EMERGING INFECTIOUS DISEASES®

Neurologic Disorders

September 2013

Franconian painter of the Nuremberg School; active late 15th century in Nuremberg. *The Miracle of Saint Vitus* (c. 1490) Oil on panel (116.8 cm x 99.1 cm)
The John and Mable Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

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

The opinions expressed by authors contributing to this journal do not necessarily reflect the opinions of the Centers for Disease Control and Prevention or the institutions with which the authors are affiliated.

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On the Cover

Franconian painter of the Nuremberg School; active late 15th century in Nuremberg

***The Miracle of Saint Vitus* (c. 1490)**

Oil on panel (116.8 cm x 99.1 cm)

The John and Mable Ringling Museum of Art, the State Art Museum of Florida, a division of Florida State University

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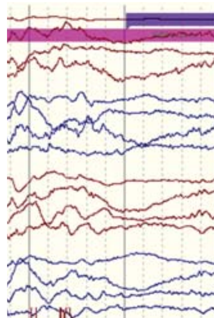

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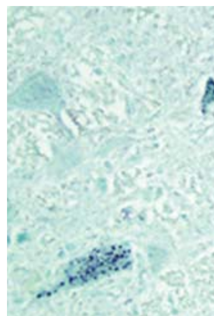

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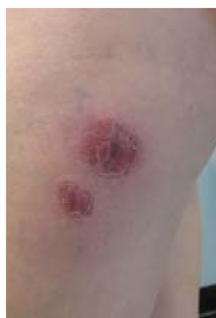

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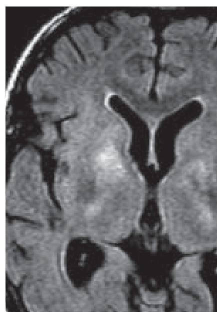

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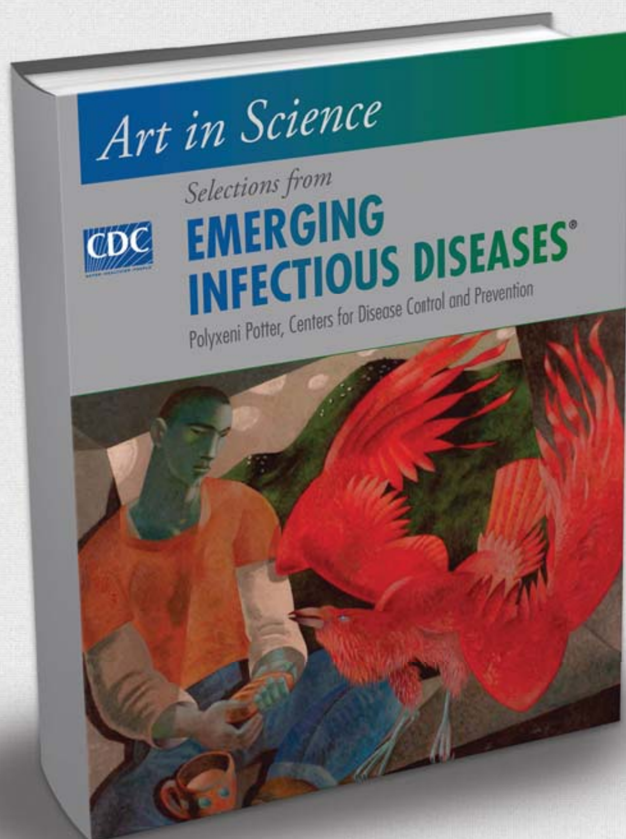

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ISBN-10: **0199315698** | ISBN-13: **978-0199315697**

Acute Encephalitis Syndrome Surveillance, Kushinagar District, Uttar Pradesh, India, 2011–2012

Manish Kakkar, Elizabeth T. Rogawski, Syed Shahid Abbas, Sanjay Chaturvedi, Tapan N. Dhole, Shaikh Shah Hossain, and Sampath K. Krishnan

In India, quality surveillance for acute encephalitis syndrome (AES), including laboratory testing, is necessary for understanding the epidemiology and etiology of AES, planning interventions, and developing policy. We reviewed AES surveillance data for January 2011–June 2012 from Kushinagar District, Uttar Pradesh, India. Data were cleaned, incidence was determined, and demographic characteristics of cases and data quality were analyzed. A total of 812 AES case records were identified, of which 23% had illogical entries. AES incidence was highest among boys <6 years of age, and cases peaked during monsoon season. Records for laboratory results (available for Japanese encephalitis but not AES) and vaccination history were largely incomplete, so inferences about the epidemiology and etiology of AES could not be made. The low-quality AES/Japanese encephalitis surveillance data in this area provide little evidence to support development of prevention and control measures, estimate the effect of interventions, and avoid the waste of public health resources.

Acute encephalitis syndrome (AES) is a clinical condition caused by infection with Japanese encephalitis virus (JEV) or other infectious and noninfectious causes. A confirmed etiology is generally not required for the clinical management of AES. Thus, surveillance for JEV infection in India has focused on identifying AES cases rather than JE cases; this approach is more feasible given the limitations of public health resources (*1*). However, identification of the etiologic agent is necessary for planning relevant interventions. The standard for determining the etiology of AES is examination of cerebrospinal fluid (CSF) during the acute phase of illness; pathogen-specific IgM capture ELISA or nucleic acid amplification techniques are used to detect pathogens in the CSF. Serologic tests for pathogen-specific antibodies and virus detection in serum are also recommended. However, examination of CSF is preferred because serologic test results may indicate the presence of antibodies in the serum, but the AES may have a cause different than the agent producing the detected antibodies (*1–3*).

A good quality surveillance system with laboratory support is essential for understanding the causes of AES and responding appropriately. Accordingly, the National Vector Borne Diseases Control Programme in New Delhi, India, has developed guidelines for AES surveillance that promote the need for a strong surveillance system as a critical component for any control activities. In these guidelines, the goals outlined for AES surveillance are to 1) assess and characterize the burden of JE, 2) detect early warning signals for an outbreak, 3) assess the effect of vaccination, and 4) guide future strategies (*1*). The National Vector Borne Diseases Control Programme has also implemented several measures to strengthen local health systems, including building on the capacity of the health workforce to provide better clinical management, extending referral diagnostic facilities by upgrading the existing Baba Raghav Das (BRD) Medical College facilities and setting up a National Institute of Virology field unit; and establishing a dedicated surveillance unit in the Department of Preventive and Social Medicine at BRD Medical College to provide improved surveillance and outbreak responses (*4*).

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DOI: http://dx.doi.org/10.3201/eid1909.121855

From the 1970s until around 2010, JEV infection was considered to be the leading cause of AES in the traditional JE belt of India, which includes Kushinagar District in the state of Uttar Pradesh (*5–11*). However, because of a large number of JE cases of unknown etiology, AES patterns alone have not suggested a clear picture of the epidemiology of the disease. In recent years, despite of the introduction of a JE vaccine, an increased number of AES cases have been reported in India, including Uttar Pradesh, and the disease has spread to new districts, urban areas, and villages without pigs, which are not usually associated with JE transmission (*12,13*). Thus, the assertion that JEV is the leading cause of AES has been questioned, and other infectious agents, such as enteroviruses, have been reported as a cause of AES in Uttar Pradesh and other parts of India (*14–19*). A substantial contributor to the ambiguity about the etiology of AES could be the fact that surveillance data for AES have not been analyzed to assess reasons for the increased cases and other reported causes. We examined the completeness and quality of AES surveillance data from Kushinagar District, an area where JEV is highly endemic. Herein, we discuss the ability to make inferences about AES epidemiology and etiology from these data and the implications of our findings for policy planning and program implementation.

Methods

Source of Surveillance Data

The sentinel site for JE surveillance in Kushinagar District is the district hospital. Using a standard format, the hospital reports all AES/JE cases that meet the standard World Health Organization case definition (*3*) to the district malaria officer; this officer then forwards compiled data to the state program officer for transmission to National Vector Borne Disease Control Programme (http://nvbdcp.gov.in/Doc/AES%20guidelines.pdf). BRD Medical College in Gorakhpur, Uttar Pradesh, is a nearby regional field laboratory of the Indian Council of Medical Research, which receives clinical samples from patients admitted to the district hospital in Kushinagar and completes most laboratory testing for JEV infections. In addition, BRD Medical College serves as a tertiary care center for patients who directly seek medical care or who are referred from nearby districts, including Kushinagar. A line list with laboratory results of these patients is reported by BRD Medical College to the district malaria officer in Kushinagar for submission to state program officers.

