits developed by China for diagnosis of imported schistosomiasis or develop new kits if necessary. Third, training programs on standardized clinical diagnostic criteria and normative case reporting operation are required in different types of healthcare organizations (e.g., hospitals, Centers for Disease Control and Prevention, entry and exit border stations) in mainland China to ensure accurate diagnosis and disease treatment. Other measures, such as multisector cooperation and coordination, could help achieve elimination of schistosomiasis in China.

This study was financially supported by the National Science and Technology Major Project (no. 2018ZX10101002-002, 2016YFC1202000).

About the Author

Miss Dai is a junior researcher in the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention, Shanghai, China. Her research interests include tropical diseases and epidemiology.

References

1. Song LG, Wu XY, Sacko M, Wu ZD. History of schistosomiasis epidemiology, current status, and challenges in China: on the road to schistosomiasis elimination. *Parasitol Res.* 2016;115:4071–81. <https://doi.org/10.1007/s00436-016-5253-5>
2. McManus DP, Dunne DW, Sacko M, Utzinger J, Vennervald BJ, Zhou XN. Schistosomiasis. *Nat Rev Dis Primers.* 2018;4:13. <https://doi.org/10.1038/s41572-018-0013-8>
3. Meier-Brook C. A snail intermediate host of *Schistosoma mansoni* introduced into Hong Kong. *Bull World Health Organ.* 1974;51:661.
4. Habib MR, Lv S, Guo YH, Gu WB, Standley CJ, Caldeira RL, et al. Morphological and molecular characterization of invasive *Biomphalaria straminea* in southern China. *Infect Dis Poverty.* 2018;7:120. <https://doi.org/10.1186/s40249-018-0505-5>
5. Wang ZQ, Wang Y, Jia LJ, Cui J. *Schistosoma haematobium* infection in workers returning from Africa to China. *J Travel Med.* 2013;20:256–8. <https://doi.org/10.1111/jtm.12032>

Address for correspondence: Jing Xu, Chinese Center for Disease Control and Prevention, National Institute of Parasitic Diseases; WHO Collaborating Centre for Tropical Diseases, National Tropical Disease Research Center, 207 Rui Jin Rd, Shanghai 200025, China; email: xfmjing@163.com

Rare Case of Enteric *Ancylostoma caninum* Hookworm Infection, South Korea

Bong-Kwang Jung, Jung-Yeop Lee, Taehee Chang, Hyemi Song, Jong-Yil Chai

Author affiliations: Korea Association of Health Promotion, Seoul, South Korea (B.-K. Jung, T. Chang, H. Song, J.-Y. Chai); Korea Association of Health Promotion, Jeonju, South Korea (J.-Y. Lee); Seoul National University College of Medicine, Seoul (J.-Y. Chai)

DOI: <https://doi.org/10.3201/eid2601.191335>

A 60-year-old man from South Korea underwent a colonoscopy. A juvenile female worm showing 3 pairs of teeth in the buccal cavity was recovered from the descending colon. Partial sequencing of the internal transcribed spacer region showed 100% identity with *Ancylostoma caninum*, the dog hookworm.

Ancylostoma duodenale and *Necator americanus* are the 2 major hookworm species causing human enteric infections around the world. However, *Ancylostoma ceylanicum*, which infects mainly dogs and cats, has become an emerging hookworm species causing human enteric infections in Southeast Asia and the Pacific Islands (1,2). Another hookworm species, *Ancylostoma caninum*, which infects dogs and rarely cats, can also infect humans, possibly as cutaneous larva migrans (3) or enteric infections in association with eosinophilic enteritis (4–6). Human enteric *A. caninum* infection has been known mainly in Australia (4,5). However, 2 sporadic human cases (serologically positive but not based on recovery of worms) were reported in the United States (6), and samples from 3 schoolchildren in South Africa were detected molecularly to be egg positive (7).

We recently encountered a case of *A. caninum* infection in a 60-year-old man who visited a health checkup center and demonstrated a moderate degree of eosinophilia. During colonoscopy, a nematode parasite (female) was found and removed with forceps. The worm was then morphologically and molecularly confirmed to be a dog hookworm, *A. caninum*.

The patient had no subjective symptoms such as abdominal pain, diarrhea, dark feces, or weight loss. However, laboratory examinations showed some abnormalities in liver functions and blood values: total bilirubin 2.11 mg/dL (reference range 0.2–1.57 mg/dL), gamma-glutamyltransferase 238 IU/L (reference range 0–56 IU/L), total serum IgE 1,265 IU/mL

(reference range 0–87 IU/mL), and peripheral blood eosinophilia 7.5% (reference range 0%–0.45%). Other blood results were within normal limits: hemoglobin 14.6 g/dL, lymphocytes 26.6%, monocytes 8.0%, basophils 0.9%, and neutrophils 57.0%. The patient owned a dog for 5 years and had regular contact with the dog but had never traveled abroad.

Colonoscopy showed a single threadlike moving worm at the descending colon (Figure, panels A, B). Under magnifying endoscopy, the worm looked like a nematode hooking its head into the colonic mucosa. We removed the worm and sent it to the laboratory. The worm was cleared in lactophenol before microscopic examinations and was found to be a juvenile female hookworm, 12 mm in length and 0.36 mm in width, morphologically identified as *A. caninum* based on the presence of its characteristic 3 pairs of teeth in the buccal cavity (Figure, panel C). We treated the patient with a 3-day course of albendazole.