In July 2012, we obtained the AES line lists data for January 2011–June 2012 that were submitted by BRD Medical College to Kushinagar District Headquarters in Padrauna. The list was originally to be used to identify blocks in Kushinagar District with high, medium, and low numbers of AES cases so that sites could be selected for a larger study of the drivers of JEV transmission. Cases recorded in the line lists represented case-patients from Kushinagar District who were 1) admitted directly to BRD Medical College, 2) referred to BRD Medical College by the district hospital in Padrauna, and 3) admitted to the district hospital in Padrauna but had serum samples referred to BRD Medical College because no diagnostic kits were available and laboratory testing was limited at the district hospital. An individual AES patient tracking system does not exist in the state of Uttar Pradesh, so patients treated in the private sector or at the district hospital in Gorakhpur were not included in the surveillance lists.

Preparation of Surveillance Database for Analysis

Individual identifiers for the case-patients were removed from the line lists to ensure confidentiality. Data were cleaned (i.e., extraneous data were removed) in multiple steps, resulting in 4 changes being made to the AES line lists. First, the spelling of residential localities (block, village, or police station) were matched and standardized to result in a list of 32 residential areas. Residential areas were then categorized and combined by block (14 blocks in Kushinagar). Modifications were validated by cross-checking with village population lists provided by Savera, a local nongovernmental organization. Second, all age values were standardized to a uniform decimal system (e.g., 1 year and 6 months was changed to 1.5 years, and 8 months was changed to 0.67 year). Third, the dates of symptom onset, hospital admission, sample collection, and outcome (i.e., discharged, left against medical advice, or death) were standardized to 1 format. In the original list, dates were variously coded by using different notations (e.g., 4 March 2011 was mentioned within the same row as both 04/03/11 and 03/04/11). Assuming that the case data were entered in chronological order, we standardized dates by using, as a cue, the dates of the preceding and following AES cases. Fourth, case-patients who died, were absent, or left against medical advice were considered to have been discharged.

Data Analysis

Cleaned data were imported into SAS version 9.2.2 (SAS Institute, Inc., Cary, NC, USA) for analysis. AES cases were plotted by week and month by using the recorded dates of symptom onset. We tabulated demographic characteristics of AES case-patients for 2011 and 2012 and stratified case-patients by block, vaccination status, laboratory test result for JEV infection, and clinical outcome. Incidence for 2011 was calculated by using population denominators from the 2011 Census of India (*20*). We calculated incidence overall, by sex, and for children 0–6 years of age by using the age stratification available in the

district-level census data. We estimated the incidence for each block by using block-specific population denominators projected with the decadal growth rate from the 2001 Census of India (*20*). Crude incidence rate ratios were estimated with Wald-based CIs.

We also determined the median number of days between key points in AES disease progression and diagnosis: time between onset of symptoms and hospital admission, onset of symptoms and serum sample collection, hospital admission and serum sample collection, and hospital admission and discharge or death. Using previously described methods (*21*), we evaluated the quality of the surveillance data by assessing the amount of data cleaning required, the proportion of missing or incomplete values in line list fields, and inconsistencies in dates recorded for key points in AES disease progression and diagnosis.

Results

AES Epidemiology

In 2011, a total of 721 AES cases from Kushinagar District were identified through BRD Medical College; in 2012 (January–June), 91 cases were identified. Using the cleaned line lists, we determined the weekly number of AES cases reported during January 2011–June 2012 (Figure 1). Cases peaked during August–October 2011; >150 cases were identified in each of these 3 months. This seasonal trend corresponds with an expected increase in cases during the monsoon season, when transmission of both waterborne and vector-borne diseases increases (vector density is at its maximum).

In 2011 and 2012, most case-patients were male (57.4% and 59.3%, respectively) (Table 1). In 2011, almost half of the AES cases were in children <5 years of age (44.7%); the distribution of cases by age group was not substantially different in 2012. The case-fatality rate was 18.0% in 2011 and 19.8% in 2012.

Table 1. Characteristics of case-patients with acute encephalitis syndrome, Kushinagar District, Uttar Pradesh, India, 2011–2012*

Characteristic	No. (%) cases	
	2011, n = 721	2012, n = 91
Age, y		
0–4	322 (44.7)	36 (39.6)
5–9	205 (28.4)	22 (24.2)
10–14	83 (11.5)	12 (13.2)
≥15	111 (15.4)	21 (23.1)
Sex		
M	414 (57.4)	54 (59.3)
F	307 (42.6)	37 (40.7)
Religion		
Hindu	632 (87.7)	80 (87.9)
Muslim	89 (12.3)	11 (12.1)
Vaccinated against JEV		
Yes	3 (0.4)	0
No	116 (16.1)	0
Unknown	602 (83.5)	91 (100)
Outcome		
Died	130 (18.0)	18 (19.8)
Absent	18 (2.5)	0
LAMA	16 (2.2)	0
Discharged	557 (77.3)	73 (80.2)
Result for JEV laboratory test		
Positive	3 (0.4)	0
Negative	128 (17.8)	0
Awaited†	590 (81.8)	91 (100)

*Based on data obtained from Baba Raghav Das Medical College, Gorakhpur, Uttar Pradesh, India. JEV, Japanese encephalitis virus; LAMA, left against medical advice.
†Clinical samples awaiting laboratory test results.

Using 2011 population data (*20*), we estimated that there were 20.2 AES cases/100,000 population in Kushinagar District in 2011 (Table 2). The incidence was higher among male residents than female residents (incidence rate ratio 1.29, 95% CI 1.11–1.49), and it was highest among children 0–6 years of age. The crude incidence rate ratio, comparing case-patients 0–6 years of age with those >6 years of age, was 7.97 (95% CI 6.87–9.25). Boys 0–6 years of age were at highest risk for AES. The incidence among 0- to 6-year-old boys was almost 50% greater than that among girls of the same age.

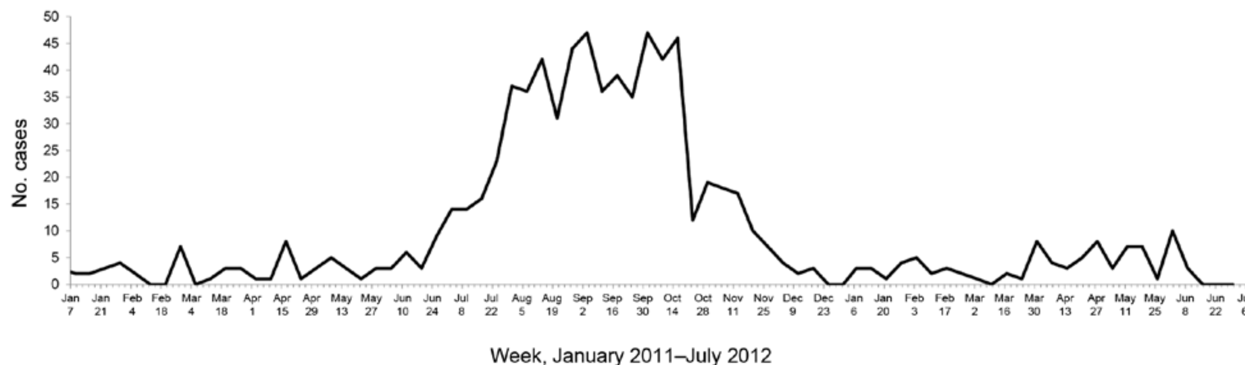

Figure 1. Weekly number of acute encephalitis syndrome cases, by month, in Kushinagar District, Uttar Pradesh State, India, 2011–2012. Numbers are based on data obtained from Baba Raghav Das Medical College, Gorakhpur, Uttar Pradesh, India.

Table 2. Incidence of acute encephalitis syndrome, Kushinagar District, Uttar Pradesh, India, 2011–2012*

Age, sex of population	2011 population†	No. cases	Incidence‡	Incidence rate ratio (95% CI)
All ages	3,560,830	721	20.2	
M	1,821,242	414	22.7	1.29 (1.11–1.49)
F	1,739,588	307	17.6	1.0
0–6 y	551,467	428	77.6	
M	287,672	260	90.4	1.42 (1.17–1.72)
F	263,795	168	63.7	1.0

*Based on data obtained from Baba Raghav Das Medical College, Gorakhpur, Uttar Pradesh, India.
†From 2011 Census of India (*20*).
‡Per 100,000 population.