After morphologic examinations, we cut the middle part of the nematode and preserved it in 70% ethanol for molecular studies. We isolated genomic DNA from the worm segment using the DNeasy Blood and Tissue Kit (QIAGEN, <https://www.qiagen.com>), according to the manufacturer's instructions. We partially amplified the internal transcribed spacer 1 and 5.8S rDNA regions (312 bp) using the standard PCR protocol with 2x MasterMix (MGmed, <https://www.mgmed.com>) and 10 pmol of forward and reverse primers to detect *Ancylostoma* species (8). The PCR product obtained was directly sequenced at Macrogen Inc. (Seoul, South Korea). The results revealed 100% homology with the sequences of *A. caninum* deposited in GenBank (accession no. MG589635) (Figure, panel D). Our sample also revealed 98.1% homology with *A. duodenale*, 97.1% with *A. ceylanicum*, and 92.4% with *Ancylostoma braziliense*.

Human enteric *A. caninum* infection is extremely rare except in northeastern semitropical regions of Queensland, Australia, where several hundred cases in the form of eosinophilic enteritis have been detected (5). Most of these human cases came from a typical suburban environment; in Townsville district, Queensland, 50% of the population have a dog at home, and 69% of adults regularly engage in high-risk behavior, such as walking barefoot on damp grass frequented by dogs (5). Human infections with infective-stage larvae appear to occur through a cutaneous route (5) or ingestion of infective larvae (9).

Signs of human infections include ambiguous abdominal pain that may or may not be associated with eosinophilia (5). This patient had no remarkable subjective symptoms except for circulating eosinophilia

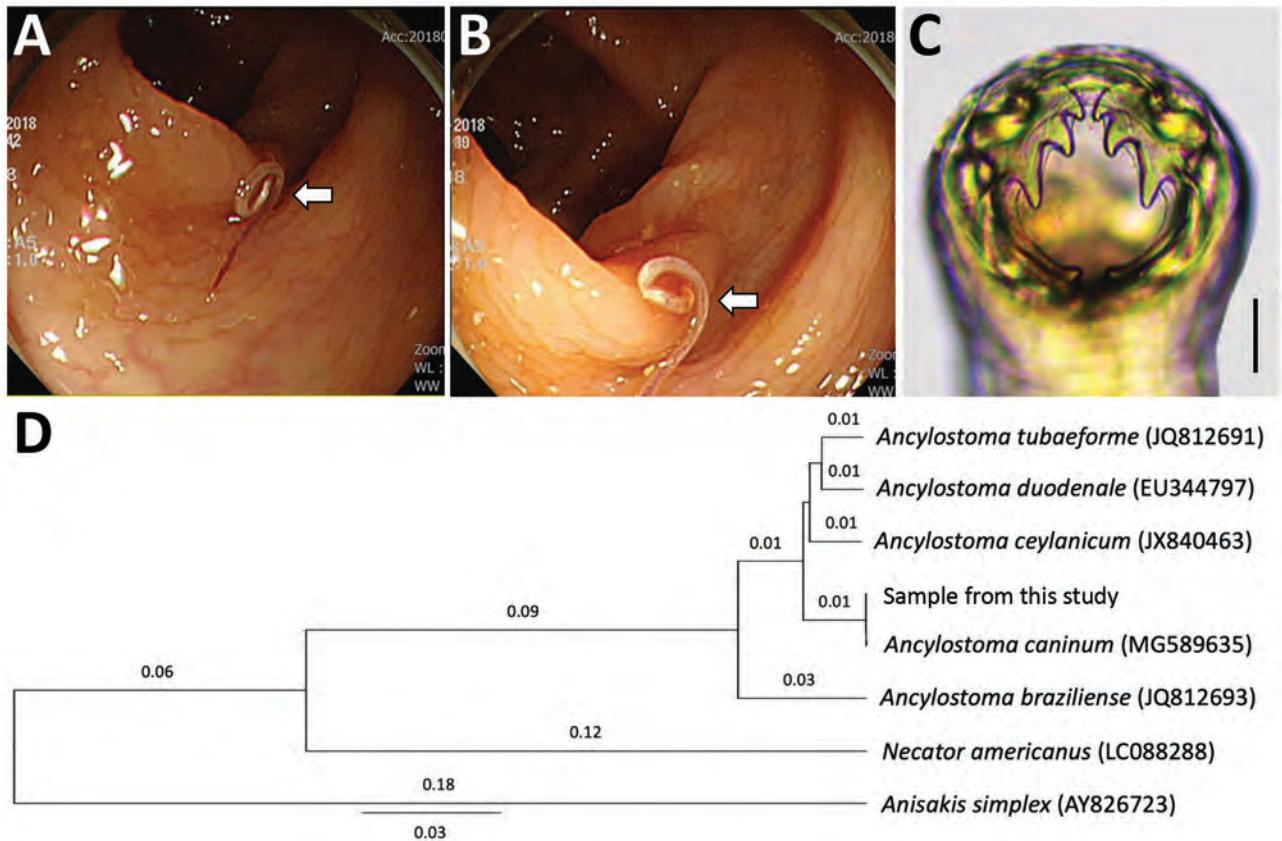


Figure. Analysis of a worm from a 60-year-old man in South Korea that was identified as *Ancylostoma caninum*, the dog hookworm. A, B) Colonoscopy images showing a moving threadlike nematode in the mucosa of the descending colon. This nematode (arrows) is seen hooking its head into the colonic mucosa. C) The head part of the worm, showing its characteristic morphology of 3 pairs of teeth in the buccal cavity, by which it could be morphologically identified as *A. caninum*. Scale bar indicates 0.1 mm. D) Phylogenetic tree based on nucleotide sequences of the internal transcribed spacer 1 region and 5.8S rDNA of the worm from this study in comparison with various hookworm species deposited in GenBank (accession numbers indicated in parentheses), inferred by using the UPGMA method. MEGA-X (<https://www.megasoftware.net>) was used as the software. Scale bar indicates evolutionary distance.

and markedly elevated total serum IgE. Some abnormalities were noted in liver functions, including slightly elevated total bilirubin and markedly elevated gamma-glutamyltransferase levels. However, these findings seem to have little meaning in relation to *A. caninum* infection.

The worm from this case was a juvenile female that did not contain eggs in its uterus. Thus, our result agrees with previous reports from Australia (4,5) that these worms do not mature sufficiently to cause infection; they survive probably for only a few weeks. However, recent studies in South Africa (7) and India (10) detected hookworm eggs in human feces; these were molecularly confirmed to be *A. caninum*. This finding apparently showed that *A. caninum* hookworms can fully develop into egg-producing adults within the human intestine, although occurrence may be rare.

Our study is epidemiologically noteworthy because human enteric *A. caninum* infection may also

occur in countries in Asia where this hookworm species is common among dogs, as well as in Australia, the United States, India, and South Africa. Even symptomatic infections with *A. caninum* hookworms may often be missed or misdiagnosed as other conditions or diseases.

Acknowledgments

We thank the staff in Jeonbuk Branch of Korea Association of Health Promotion, Seoul, South Korea, who helped with the management of this patient.

About the Author

Dr. Jung is a senior researcher at the Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul, South Korea. His primary research interest is parasite fauna study. He also studies toxoplasmosis of the central nervous system in humans and animals.

References

- Hsu YC, Lin JT. Images in clinical medicine: intestinal infestation with *Ancylostoma ceylanicum*. N Engl J Med. 2012;366:e20. <https://doi.org/10.1056/NEJMicm1101717>
- Bradbury RS, Hii SF, Harrington H, Speare R, Traub R. *Ancylostoma ceylanicum* hookworm in the Solomon Islands. Emerg Infect Dis. 2017;23:252-7. <https://doi.org/10.3201/eid2302.160822>
- Liu Y, Zheng G, Alsarakibi M, Zhang X, Hu W, Lu P, et al. Molecular identification of *Ancylostoma caninum* isolated from cats in southern China based on complete ITS sequence. BioMed Res Int. 2013;2013:868050. <https://doi.org/10.1155/2013/868050>
- Prociv P, Croese J. Human eosinophilic enteritis caused by dog hookworm *Ancylostoma caninum*. Lancet. 1990;335:1299-302. [https://doi.org/10.1016/0140-6736\(90\)91186-E](https://doi.org/10.1016/0140-6736(90)91186-E)
- Prociv P, Croese J. Human enteric infection with *Ancylostoma caninum*: hookworms reappraised in the light of a "new" zoonosis. Acta Trop. 1996;62:23-44. [https://doi.org/10.1016/S0001-706X\(96\)00016-2](https://doi.org/10.1016/S0001-706X(96)00016-2)
- Khoshoo V, Craver R, Loukas A, Prociv P, Schantz P. Abdominal pain, pan-gut eosinophilia, and dog hookworm infection. J Pediatr Gastroenterol Nutr. 1995;21:481-3. <https://doi.org/10.1097/00005176-199511000-00022>
- Ngcamphalala PI, Lamb J, Mukaratirwa S. Molecular identification of hookworm isolates from stray dogs, humans and selected wildlife from South Africa. J Helminthol. 2019:e39. <https://doi.org/10.1017/S0022149X19000130>
- Traub RJ, Robertson ID, Irwin P, Mencke N, Thompson RC. Application of a species-specific PCR-RFLP to identify *Ancylostoma* eggs directly from canine faeces. Vet Parasitol. 2004;123:245-55. <https://doi.org/10.1016/j.vetpar.2004.05.026>
- Landmann JK, Prociv P. Experimental human infection with the dog hookworm, *Ancylostoma caninum*. Med J Aust. 2003;178:69-71. <https://doi.org/10.5694/j.1326-5377.2003.tb05066.x>
- George S, Levecke B, Kattula D, Velusamy V, Roy S, Geldhof P, et al. Molecular identification of hookworm isolates in humans, dogs and soil in a tribal area in Tamil Nadu, India. PLoS Negl Trop Dis. 2016;10:e0004891. <https://doi.org/10.1371/journal.pntd.0004891>

Address for correspondence: Jong-Yil Chai, Korea Association of Health Promotion, Institute of Parasitic Diseases, 333 Hwagok-ro, Gangseo-ku, Seoul 07649, South Korea; e-mail: cjy@snu.ac.kr

etymologia revisited

Angiostrongylus [an"je-o-stron'jĭ-ləs]

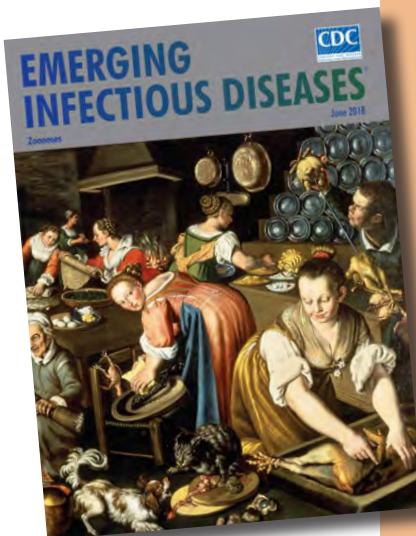
From the Greek *angeion* ("vessel") + *strongylos* ("round"), *Angiostrongylus* is a genus of parasitic nematodes (roundworms) in the family *Angiostrongylidae*, 2 species of which are known to parasitize humans. *A. cantonensis* (commonly known as rat lungworm) was first described in 1935 (as *Pulmonema cantonensis*) from rats in Canton, China. It is the most common cause of eosinophilic meningitis in Asia and the Pacific Basin, but cases have been reported in many parts of the world. *A. costaricensis* roundworms were first described in 1971 in Costa Rica from surgical specimens from children with eosinophilic infiltration in the abdominal cavity. The distribution of this species ranges from the southern United States to northern Argentina.