The weekly numbers of AES cases, classified by JE IgM laboratory result (positive, negative, awaiting determination), is shown in Figure 2. Only 3 (4.2%) cases of JEV infection were identified in 2011: two cases were in 55-year-old men, 1 of whom died, and 1 case was in a 14-year-old girl. Vaccination status was reported for 119 case-patients, of whom 3 (2.6%) had been vaccinated. The case-patients who had received vaccine were boys 6, 7, and 8 years of age; they began experiencing symptoms in July 2011 and were discharged within 3 weeks of hospital admission. None of the 3 JE case-patients had been vaccinated.

Quality of Surveillance Data

During data cleaning, we modified 25% of the 2011 and 5% of the 2012 line list values for residential locality, age, and date parameters (Table 3). Nearly one fifth of the age data and more than one fourth of the dates were edited. For 3.2% and 13.2% of cases in 2011 and 2012, respectively, the block name could not be determined from the residential locality provided and was marked as "unknown" because the village or police station name was not found or was present in multiple blocks. In addition, several fields in the database were incomplete. As of July 2012, laboratory results for JEV infection were still classified as "awaited" (i.e., awaiting determination) in the line lists for 82% (590) of the 721 cases in 2011 and for all 91 cases in 2012 (Table 1). The line lists indicated the date of sample collection, but the type of sample collected (CSF and/or serum) and the laboratory test used (IgM ELISA, PCR, and/or cell culture) were not recorded for any of the case-patients. Most samples submitted after July 2011 were still awaiting laboratory results at the time of our study (Figure 2). In addition, for 602 (83.5%) of the 721 case-patients in 2011 and all 91 case-patients in 2012, JEV vaccination status was marked as "unknown" in the line lists (Table 1).

Key epidemiologic and programmatic indicators, such as the time between key points in AES disease progression and diagnosis, varied widely among case-patients (Table 4). In some instances, the dates were illogical (e.g., dates of symptom onset and sample collection following and preceding the dates of hospital admission, respectively). It was not possible to rectify these inconsistencies on the basis of the available data. In addition, the range of values was often large. For example, median time from onset of symptoms to hospital admission was 4 days in 2012, but some patients were admitted >2 months after symptom onset. Because AES is an acute syndrome, a long interval is not expected between symptoms and may indicate reporting of unrelated symptoms or misclassification of AES.

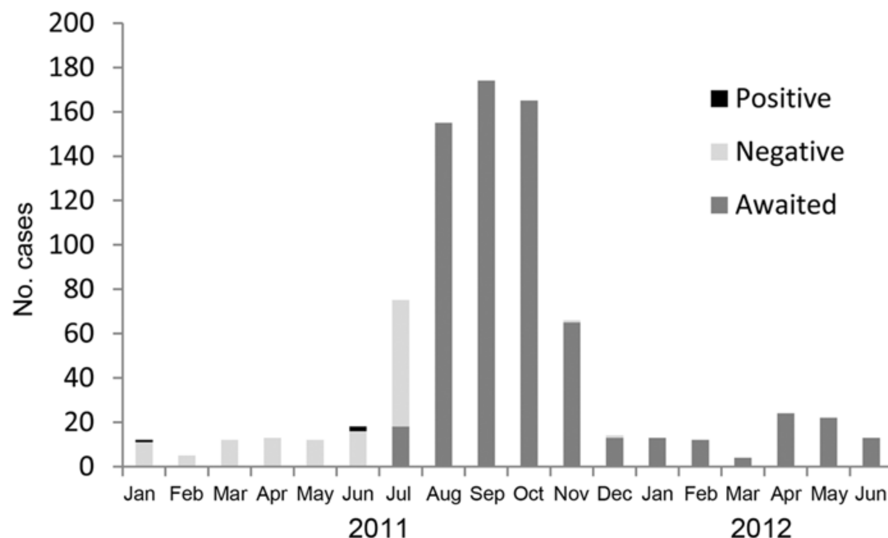

Figure 2. Weekly number of acute encephalitis syndrome cases, by month, in Kushinagar District, Uttar Pradesh State, India, 2011–2012. Numbers represent results of laboratory testing for Japanese encephalitis and are based on data from Baba Raghav Das Medical College, Gorakhpur, India. In the key, "awaited" refers to samples that were awaiting laboratory test results.

Table 3. Values modified in line lists of case-patient data used in a study of acute encephalitis syndrome, Kushinagar District, Uttar Pradesh, India, 2011–2012*

	No. (%) modified		
Case-patient value	2011, n = 721	2012, n = 91	Combined, n = 812
Name of block of residence	192 (26.6)	2 (2.2)	194 (23.9)
Age	128 (17.8)	18 (19.8)	146 (18.0)
Date of symptom onset	221 (30.7)	4 (4.4)	225 (27.7)
Date of fever onset	213 (29.5)	4 (4.4)	217 (26.7)
Date of admission	193 (26.8)	2 (2.2)	195 (24.0)
Date of sample collection	186 (25.8)	1 (1.1)	187 (23.0)
Out come	139 (19.3)	2 (2.2)	141 (17.4
All fields	183 (25.4)	33 (36.3)	216 (26.6)

*Based on data obtained from Baba Raghav Das Medical College, Gorakhpur, Uttar Pradesh, India.

Discussion

Despite the introduction of JE vaccine, an increased number of AES cases have been reported in Kushinagar District in recent years, and AES has been reported in areas not previously associated with the disease (*12,13*). Because of this apparently changing epidemiology of AES/JE in Kushinagar District, the importance of quality surveillance for guiding local and stratified caseload predictions for patient management and decision-making at policy and program levels cannot be overemphasized. However, our analyses show that despite recent attention regarding AES and public health interventions, constraints imposed by the district's surveillance capacity hinder patient management, policy decisions, and implementation of prevention and control measures. The surveillance line list from BRD Medical College is representative of the district data on AES/JE (district malaria officer, pers. comm.). However, the current surveillance system captures only data for case-patients/samples that have been admitted/referred to BRD Medical College; thus, AES patients from Kushinagar District who seek medical care in the private sector are not represented in the surveillance data.

It seems that the surveillance database is not currently used for analysis at the district level; instead, the database is maintained only for administrative purposes, which include reporting to the State Directorate. However, the number of modifications that we had to make to the AES line lists for our analyses indicates that the quality of data collection is poor. We corrected some errors by making comparisons with other fields in the database, but other entries remain illogical (e.g., dates of hospital admission that proceed the dates of symptoms onset). Despite our thorough review and use of standardized protocol for cleaning the line lists, the results presented here should be viewed with regard to the inconsistencies in the original surveillance data. Regardless of the practical difficulties in coordination, collation, recording, and reporting of data, the high level of incomplete records, especially for JEV vaccination status and laboratory result, suggests a lack of initiative by data collectors to record complete and accurate information.

Current surveillance data provide little credible information to guide program planning and policy making for AES/JE in Kushinagar District. We could not determine if JE is etiologically responsible for AES in this area because reporting for JE laboratory testing was vague and incomplete. In addition, it is likely that only serum samples were collected to determine if AES was caused by JEV infection because the sampling procedure for serum is simpler than that for CSF. However, serum is a suboptimal sample for determining the cause of AES. Many JE infections are asymptomatic, so AES may be caused by an agent other than JEV even if JEV–specific IgM is present in the serum (*2,3*). In addition, a live, attenuated JEV vaccine is used in India, so the presence of JEV IgM in serum may be the effect of previous vaccination. CSF is preferred over serum samples for JEV testing because the presence of JE antibody in CSF provides a definitive diagnosis of JEV infection. A record of the type of sample collected is also essential for assessing the diagnostic yield of the sample and determining whether the sample was collected at the appropriate time after symptom onset (*2*). This need for a complex diagnostic process may have contributed to the incompleteness of laboratory results.

Regardless of the type of sample collected, the recording of JEV laboratory test results was inconsistent during the latter half of 2011 and nonexistent during 2012, despite collection of clinical samples soon after hospital admission. It is unknown whether the delays were caused by laboratory constraints or miscommunication in reporting the results. However, lack of timeliness in reporting surveillance data hinders its utility for guiding interventions and responding to outbreaks. JEV laboratory test results were available only during the low-transmission period, thus excluding any analysis for peak-transmission periods. JEV vaccination has been variously reported at 52% (*22*) to >95% (district health authorities, pers. comm.). We could not use the current surveillance data to estimate or validate the reported coverage figures because most vaccination histories were unknown.