There is still debate about what taxonomic name should be used. *A. cantonensis* remains in general use, but some researchers suggest it should be changed to *Parastrongylus cantonensis* on the basis of the morphology of the adult male bursa and the definitive host being rats.

Sources: see web link for more information.



Figure. Adult *Angiostrongylus cantonensis* nematode recovered from rat lung.



Originally published
in June 2018

https://wwwnc.cdc.gov/eid/article/24/6/et-2406_article

Vaccine Effectiveness against DS-1-Like Rotavirus Strains

Toyoko Nakagomi¹

Author affiliation: Retired from Nagasaki University, Nagasaki, Japan

DOI: <https://doi.org/10.3201/eid2601.191377>

To the Editor: Global emergence of reassortant rotavirus A (RVA) strains possessing the DS-1 backbone genes, such as DS-1-like G1P[8] strains (1–3), raises 2 key questions of public health importance. First, is the G1P[8] monovalent vaccine (RV1) effective against the DS-1-like G1P[8] strain? Second, does this strain cause more severe disease than the Wa-like G1P[8] strain?

For the first question, Jere et al. (1) showed that RV1 was highly effective against DS-1-like G1P[8] RVAs (vaccine effectiveness [VE] 85.6% [95% CI 34.4%–96.8%]). For the second question, we conducted a study in Hanoi, Vietnam, during the 2012–2013 rotavirus season in which 20 DS-1-like G1P[8] RVAs co-circulated with 49 Wa-like G1P[8] RVAs and 50 G2P[4] RVAs among children <2 years of age who had diarrhea. We found no evidence of increased virulence of DS-1-like G1P[8] strains as measured by Vesikari's severity scores (3).

However, Jere et al. (1) were unable to demonstrate statistically significant VE against DS-1-like G2P[4] or Wa-like G1P[8] strains and were concerned about the low point estimate of the VE against heterotypic G2P[4] strains. Using the previous dataset (3), we conducted a post hoc logistic regression analysis to compare any RV1 vaccination status versus 0-dose RV1 vaccination status between strain-specific rotavi-

rus diarrhea case-patients and 127 acute respiratory infection control patients whose RV1 coverage was 49.6%. We calculated VE against DS-1-like G1P[8] as 72.5% (95% CI 10.8%–91.5%; $p = 0.045$), VE against Wa-like G1P[8] as 90.8% (95% CI 72.9%–96.9%), and VE against G2P[4] as 78.1% (95% CI 49.1%–90.6%). These findings confirm the effectiveness of RV1 against fully heterotypic G2P[4] strains, as shown elsewhere (4,5), and supplement the study by Jere et al. (1) in helping dismiss concern about continued use of the monovalent vaccine, even in places where RVAs with the DS-1 backbone are not uncommon.

References

1. Jere KC, Bar-Zeev N, Chande A, Bennett A, Pollock L, Sanchez-Lopez PF, et al. Vaccine effectiveness against DS-1-like rotavirus strains in infants with acute gastroenteritis, Malawi, 2013–2015. *Emerg Infect Dis*. 2019;25:1734–7. <https://doi.org/10.3201/eid2509.190258>
2. Fujii Y, Nakagomi T, Nishimura N, Noguchi A, Miura S, Ito H, et al. Spread and predominance in Japan of novel G1P[8] double-reassortant rotavirus strains possessing a DS-1-like genotype constellation typical of G2P[4] strains. *Infect Genet Evol*. 2014;28:426–33. <https://doi.org/10.1016/j.meegid.2014.08.001>
3. Nakagomi T, Nguyen MQ, Gauchan P, Agbemabiese CA, Kaneko M, Do LP, et al. Evolution of DS-1-like G1P[8] double-gene reassortant rotavirus A strains causing gastroenteritis in children in Vietnam in 2012/2013. *Arch Virol*. 2017;162:739–48. <https://doi.org/10.1007/s00705-016-3155-6>
4. Correia JB, Patel MM, Nakagomi O, Montenegro FM, Germano EM, Correia NB, et al. Effectiveness of monovalent rotavirus vaccine (Rotarix) against severe diarrhea caused by serotypically unrelated G2P[4] strains in Brazil. *J Infect Dis*. 2010;201:363–9. <https://doi.org/10.1086/649843>
5. Braeckman T, Van Herck K, Meyer N, Pirçon JY, Soriano-Gabarró M, Heylen E, et al.; RotaBel Study Group. Effectiveness of rotavirus vaccination in prevention of hospital admissions for rotavirus gastroenteritis among young children in Belgium: case-control study. *BMJ*. 2012;345:e4752. <https://doi.org/10.1136/bmj.e4752>

Address for correspondence: Toyoko Nakagomi, 14-1-604, Ohashi, Nagasaki 852-8134, Japan; email: tnakagom@gmail.com

¹Retired.

In Memoriam: Jay Stephen Keystone (1943–2019)

David O. Freedman

After a year-long struggle with cancer, Jay S. Keystone, CM, MD, MSc (TM London), died at 76 years of age on September 3, 2019, surrounded by his devoted wife Margaret and his 5 beloved children. He talked about his children constantly, even in academic presentations in front of thousands of strangers. Jay always said, “Humor is an important tool in the practice of medicine. In teaching, I use it to engage the learner; in practice, it creates a relationship between me and the patient, which levels the playing field and puts them at ease.” To clinical colleagues, he was the go-to clinician for tropical medicine cases; to trainees, he was the master professor to whom those from all over Canada came to work and learn; and to audiences worldwide, he was a marquee attraction for his legendary humor and wit (1). His clever political incorrectness never failed to augment the delivery of the key points on tropical and emerging exotic diseases.