Even basic epidemiologic analyses of demographic characteristics cannot be confidently interpreted as true or

Table 4. Time between key points in disease progression and diagnosis for case-patients with acute encephalitis syndrome, Kushinagar District, Uttar Pradesh, India, 2011–2012*

Key points	Median time, d, between key points (range) 2011	2012
Symptom onset to hospital admission	7 (–4 to 39)	4 (1 to 63)
Symptom onset to sample collection	8 (1 to 40)	6 (1 to 64)
Hospital admission to sample collection	1 (–5 to 31)	1 (0 to 10)
Hospital admission to discharge or death	8 (0 to 70)	4 (1 to 17)

*Based on data obtained from Baba Raghav Das Medical College, Gorakhpur, Uttar Pradesh, India.

simply as the results of poor data collection. In the absence of reliable data, technical discussions have been overshadowed by shifting hypotheses about the etiology and epidemiology of AES, but these discussions are without a strong evidence base. For example, recent debates centered on the role of pigs in JEV transmission, yet the debates lacked relevant data about pigs in the area. In a similar manner, focus has shifted to waterborne causes of encephalitis, even though studies that have identified enteroviruses in patient samples have not concluded that waterborne pathogens are the main cause of AES incidence (*14–16*,*18*). These studies used different sampling methods and had different results, so the relative contributions of waterborne and vector-borne agents to AES cases in Kushinagar District remain unknown. Interventions cannot be planned when, depending on the etiology of AES, strategies as diverse as strengthening vaccination to improving water quality and sanitation may be appropriate. Quality surveillance data, including laboratory results and vaccination history, would resolve the inconsistencies between studies and inform intervention strategies.

Conclusion

The current AES/JE surveillance system has a complicated specimen referral and reporting system at the district level, and the available line lists suggest that data are of low quality. Without evidence to estimate the effect of interventions, AES prevention and control measures may be ineffective and public health resources may be wasted. In 2011, AES and JE were highlighted in the national media, leading to a declaration for several policy initiatives, including formation of a multisectorial and interministerial National Encephalitis Control Programme (*23*). Despite the high profile of AES, the importance of surveillance data for guiding these initiatives has not been realized or translated to action. Gaps in surveillance capacity that were identified in this study indicate the need for a systematic evaluation of the AES/JE surveillance system in Kushinagar District and constitute key lessons that need to be incorporated as strategic planning is undertaken for this new initiative.

Acknowledgment

We thank the District Health Authorities of Kushinagar District, Uttar Pradesh, India, for providing the epidemiologic data used in this study.

This study was part of a larger project supported by an International Development Research Centre grant (no. 105509-037).

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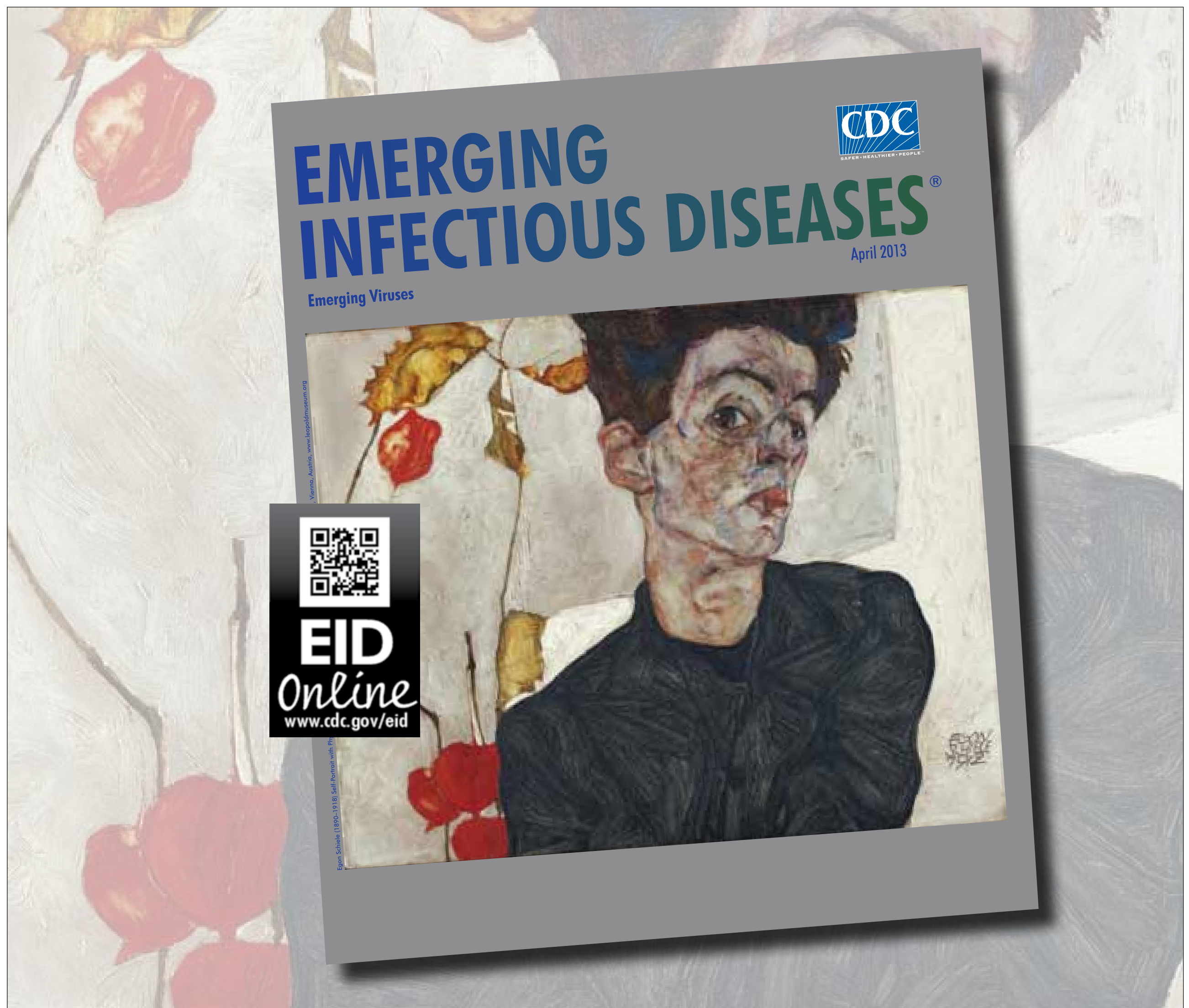

Detection of Diphtheritic Polyneuropathy by Acute Flaccid Paralysis Surveillance, India

Farrah J. Mateen,[1] Sunil Bahl, Ajay Khera, and Roland W. Sutter

Diphtheritic polyneuropathy is a vaccine-preventable illness caused by exotoxin-producing strains of *Corynebacterium diphtheriae*. We present a retrospective convenience case series of 15 children (6 girls) <15 years of age (mean age 5.2 years, case-fatality rate 53%, and 1 additional case-patient who was ventilator dependent at the time of last follow-up; median follow-up period 60 days) with signs and symptoms suggestive of diphtheritic polyneuropathy. All cases were identified through national acute flaccid paralysis surveillance, which was designed to detect poliomyelitis in India during 2002–2008. We also report data on detection of diphtheritic polyneuropathy compared with other causes of acute flaccid paralysis identified by this surveillance system.

Diphtheria is caused by toxin-producing strains of the bacterium *Corynebacterium diphtheriae* and is spread by human-to-human contact (respiratory secretions and cutaneous lesions). Before the advent of vaccination with diphtheria toxoid in the 1940s, ≈1 in 20 persons in temperate zones had diphtheria in their lifetime and 5%–10% of cases led to death (*1*). The last case of diphtheria in the United States was in 2003 in a traveler who returned from Haiti (*2*). However, diphtheria remains a health concern among immigrants, travelers, and those with incomplete immunity and vaccination coverage in many regions. Adults may also be increasingly at risk for diphtheria because of waning immunity or incomplete immunization, especially in outbreak situations (*3*).

Although diphtheria is preventable by vaccination, only 24% of countries worldwide reached the targeted >80% routine coverage of all districts for diphtheria-tetanus-pertussis (DTP3) vaccine in 2011 (*4*). In 2004, the World Health Organization (WHO) reported 5,000 deaths caused by diphtheria, all of which were in children <5 years of age (*5*). However, reporting of diphtheria is variable, and some countries report cases inconsistently because of limited recognition among health care workers and no dedicated surveillance systems (*5*). It is likely that many cases are not reported.

Diphtheria is clinically considered to be a biphasic illness with initial symptoms of low-grade fever, sore throat, neck swelling, nasal twang, and usually ipsilateral palatal paralysis. The time between the first symptoms of diphtheria and the onset of polyneuropathy is deemed the latency period. Diphtheritic polyneuropathy occurs in ≈20% of patients with diphtheria. It is considered more likely with higher release of exotoxin (*6*,*7*) The classic features of diphtheritic polyneuropathy include sensory and motor signs and symptoms, most notably acute flaccid paralysis (AFP) with reduced or absent deep tendon reflexes. Limb paralysis from a segmental demyelinating process occurs with temporal regularity, with onset and resolution 35–140 days after the onset of bulbar signs and symptoms (*6*,*7*). Although serious, diphtheritic polyneuropathy is not consistently fatal and may resolve. Death from diphtheria occurs by parasympathetic dysfunction of the vagal nerve with cardiac arrhythmias, myocarditis, or from respiratory paralysis caused by laryngeal involvement (*2*).

We report a case series of diphtheritic polyneuropathy in children in India identified by routine screening for AFP, which was performed to achieve eradication of poliomyelitis in India. The clinical characteristics of diphtheritic polyneuropathy are presented in detail to remind clinicians of the key diagnostic features of this major cause of neuropathy and the value of a throat examination in persons with AFP.

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DOI: http://dx.doi.org/10.3201/eid1909.130117

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Cases of diphtheria with associated flaccid paralysis are reported to the polio eradication program in India as part of routine surveillance of AFP in children ≤15 years of age. All children with new-onset weakness in ≥1 limbs or facial weakness are reported by a network of health facilities that have been established in all districts of India for AFP reporting. Cases are reviewed for poliomyelitis; this review includes results of fecal testing for poliovirus serotyping. Case-patients, or primary providers for young or severely ill children, are interviewed. All case-patients with AFP are examined in detail by district medical officers trained by government/WHO surveillance officers.

On the basis of results of fecal testing, preliminary clinical findings, and course of the illness, a final diagnosis is established by the district government/WHO medical officers in tandem with local physicians. For patients from whom fecal samples are not collected in time to diagnose poliomyelitis (cases with inadequate samples or inadequate signs and symptoms), the final diagnosis is made by an expert review committee of neurologists, virologists, pediatricians, and epidemiologists convened by the WHO–India National Polio Surveillance Unit in New Delhi, India (*8*,*9*). This committee comprises a minimum of 3 senior experts, usually professors of major academic centers in India, to determine the final diagnosis of children with AFP and inadequate fecal specimens. The committee uses no specific algorithm or diagnostic criteria for every case but comes to a consensus on the most likely diagnosis on the basis of clinical, laboratory, and epidemiologic data.

All cases in this series were given a final clinical diagnosis of diphtheritic neuropathy. The European Union case definition (2002) for the National Diphtheria Surveillance category of probable diphtheria was used to look for diphtheria: "a clinically compatible case that is not laboratory confirmed and does not have an epidemiological link to a laboratory case." Diphtheria is clinically defined "as an upper respiratory tract illness characterized by sore throat, low grade fever, and an adherent membrane of the tonsils, pharynx or nose or non-respiratory diphtheria; cutaneous, conjunctival, otic, and genital lesions" (*10*). The cases reported represent a convenience sample of cases suggestive of diphtheritic polyneuropathy during 2002–2008 in which Guillain-Barré syndrome or poliomyelitis was first suspected. For many case-patients, details of antecedent sore throat, adherent membrane, and respiratory and systemic features were not available because the investigation began only after reporting of AFP and surveillance dedication to poliomyelitis. All cases were diagnosed as diphtheria clinically by at least the treating physicians and the expert review committee in New Delhi.

The cases reported were included because of chart accessibility at the National Polio Surveillance Unit and accessibility of paper files from large storage containers in off-site warehouses. All cases were classified as cases having inadequate fecal specimens for poliovirus testing.

Clinical features were extracted from information obtained by medical officers at the time of clinical presentation. Preselected clinical history, physical examination, baseline demographics, and laboratory findings were obtained from standardized reporting forms. All surviving case-patients were evaluated and caregivers were interviewed at a follow-up visit 60 days after diagnosis per usual protocols during AFP surveillance in India. Death information was also ascertained at 60 days. A neurologist (F.J.M.) extracted all relevant and available clinical information from the medical and surveillance records and retrospectively assigned a Guillain-Barré syndrome disability outcome score (*11*).

Fifteen children with features suggestive of diphtheritic paralysis were identified (6 girls, average age 5.2 years, age range 2.3–14.5 years) in 9 states and union territories of India (Table 1, Appendix, wwwnc.csdc.gov/EID/article/19/9/13-0117-T1.htm). The proportion of AFP cases with inadequate signs or symptoms believed to represent diphtheria compared with other causes detected in a single year is shown in the Figure.

The average time from paralysis onset to maximal weakness was 10 days (SD 9 days, range 1–30 days).

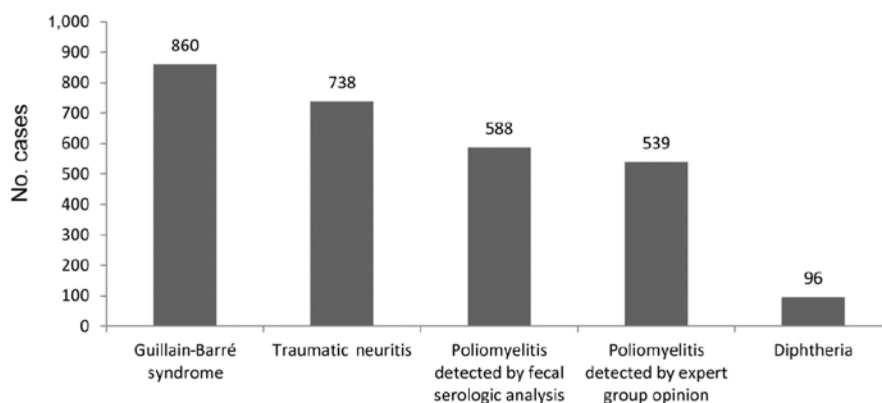

Figure. Reported cases of acute flaccid paralysis in children <15 years of age in India caused by selected factors affecting the peripheral nerve and anterior horn cell, taken from discarded cases in which fecal samples were inadequate to confirm or refute poliomyelitis on the basis of timing of samples or other reasons, 2008. Cases indicated as diphtheria were deemed diphtheritic polyneuropathy by the Expert Review Committee and were suggestive of diphtheritic polyneuropathy but may not meet standard case definitions such as those derived in the European Union. Values above bars are numbers of cases.

Clinical features included fever (60%, n = 9) and laryngeal, pharyngeal, or nasal symptoms (100%, n = 15). Data on exudative pseudomembranes in the pharynx were incomplete at the time of evaluation of AFP and were not part of the screening examination in poliomyelitis surveillance. There were no cases in which cutaneous diphtheria was noted before the onset of paralysis.

Paralysis was ascending (33%, n = 5), descending (27%, n = 4), or uncertain (40%, n = 6). It was most often symmetric (93%, n = 14). The number of paralytic limbs involved was 4 in 73% (n = 11), 2 in 20% (n = 3), and 0 in 13% of cases (n = 2, i.e., palatal and pharyngeal paralysis only). The mean lowest motor power was 3.3/5 in the lower extremities (range 0–5) and 3.7/5 in the upper extremities (range 0–5). Most case-patients also had hyporeflexia or areflexia (73%, n = 11) and hypotonia of the arms (60%, n = 9) and legs (80%, n = 12).