Jay’s patients worshipped him for his diagnostic acumen and ability to listen. Jay was always in a good mood; full of life; and a caring, warm, and compassionate teacher, mentor, physician, and research collaborator.

As one of Jay’s earliest mentees and professional friends, I appreciated this visionary as a first-year medical student at the University of Toronto in 1977. He was in his first year as director of the Tropical Disease Unit after returning from the London School of Hygiene and Tropical Medicine and fieldwork in Africa and South America. He was clearly on track to lead the divergence of travel medicine from tropical medicine. Air travel was changing that. His famous teaching slide was a response to the question, “What is the most dangerous vector in the world?” The slide answered, “*Anopheles Air canadensis*” and showed the appropriately branded 747 in midflight. He was among the first to recognize that the rapid movement of infected and potentially infectious persons away from the point of infection acquisition would require

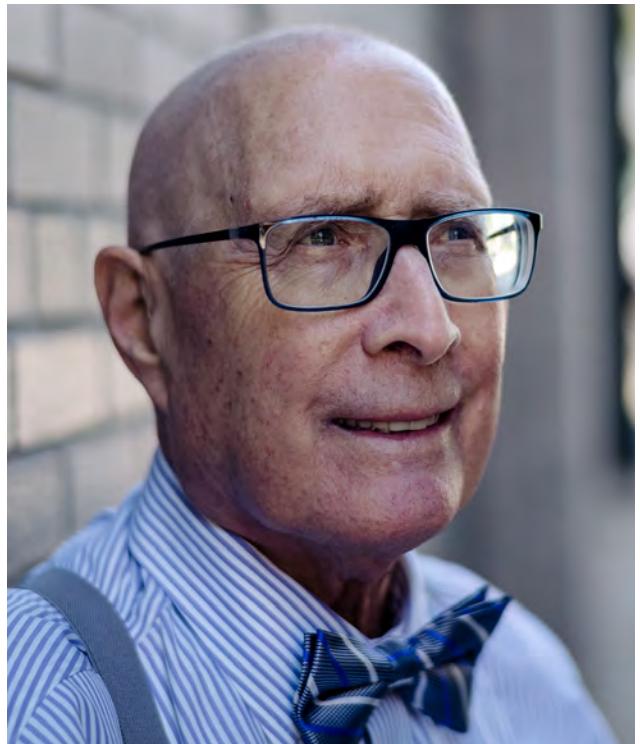


Figure. Jay S. Keystone

Keystone-level clinical insights to differentiate the usual tropical diseases from the novel. He imparted to his large cadre of trainees, mentees, and collaborators the use of people skills to forge the links with the laboratory and public health colleagues necessary to complete these puzzles.

Jay authored approximately 200 publications with more than 5,000 citations, remarkable considering the relative obscurity of some of the diseases investigated. His most cited papers were collaborative across multiple disciplines, and none would have been possible without Jay’s acumen at sorting the clinically relevant from the irrelevant. In 1984, working with Canadian surgeon Bernie Langer, Jay published an often-cited case series on the surgical approach to hepatic hydatid disease in immigrants from Greece and Italy to Canada, which forms the basis for our current approaches and understanding (2). In 1998, working with Kevin Kain, Jay reported the problems encountered when

Author affiliation: University of Alabama at Birmingham, Birmingham, Alabama, USA

DOI: <https://doi.org/10.3201/eid2601.191500>

diagnosing and managing imported malaria, which codified issues we still struggle with today (3). A 2004 paper articulated the difficulties of dealing with infection in immigrants and their descendants returning home to visit friends and relatives (4). Jay's legacy includes a superb cadre of clinicians in Toronto, poised in prescient preparation for the next emergence event. Past sentinel events include outbreaks of travelers' leptospirosis, severe acute respiratory syndrome, Zika, and drug-resistant malaria.

Jay's vision was instrumental in founding the GeoSentinel Surveillance Network in 1995; while president of the then-fledgling International Society of Travel Medicine, Jay committed a significant portion of the Society's assets to a \$50,000 seed grant to found GeoSentinel. Returning travelers are often sentinels of emerging infectious diseases. GeoSentinel continues to be the largest available database of travel-related illness. With the initial efforts by Phyllis Kozarsky, Hans Lobel, Marty Cetron, and me, and with Jay's stature behind us, the eventual results were the continuous Centers for Disease Control and Prevention funding of the network to this day. Jay co-authored 2 of the landmark GeoSentinel papers (5,6).

Of Jay's many honors, he was most proud of a few. They were the Cody gold medal for standing first in his class through all 4 years of medical school in Toronto, serving as president of the clinical group of the American Society of Tropical Medicine and Hygiene, and receiving the 2008 American Society of Tropical Medicine and Hygiene Ben Kean medal for excellence in teaching and mentoring. In 2015, he was made a Member of the Order of Canada, one of the highest civilian honors in the country, for his outstanding contributions as a pioneer of travel and tropical medicine in Canada.

Jay continued teaching and seeing his patients until a few weeks before his death. He worried about

who would follow his cohort of delusional parasitosis patients, a group that few but Jay would listen to and treat with skill and compassion.

Jay taught me to never take my work overly seriously yet to thrive by enjoying the meaning and relevance of contributions to the health of patients and of the increasingly mobile public. Jay's life was remarkable not only for his accomplishments and the inspiration that he engendered in others but also for his self-deprecating humor. He maintained close friendships with colleagues from all over the world, who surely share our feelings of loss.

References

1. Bogoch II, Chakrabarti S, Sharkawy A. In Memoriam: Jay Keystone (1943–2019). *Am J Trop Med Hyg.* 2019;101:953–4. <https://doi.org/10.4269/ajtmh.19-1943>
2. Langer JC, Rose DB, Keystone JS, Taylor BR, Langer B. Diagnosis and management of hydatid disease of the liver. A 15-year North American experience. *Ann Surg.* 1984;199:412–7. <https://doi.org/10.1097/0000658-198404000-00007>
3. Kain KC, Harrington MA, Tennyson S, Keystone JS. Imported malaria: prospective analysis of problems in diagnosis and management. *Clin Infect Dis.* 1998;27:142–9. <https://doi.org/10.1086/514616>
4. Bacaner N, Stauffer B, Boulware DR, Walker PF, Keystone JS. Travel medicine considerations for North American immigrants visiting friends and relatives. *JAMA.* 2004;291:2856–64. <https://doi.org/10.1001/jama.291.23.2856>
5. Freedman DO, Weld LH, Kozarsky PE, Fisk T, Robins R, von Sonnenburg F, et al.; GeoSentinel Surveillance Network. Spectrum of disease and relation to place of exposure among ill returned travelers. *N Engl J Med.* 2006;354:119–30. <https://doi.org/10.1056/NEJMoa051331>
6. Leder K, Torresi J, Libman MD, Cramer JP, Castelli F, Schlagenhauf P, et al.; GeoSentinel Surveillance Network. GeoSentinel surveillance of illness in returned travelers, 2007–2011. *Ann Intern Med.* 2013;158:456–68. <https://doi.org/10.7326/0003-4819-158-6-201303190-00005>

Address for correspondence: David O. Freedman, University of Alabama at Birmingham, BBRB 203, 1720 2nd Ave S, Birmingham, AL 35294, USA; email: dfreedman@uabmc.edu



Charles Burchfield, *Orion in December, 1959* (detail). Watercolor and pencil on paper. 39 7/8 in x 32 7/8 in/101.2 x 83.4 cm. Smithsonian American Art Museum, Gift of S.C. Johnson & Son, Inc., 1969.47.53.

Hunters Searching among Starry Nights and at the Edges of Life

Byron Breedlove

Astronomers and astrophysicists work with immense, mind-boggling numbers. Caleb A. Scharf, Director of Astrobiology at Columbia University, writes “On a finite world a cosmic perspective isn’t a luxury, it’s a necessity.” Consider that the distance between the earth and sun equals ≈ 100 million miles. One of the nearest stars to Earth, Alpha Centauri shimmers some 4.4 light-years away. Put another way, if the distance from the earth to the sun were fixed at 1 inch, then one of Earth’s closest neighbors would be 4.4 miles (7 km) distant. The Milky Way, our home galaxy, comprises something in the range of 200–300 billion stars. It is nestled among the other 2 trillion or so galaxies estimated to populate the observable universe, postulated to have a radius of 13.8 billion light-years.

However, the figure ascribed to the quantity of terrestrial viruses, an estimated 10^{31} , eclipses even those immense numbers. That means there are potentially 5–10 million times more viruses on Earth than stars thought to exist in the observable universe. Science writer Carl Zimmer offers this perspective: “If you were to stack one virus on top of another, you’d create a tower that would stretch beyond the moon, beyond the sun, beyond Alpha Centauri, out past the

edge of the Milky Way, past neighboring galaxies, to reach a height of 200 million light years.”

Viruses, which have no cell nucleus and consist of DNA or RNA enveloped in a protein, are not classified among the 5 kingdoms of living things: bacteria, fungi, protists, plants, and animals. Debate lingers about whether viruses are alive. Virologists Marc H.V. van Regenmortel and Brian W.J. Mahy explain that “A virus becomes part of a living system only after it has infected a host cell and its genome becomes integrated with that of the cell. Viruses are replicated only through the metabolic activities of infected cells, and they occupy a unique position in biology. They are nonliving infectious entities that can be said, at best, to lead a kind of borrowed life.”

Viruses exist at the edge of life and do not themselves exhibit life functions. They replicate and mutate efficiently and quickly, enabling them to readily adapt to new hosts. Some viruses can survive in the most extreme conditions. Their vast, diverse population outnumbers other cellular life-forms by at least tenfold. Viruses have played a crucial role in the evolution and adaptability of life, and although viruses cause many human illnesses, most do not cause disease and death.

In the past few decades, emerging infectious diseases researchers have identified a number of deadly viruses that infect humans. Recently discovered viruses include dengue viruses, Ebola virus, Marburg

Author affiliation: Centers for Disease Control and Prevention, Atlanta, Georgia, USA

DOI: <https://doi.org/10.3201/eid2601.AC2601>

virus, many hantavirus, HIV, influenza viruses, rotaviruses, Nipah virus, and Middle East respiratory syndrome coronavirus.

Viruses also cause myriad diseases in animals, including rabies, foot-and-mouth disease, bluetongue, avian influenza, peste des petits ruminants, and swine flu. A number of diseases in plants, such as various leaf roll and leaf curl diseases, mosaic diseases, and ring spot, are caused by viruses. The bottom line is that viruses infect organisms at every scale, from bacteria to blue whales.

As virology enters its second century, some researchers are starting to investigate whether viruses can survive extraterrestrially. Berliner, Mochizuki, and Stedman, authors of a review article in *Astrobiology*, lay out some short-term and long-term priorities for astrovirology research and discuss difficulties and stratagems for finding viral biosignatures on Earth and in extraterrestrial environments. As they point out, anywhere life exists on Earth, viruses also exist in abundance, and they note “we do need to learn more about viruses on modern Earth before we look elsewhere, but let’s start looking.”