All case-patients had been vaccinated with oral poliovirus vaccine; these case-patients had a mean of 16 doses before AFP onset (range 4–40 doses). No patients had poliovirus identified in fecal samples. Cerebrospinal fluid was analyzed for 6 patients, and results were within reference ranges for all patients (mean ± SD protein level 32 ± 12.8 mg/dL, range 16–49 mg/dL; mean ± SD glucose level 71 ± 17 mg/dL, range 40–89 mg/dL; mean ± SD leukocyte count 2 ± 2 cells/mL, range 0–5 cells/mL). Nerve conduction studies were performed for 6 patients. Results were categorized by the examining physician in India as demyelinating (n = 4), mixed demyelinating and axonal (n = 1), and normal (n = 1) at the time of the study.

Cardiorespiratory failure occurred in 10 (67%) patients and was the attributed cause of death for 8 (53%) patients; an additional patient was ventilator dependent at the time of last follow-up evaluation. Mean ± SD time to death was 24 ± 14 days from AFP onset (range 6–46 days). Guillian-Barré syndrome disability scores among survivors showed persistent disability without major improvement in most patients (median disability score 4 [bedridden or chair bound], median follow-up 60 days, mean 54 days).

Diphtheria in India

During 2007–2011, a total of 55 countries worldwide, including 10 high-income countries, reported >20,000 cases of diphtheria to WHO (*4*). India reported the most cases of diphtheria of any country (n = 17,926) and more than all of the other highest reporting countries combined (Table 2). During 1998–2008, India had 19%–84% of the global incidence of diphtheria. Since the Expanded Program on Immunization was introduced in the late 1970s, the number of diphtheria cases in India has decreased but diphtheria remains a major cause of illness and death. There were 39,231 reported cases in 1980, 8,425 in 1990, 5,125 in 2000, and 3,485 in 2011 (*12*).

Most persons with diphtheria in India are either not vaccinated or have only partial immunization from DTP or DTP combination vaccines (*13*). In this case series, vaccination status for DTP was not unreported during routine AFP surveillance because the programmatic focus was on poliomyelitis. In India in 2011, >4 million children did not receive a first dose of DTP vaccination and >2.5 million did not receive a third dose (*12*,*14*). Although this number represents the minority of all children in India, this country had the highest number of children worldwide who had not received DTP vaccine (*12*,*14*).

Clinical Aspects of Diphtheritic Polyneuropathy

Diphtheritic polyneuropathy is a toxic complication of initial infection with *C. diphtheriae* resulting from hematogenous dissemination of intracellular toxin subunit A. Transport down the axon of newly synthesized protein with eventual destruction of the myelin sheath accounts for the delay in neuropathic symptoms (mean 8 weeks) after initial infection (*15*). Diphtheritic polyneuropathy is potentially reversible, and surviving patients often report few to no neurologic symptoms (*16*). Our report attempts to underscore the key clinical features of diphtheritic polyneuropathy, including initial sore throat; nasal, laryngeal, and pharyngeal involvement; neck swelling with the classic bull neck appearance; and the potentially long time course between initial infection and neuropathic symptoms.

Diphtheritic polyneuropathy is generally considered a demyelinating neuropathy with proximal to distal spread of weakness and prominent sensory features. However, in children and in resource-limited settings, simply examining for pharyngeal exudate in the setting of AFP may help lead to a clinical diagnosis, throat swab, and appropriate management with antidiphtheroid serum and antimicrobial drugs when available (*17*,*18*). Isolation of the bacterium from a throat swab specimen is unlikely in most cases. Other reports of diphtheritic neuropathy in India have been bacteriologically confirmed for 15%–39% of patients (*19*–*21*). Indiscriminate use of antimicrobial drugs before the diagnosis of diphtheria may make culture positivity even more difficult (*17*,*19*) and is also possible in this case series because many parents in India first seek the advice of local healers and health care workers before seeking formal medical evaluation.

In the United States, diphtheria antitoxin is available only through the Centers for Disease Control and Prevention and should be given as soon as possible after diagnosis of diphtheria or suspected diphtheria (*2*,*18*). Although rapid administration of diphtheria antitoxin reduces the case-fatality rate for respiratory diphtheria, antitoxin administration after day 1 of diphtheritic polyneuropathy shows no benefit (*22*). Many countries no longer store diphtheria antitoxin for therapeutic use. A recent survey including

Table 2. Countries reporting >100 cases of diphtheria, 2007–2011*

Rank in no. reported cases	Country	World Bank gross national income per capita level (2010)	World Health Organization region	No. reported cases
1	India	Low	Southeast Asian	17,926
2	Indonesia	Lower middle	Western Pacific	1829
3	Nepal	Low	Southeast Asian	710
4	Iran	Upper middle	Eastern Mediterranean	380
5	The Philippines	Lower middle	Western Pacific	329
6	Sudan	Low	African	243
7	Bangladesh	Low	Southeast Asian	190
8	Russia	Upper middle	European	169
9	Ukraine	Upper middle	European	167
10	Haiti	Low	Americas	151
11	Pakistan	Low	Eastern Mediterranean	136
12	Brazil	Upper middle	Americas	127
13	Thailand	Lower middle	Southeast Asian	105
14	Afghanistan	Low	Southeast Asian	104

*Source: World Health Organization Immunization Assessment, Surveillance, and Monitoring: Diphtheria (www.who.int/immunization_monitoring/data/data_subject/en/index.html).

47 countries in which the diphtheria antitoxin stock was known during 2007–2008 found that only 57% stocked antitoxin, including countries in which diphtheria antitoxin is still produced (*23*). India produces and exports diphtheria antitoxin, but local patients are often unable to pay for this therapy (*20*,*23*).

Detection of Diphtheritic Neuropathy by AFP Surveillance Systems

Our study demonstrates that diphtheritic neuropathy can be detected through existing AFP surveillance systems designed to detect poliomyelitis and may be pragmatically expanded to include diphtheritic neuropathy in children <15 years of age. Several additional features make diphtheritic polyneuropathy more difficult to detect than poliomyelitis. Awareness of diphtheritic polyneuropathy may be lower than that of poliomyelitis among health care workers and surveillance officers. Laboratory testing for diphtheria requires special growth media and is less sensitive and less specific for diagnosis of diphtheria than fecal testing for poliomyelitis, especially for patients who have received antimicrobial drugs. In the case of diphtheritic polyneuropathy, nasopharyngeal involvement precedes development of neuropathy and may be resolved by the time AFP develops. Isolated cranial nerve involvement is notable in patients with diphtheritic polyneuropathy and is not currently a major focus of AFP surveillance. Case definitions were developed in higher-income settings and may not be developed for or by low-income practitioners for general use in resource-constrained settings.

Nonetheless, simple additions to the current screening for AFP in diphtheria-endemic areas, including dedicated questions about DTP or DTP combination vaccination status, contacts with known diphtheria case-patients, cutaneous lesions, and nasopharyngeal symptoms might inexpensively improve the detection of diphtheritic neuropathy. Notably, diphtheritic neuropathy, which accounts for a minority of all diphtheritic illness, can signal the need for improved routine DTP or DTP combination vaccination coverage, lead to booster vaccinations for persons in various age groups, and herald the need for release of antitoxin from storage for administration to patients. Because antitoxin is not fully available and must be administered promptly, routine and bolstered immunization strategies remain crucial for reducing the incidence of diphtheria, and early detection through existing surveillance systems is especially needed for nonimmunized community populations.

Study Strengths and Limitations

Although this study represents a convenience sample because of chart availability and may represent reporting bias, the case-patients reported had high case-fatality and disability rates at last known follow-up evaluation at 60 days. The cases have fairly complete information regarding onset and course of the neuropathy but lack details surrounding the first phase of the diphtheritic illness, infectious contacts, and community-level and person-level vaccination status. None of the case-patients reported, with the possible exception of 1 case-patient, represent definite diphtheria diagnosed without bacteriological identification. This finding is also common in outbreaks in middle-income countries because the organism may be difficult to identify (*19*,*21–24*). All cases in this report should be best considered as suggestive of diphtheria.

It is possible but unlikely that these case-patients had Guillain-Barré syndrome, poliomyelitis, or other causes of AFP (*25*,*26*). In addition to diphtheria, there are other differential diagnoses for AFP with respiratory involvement for patients in this age group. However, these case-patients displayed several classic features of diphtheria and meet the definition of having probable cases. Finally, AFP surveillance in India is targeted to persons <15 years of age. Diphtheria is not limited to persons in this age

group and may be seen in adults, including older adults. In higher-income countries, eradication of diphtheria was first achieved among children in the youngest age groups and later older children and adults became more vulnerable because of waning immunity or incomplete immunization (*27*). A study specific to healthy adults in India found that adults in the oldest age group were least likely to have protective levels of antibodies against *C. diphtheria* (*28*).