Orion in December, this month’s cover art, connects the realms of Earth and space and invokes Orion the Hunter from Greek mythology. Its creator, Charles Burchfield, may not be the most recognized name in the canon of 20th century artists but is considered by some a classic example of the “artist’s artist.” (See *Military Magic or Nature’s Fool*, the cover essay for the April 2012 EID, for more details about Burchfield.) He favored watercolors, often combined with gouache, pencil, charcoal, or pastels. Art scholar Paloma Alarcó notes that “Burchfield, who was well versed in astronomy, felt a special attraction for the sky and heavenly bodies—not only the sun and moon but also the Pleiades and certain constellations of stars, particularly Orion, which appears in winter.” Indeed, the artist featured Orion, one most prominent and recognizable constellations visible from almost everywhere on earth, in 3 other paintings.

Orion’s bright stars, including the supergiants Betelgeuse and Rigel, shimmer so brightly they seem to vibrate. The constellation fills the winter sky and dips into the trees, cinching together earth and sky. Burchfield links the points of lights within Orion using thick, hazy lines, ensuring viewers cannot miss the overall form. The spire-shaped trees stand like rockets poised to launch into the night sky. In the foreground, the underbrush, heavy with frost and ice, reaches skyward and seems to crackle like pale flames lapping at the tree trunks.

Heightened hallucinatory effects such as these were integral to Burchfield’s approach in the late stages of his career. The American Art at the Phillips Collection notes, “Burchfield’s subjects are unsophisticated but gain immediacy through energetic two-dimensional patterns that animate the surface of his pictures and evoke sensations of the subject’s particular play of light, weather conditions, and even sound.”

Orion was a hunter in Greek mythology, and the constellation Burchfield painted happens to be a focal point in the hunt for exoplanets, some of which may have conditions favorable for viruses and by association the potential for extraterrestrial life. Any such discoveries would further amplify the already gargantuan numbers associated with astronomy and virology.

Bibliography

1. Alarcó P. Charles Ephraim Burchfield. Museo Thyssen-Bornemisza [cited 26 Nov 2019]. <https://www.museothyssen.org/en/collection/artists/burchfield-charles-ephraim/orion-winter>
2. American Art at the Phillips Collection [cited 27 Nov 2019]. https://www.phillipscollection.org/research/american_art/bios/burchfield-bio.htm
3. Berliner AJ, Mochizuki T, Stedman KM. Astrovirology: viruses at large in the universe. *Astrobiology*. 2018;18:207–23. <https://doi.org/10.1089/ast.2017.1649>
4. Burchfield Penny Art Center. The visionary Charles Burchfield [cited 2019 Dec 6]. <https://www.burchfield-penny.org/general/news-and-updates/news-releases/article:10-01-2009-12-00am-the-visionary-charles-burchfield>
5. Conselice CJ, Wilkinson A, Duncan K, Mortlock A. The evolution of galaxy number density at $z < 8$ and its implications. *Astrophys J*. 2016;830:2. <https://doi.org/10.3847/0004-637X/830/2/83>
6. Harrington J. The moon, the stars, and the artist: astronomy in the works of Charles E. Burchfield. *Am Art J*. 1990; 22:33–59. <https://doi.org/10.2307/1594560>
7. Potter P. Military magic or nature’s fool. *Emerg Infect Dis*. 2012;18:709–10. <https://doi.org/10.3201/eid1804.AC1804>
8. Scharf CA. Will we find extraterrestrial life in 2015? *Scientific America* [cited 26 Nov 2019]. <https://blogs.scientificamerican.com/life-unbounded/will-we-find-extraterrestrial-life-in-2015>
9. Strauss M. Our universe is too vast for even the most imaginative sci-fi. *Aeon* [cited 26 Nov. 2019]. <https://aeon.co/ideas/our-universe-is-too-vast-for-even-the-most-imaginative-sci-fi>
10. van Regenmortel MH, Mahy BW. Emerging issues in virus taxonomy. *Emerg Infect Dis*. 2004;10:8–13. <https://doi.org/10.3201/eid1001.030279>
11. Villarreal LP. Are viruses alive? *Scientific America* [cited 25 Nov 2019]. <https://www.scientificameric.n.com/article/are-viruses-alive-2004>
12. Zimmermann KA. Orion constellation: facts about the hunter [cited 2019 Dec 2] <https://www.space.com/16659-constellation-orion.html>

Address for correspondence: Byron Breedlove, EID Journal, Centers for Disease Control and Prevention, 1600 Clifton Rd NE, Mailstop HI16-2, Atlanta, GA 30329-4027, USA; email: wbb1@cdc.gov

EMERGING INFECTIOUS DISEASES®

Upcoming Issue

- Acute Toxoplasmosis among Canadian Deer Hunters Associated with Consumption of Undercooked Deer Meat Hunted in the United States
- Interspecies Transmission of Swine Reassortant Influenza A Virus Containing Genes from Influenza A(H1N1)pdm09 and Swine Influenza A(H1N2) Viruses
- Neutralizing Antibodies against Enterovirus and Coxsackieviruses in Patients with Hand, Foot and Mouth Disease
- Cost-Effectiveness of Screening Program for Chronic Q Fever, the Netherlands
- Infection and Transmission of Porcine Deltacoronavirus in Commercial Poultry, United States
- Characteristics of Patients with Acute Flaccid Myelitis, USA, 2015–2018
- Multiplex Mediator Displacement LAMP for Detection of *Treponema pallidum* and *Haemophilus ducreyi* in Clinical Samples
- Chronic Human Pegivirus-2 and Hepatitis C Virus Co-infection
- Global Expansion of Pacific Northwest *Vibrio parahaemolyticus* Sequence Type 36
- Systematic Hospital-Based Travel Screening to Assess Exposure to Zika Virus
- Human Norovirus Infection in Dogs, Thailand
- Emergence of Chikungunya Virus, Pakistan, 2016–2017
- Surge in Anaplasmosis Cases in Maine, USA, 2013–2017
- Early Detection of Public Health Emergencies of International Concern through Undiagnosed Disease Reports in ProMED-mail
- Ocular *Spiroplasma ixodetis* in Newborns, France
- Influence of Rainfall on *Leptospira* Infection and Disease in a Tropical Urban Setting, Brazil
- *Elizabethkingia anophelis* Infection in Infants, Cambodia, 2012–2018
- Astrovirus in White-Tailed Deer, United States, 2018
- New Delhi Metallo- β -Lactamase-5–Producing *Escherichia coli* in Companion Animals, United States

Complete list of articles in the February issue at
<http://www.cdc.gov/eid/upcoming.htm>

Upcoming Infectious Disease Activities

January 28–30, 2020
 American Society for Microbiology
 2020 ASM Biothreats
 Arlington, VA, USA
<https://www.asm.org/Events/ASM-Biothreats/Home>

February 20–23, 2020
 International Society for Infectious Diseases
 Kuala Lumpur, Malaysia
<https://www.isid.org/>

March 8–11, 2020
 CROI
 Conference on Retroviruses and Opportunistic Infections
 Boston, MA, USA
<https://www.croiconference.org/>

March 9–13, 2020
 ASLM
 African Society for Laboratory Medicine
 7th African Network for Influenza Surveillance Epidemiology
 Livingstone, Zambia
<https://www.anise2020.org>

March 18–20, 2020
 International Conference on Reemerging Infectious Diseases
 Addis Ababa, Ethiopia
<http://www.icreid.com/>

March 26–30, 2020
 Society for Healthcare Epidemiology of America
 Decennial 2020
 6th International Conference on Healthcare Associated Infections
 Atlanta, GA, USA
<https://decennial2020.org>

April 18–21, 2020
 ECCMID
 The European Congress of Clinical Microbiology and Infectious Diseases
 Paris, France
https://www.eccmid.org/eccmid_2020/

May 3–6, 2020
 ASM Clinical Virology Symposium
 West Palm Beach, FL, USA
<https://asm.org/Events/2019-Clinical-Virology-Symposium/Home>

June 18–22, 2020
 American Society for Microbiology
 ASM Microbe 2020
 Chicago, IL, USA
<https://asm.org/Events/ASM-Microbe/Home>

Announcements

Email announcements to EIDEditor (eideditor@cdc.gov). Include the event's date, location, sponsoring organization, and a website.

Earning CME Credit

To obtain credit, you should first read the journal article. After reading the article, you should be able to answer the following, related, multiple-choice questions. To complete the questions (with a minimum 75% passing score) and earn continuing medical education (CME) credit, please go to <http://www.medscape.org/journal/eid>. Credit cannot be obtained for tests completed on paper, although you may use the worksheet below to keep a record of your answers.

You must be a registered user on <http://www.medscape.org>. If you are not registered on <http://www.medscape.org>, please click on the “Register” link on the right hand side of the website.

Only one answer is correct for each question. Once you successfully answer all post-test questions, you will be able to view and/or print your certificate. For questions regarding this activity, contact the accredited provider, CME@medscape.net. For technical assistance, contact CME@medscape.net. American Medical Association’s Physician’s Recognition Award (AMA PRA) credits are accepted in the US as evidence of participation in CME activities. For further information on this award, please go to <https://www.ama-assn.org>. The AMA has determined that physicians not licensed in the US who participate in this CME activity are eligible for AMA PRA Category 1 Credits™. Through agreements that the AMA has made with agencies in some countries, AMA PRA credit may be acceptable as evidence of participation in CME activities. If you are not licensed in the US, please complete the questions online, print the AMA PRA CME credit certificate, and present it to your national medical association for review

Article Title

Paid Leave and Access to Telework as Work Attendance Determinants during Acute Respiratory Illness, United States, 2017–2018

CME Questions

1. You are advising a large corporation about how to minimize spread of influenza and acute respiratory illness (ARI) in the workplace. According to the survey study by Ahmed and colleagues, which of the following statements about work attendance during ARI and associated characteristics among workers ages 19 to 64 years with medically attended ARI or influenza during the 2017–2018 influenza season is correct?

- A. During the first 3 days of illness, two-thirds reported they did not work at all
- B. Median interval was 12 days from illness onset to return to normal activities and 21 days from illness onset to recovery from illness
- C. During the first 3 days of ARI, mean number of days worked was 1.14 (range, 0–3), days absent from work because of illness was 1.06 (range, 0–3) days, and days missed for any other reason was 0.8 (range, 0–3) days
- D. One-third had access to telework, and half received paid leave benefits

2. According to the survey study by Ahmed and colleagues, which of the following statements about determinants of work attendance during the first 3 days after ARI onset among workers ages 19 to 64 years with medically attended ARI or influenza during the 2017–2018 influenza season is correct?

- A. Persons with access to paid leave were significantly less likely to work during the first 3 days of illness (adjusted ratio [AR] of days worked = 0.81 [95% CI: 0.68, 0.96])
- B. Working in an organization that discouraged employees from coming to work if they had influenza-like symptoms was not significantly linked to likelihood of working during the first 3 days of illness
- C. Access to telework was significantly associated with the number of days worked at the usual workplace
- D. Determinants of work attendance were significantly different for persons with vs persons without laboratory-confirmed influenza

3. According to the survey study by Ahmed and colleagues, which of the following statements about clinical implications of work attendance during the first 3 days after ARI onset among workers ages 19–64 years with medically attended ARI or influenza during the 2017–2018 influenza season is correct?

- A. Lowering workplace transmission of ARI and influenza requires only paid leave benefits
- B. The public health benefit of employee access to paid leave and telework is an increased ability to comply with the public health recommendation to stay home when ill
- C. Employees who stay home with an ARI should be censured for lowering workplace productivity and shirking responsibility to coworkers
- D. Macro-level policy intervention is the only level at which reducing ARI transmission is likely to be effective