Given resource constraints, the high reported mortality rate in this case series approximates the mortality rate for reports of diphtheria outbreaks in India in general but represents selection bias for more severe cases. The mortality rate was 73% in a case series from Assam and was maximal in persons 3–5 years of age (*27*). Another report for Assam found that 31% of patients with diphtheria died (*20*). These case series identified polyneuropathy in a minority of cases, suggesting that diphtheritic polyneuropathy in low-income settings might represent more severe presentations of the disease and, by the time of presentation, might be less responsive to standard interventions.

Conclusions

The present study reports a sample of diphtheritic polyneuropathy that occurred in India over a 7-year time frame. Diphtheria represents a small proportion of AFP cases in India. AFP is the most severe presentation of polyneuropathy. If polyneuropathy also occurs in 20% of patients with diphtheria in this region, then surveillance of diphtheritic polyneuropathy by existing networks identifies a small but major fraction of severe diphtheria cases in India.

Diphtheria can be eliminated for several reasons: the reservoir is only humans; there is a safe, effective, and affordable vaccine; and seasonal outbreaks make transmission interruption possible (*27*). However, a low level of transmission of diphtheria, a fully vaccine-preventable disease, leaves the possibility for long-term reemergence of diphtheria in many countries in which vaccination has been inadequate. Asymptomatic carriers persist. Partial immunization; increased numbers of diphtheria cases in certain groups, such as women and certain religious groups; and changing demographic patterns, such as cases in older children, are all concerning features that lead to continued disease transmission (*13*). Poor awareness for additional protection of DTP and DTP combination vaccines compared with other vaccinations and societal and individual-level fatigue with vaccinations in general can reduce free and available vaccination against diphtheria (*19*). Late and missed diagnoses and limited expertise in sample collection may contribute to the high case-fatality rate. Case-based surveillance and mandatory booster vaccination at the time of primary school entry have been suggested for children in India (*13*).

Acknowledgment

F.J.M. was responsible for study conception, study design, data interpretation, drafting, and editing of the manuscript; S.B participated in the study design and manuscript editing; A.K. participated in study design and manuscript editing; and R.W.S. participated in data interpretation and manuscript editing.

F.J.M. was supported by a research fellowship from the American Brain Foundation.

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Nodding Syndrome

Scott F. Dowell, James J. Sejvar, Lul Riek, Katelijn A.H. Vandemaele, Margaret Lamunu, Annette C. Kuesel, Erich Schmutzhard, William Matuja, Sudhir Bunga, Jennifer Foltz, Thomas B. Nutman, Andrea S. Winkler, and Anthony K. Mbonye

An epidemic illness characterized by head nodding associated with onchocerciasis has been described in eastern Africa since the early 1960s; we summarize published reports and recent studies. Onset of nodding occurs in previously healthy 5–15-year-old children and is often triggered by eating or cold temperatures and accompanied by cognitive impairment. Its incidence has increased in Uganda and South Sudan over the past 10 years. Four case–control studies identified modest and inconsistent associations. There were nonspecific lesions seen by magnetic resonance imaging, no cerebrospinal fluid inflammation, and markedly abnormal electroencephalography results. Nodding episodes are atonic seizures. Testing has failed to demonstrate associations with trypanosomiasis, cysticercosis, loiasis, lymphatic filariasis, cerebral malaria, measles, prion disease, or novel pathogens; or deficiencies of folate, cobalamin, pyridoxine, retinol, or zinc; or toxicity from mercury, copper, or homocysteine. There is a consistent enigmatic association with onchocerciasis detected by skin snip or serologic analysis. Nodding syndrome is an unexplained epidemic epilepsy.

Nodding syndrome as a distinctive entity was reported from southern Sudan in the 1990s and investigated by local authorities and the World Health Organization (WHO) during 2001–2002 (*1*,*2*). In retrospect, children with head nodding, or rhythmic dorsoventral movements of the head (*3*), as 1 characteristic feature of epilepsy syndromes, had been observed in Tanzania, Liberia, and western Uganda as far back as the 1960s but were not studied separately or described as a distinctive clinical group (*3–5*). The term nodding disease was first applied in southern Sudan in the 1990s to describe the occurrence of repetitive head nodding, characteristically occurring among children while eating, and variably associated with other seizure activity, neurologic and cognitive impairment, delayed puberty, and growth retardation.

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DOI: http://dx.doi.org/10.3201/eid1909.130401

Thousands of cases have been reported from southern Tanzania, northern Uganda, and South Sudan, although much smaller numbers have been documented and investigated in any detail (*6–13*). The effect of the disease on families and communities can be devastating because previously healthy young children drop out of school, lose the ability to eat, and require constant oversight because they might fall into a cooking fire or wander off and drown. Local authorities and national governments requested assistance from WHO, the US Centers for Disease Control and Prevention (CDC), and other agencies.

Investigations have confirmed a similar syndrome in Uganda and southern Sudan, in which the syndrome produced the characteristic clinical features, the age of onset was tightly clustered among children 5–15 years of age, and the reported incidence became higher during recent years (Figure 1) (*7*,*10–14*). Case series of patients have been intensively described and investigated by evaluations of cerebrospinal fluid (CSF), brain imaging, and video electroencephalography (EEG), and 4 case–control studies have been conducted to assess risk factors for the disease and test for infectious pathogens, toxin exposures, and nutritional deficiencies (*2*,*6–10*). Associations with onchocerciasis and nutritional deficiencies have been consistent features, but no definitive underlying cause has been identified.

Major unanswered questions remain about the reason for the persistent association with onchocerciasis, possible contributions of nutritional deficiencies or unidentified toxin exposures, and optimal treatment and prognosis. Some of the most detailed investigations have been conducted recently or are ongoing, and much of what is known about the syndrome remains unpublished. In this review, we aimed to include all available information, including unpublished reports of earlier investigations and major recent findings, to provide the fullest possible picture (Table 1).

Descriptive Epidemiology

According to media reports and assessments from local officials, there may be as many as 3,000–8,000 cases of nodding syndrome in the districts of Kitgum, Pader, and Lamwo in northern Uganda, and Western and Central

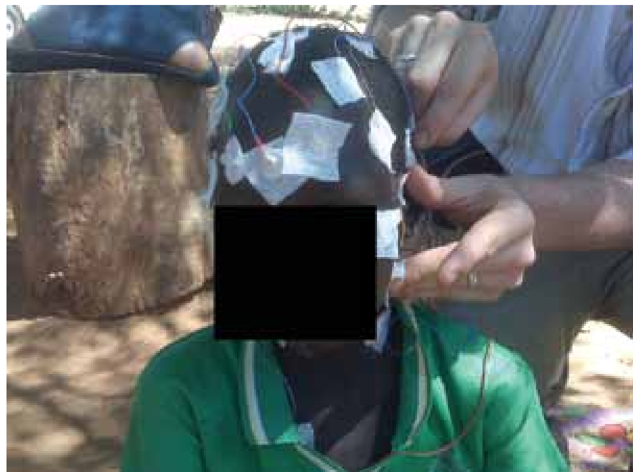

Figure 1. Child with nodding syndrome, on whom electroencephalographic leads are being attached, Uganda, 2009.

Equatoria States in South Sudan (*1*,*15*). In March 2012, the Uganda government actively sought to register cases for the purpose of providing services and recorded >3,000 names, although a standardized and consistently applied case definition was not used. Detailed investigations that used consistently applied case definitions and active community outreach have identified ≥224 cases across Kitgum District in Uganda and 260 cases in Western Equatoria State in South Sudan; complete ascertainment of all cases was not the primary objective in either investigation (*6*,*8*,*11*). Widespread registration of cases has not been completed in Tanzania, but in a study conducted in 2005, a total of 62 cases were documented and investigated in detail (*9*). What seems clear is that there are at least several hundred affected children currently in the 3 geographic areas, and the actual numbers might be much higher. In July 2012 a standard set of case definitions (Table 2) was developed during an international meeting on nodding syndrome in Kampala, Uganda (*16*), and applied in an extensive community survey across affected districts of northern Uganda.

Deaths among nodding syndrome patients also are commonly reported but incompletely ascertained. A Uganda news report in May 2012 listed the number of deaths at 205, but the Ministry of Health could not confirm that all of these deaths were a result of nodding disease (*15*). Anecdotal reports of deaths from drowning, burns, and other causes among nodding syndrome patients are common, but a collaborative effort between the Uganda Ministry of Health and CDC to register deaths and obtain autopsy specimens resulted in 1 autopsy over an 18-month period during 2011–2012. A 2009 follow-up investigation of 62 patients with nodding syndrome in Tanzania first evaluated in 2005 identified 2 deaths that occurred in the interim (*9*,*10*). A follow-up investigation of 12 patients in Uganda evaluated in 2009 and 2010 identified interval worsening in 6 patients,

Table 1. Studies of nodding syndrome and major findings

Location, author, date (reference)	Major finding*
Tanzania, Aall-Jilek, 1965 (*4*)	Reported nodding as symptom in a description of epilepsy
Liberia, Van der Waals et al., 1983 (*3*)	Described seizure disorders as dorsoventral movements of the head
Uganda, Kaiser et al., 2000 (*5*)	Reported head nodding as 1 feature of complex partial seizures
Sudan, Tumwine, et al.2001–2002 (*2*)	Described nodding disease as a progressive epileptic encephalopathy; weak associations with measles, sorghum, and baboon brain consumption; stronger associations with testing for onchocerciasis and *Mansonella perstans* nematodes
Tanzania, Winkler et al., 2008 (*9*)	Reported clinical description of 62 patients; 48 CSF samples mostly clear, 2/10 EEG interictal changes (no recording of nodding episodes), and 8/12 nonspecific MRI changes
Uganda, Sejvar et al., 2009 (*7*)	Reported neurologic and clinical characterization of the syndrome, EEG documenting atonic seizure as cause for nodding, and negative CSF and MRI findings
Uganda, Foltz et al., 2009 (*6*)	Reported descriptive epidemiology and case–control results, and associations with munitions, crushed roots, and antibodies against *Onchocerca* spp. nematodes
Uganda, unpub. data, 2010	Reported follow up case–control results; associations with gun raids and antibodies against *Onchocerca* spp. nematodes; no differences for questions regarding consumption of crushed roots
Tanzania, Winkler et al., 2010 (*10*)	Provided additional detail on 62 aforementioned patients; unsatisfactory seizure control and cognitive impairment
South Sudan, Nyungura et al., 2011 (*11*)	Described features of 96 cases
South Sudan, Riek, 2011 (*8*)	Reported skin snip specimens with microfilaria more common among patients than controls.

*CSF, cerebrospinal fluid; MRI, magnetic resonance imaging; EEG, electroencephalography.

improvement in none, and no deaths (*7*). Although the mortality rate associated with nodding syndrome remains to be accurately defined, media reports from affected communities imply that this rate is high, and long-term studies of childhood epilepsy also suggest that it will probably increase (*17*).

On the basis of reports for Uganda and probably for South Sudan, the incidence of nodding syndrome appears to be increasing (Figure 2). The earliest cases among the 224 documented patients in Uganda occurred in 2000, except for 1 possible onset in 1997, and there was a steady increase in cases identified through the study in 2009. In South Sudan, the earliest cases recognized were in 1991 in Mundri County and 1995 in Lui Township. Community reports from a village administrator in Sudan (*1*) and focus groups in Uganda

Table 2. Recommended case definitions for nodding syndrome

Case	Characteristics*
Suspected	Reported head nodding in a previously healthy person†
Probable	Suspected case, with at least 2 major and 1 minor criteria
	Major criteria
	Age 3–18 y at onset of head nodding
	Nodding frequency 5–20 times/min
	Minor criteria
	Other neurologic abnormalities (cognitive decrease, school dropout due to cognitive/behavioral problems, other seizures or neurologic abnormalities)
	Clustering in space or time with similar cases
	Triggering by eating or cold weather
	Delayed sexual or physical development
	Psychiatric manifestations
Confirmed	Probable case, with documented head nodding episodes:
	Observed and recorded by a trained health care worker
	Videotaped head nodding episode
	Video/EEG/EMG documenting head nodding as atonic seizures

*As agreed upon at the first International Conference on Nodding Syndrome, Kampala, Uganda, July 2012 (*16*). EEG, electroencephalography; EMG, electromyography.
†Repetitive involuntary drops of the head toward the chest on ≥2 occasions.

(*6*) indicated that previous generations had not been affected by this disease. In contrast, reports of head nodding in Tanzania date back >50 years (*4*), and it is not clear from available reports whether the incidence has increased.

The disease appears to be localized in 3 noncontiguous areas in South Sudan, Uganda, and Tanzania (Figure 3). Although head nodding as 1 feature of seizure disorders has been reported from Liberia (*3*), Taiwan (*18*), and elsewhere, clustering of hundreds of cases of this syndrome and the same manifestations has not been described elsewhere. Although onchocerciasis is endemic to all 3 areas, the distribution of this parasitic disease is much wider, extending to across much of eastern and western Africa (*19*,*20*) and Central and South America (*21*), which are huge areas with populations apparently unaffected by nodding syndrome (Figure 3).

Most of the populations affected by nodding syndrome were internally displaced; in Uganda and Sudan, the conflict with the Lord's Resistance Army during the 1990s resulted in dependence on refugee camps and in widespread food shortages during the years preceding nodding syndrome. In Tanzania, most (58/62) of the described patients with nodding syndrome were members of the Pogoro tribe (*9*). Although the Pogoro were not recently internally displaced refugees, they are among the poorest of the region, and therefore susceptible to food shortages (*22*). Potential associations of nodding syndrome with hunger, specific micronutrient malnutrition, or ingestion of toxic substances or contaminated relief foods have been explored in 4 case–control studies, as detailed below.

The distinctive age distribution (tight clustering among persons 5–15 years of age) is a consistent feature of nodding syndrome (Figure 4). Caregivers report that the children were unaffected as infants and had apparently normal growth; most of these children achieved their developmental milestones until the onset of nodding (*6*,*8*,*10*). Although persons of other ages with onset of nodding are occasionally identified, the disease is rare among younger children or adults. Onset in late childhood or early adolescence is seen in certain epilepsy syndromes (*3*,*5*), autoimmune diseases such as juvenile idiopathic arthritis (*23*), Sydenham chorea and other complications of group A streptococcal infections (*24*), and some nutritional toxicities such as konzo (*25*) and neurolathyrism (*26*). Many epidemic infectious diseases predispose the very young or elderly, but some clustering of infections among persons 5–15 years of age are occasionally seen in neurologic infections such as meningococcal meningitis (*27*), parasitic infections such as urinary schistosomiasis (*28*), or epidemic viral infections such as mumps (*29*).

Clinical Evaluations

Clinical evaluations of nodding disease as a distinctive entity began with the more formal description of the syndrome in South Sudan in the late 1990s and have included at least 4 detailed investigations (Table 3). Because case definitions were not standardized, studies might include clinical entities that were not identical, but repetitive head

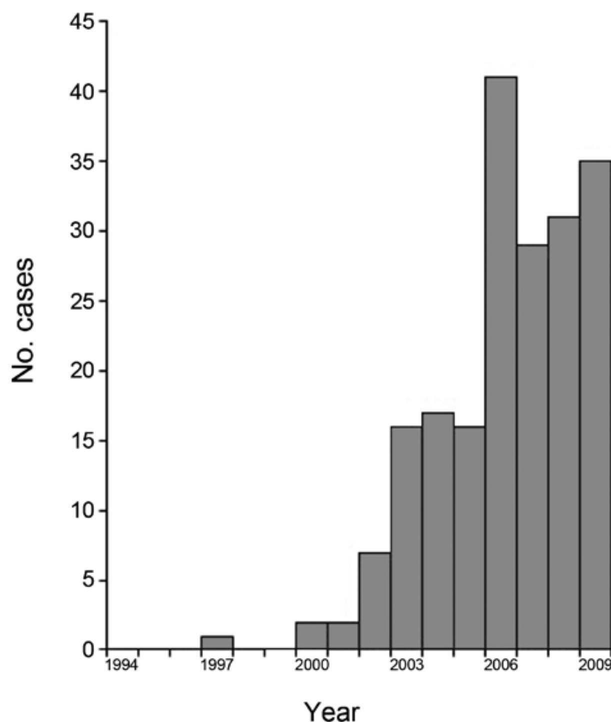

Figure 2. Epidemic curve of nodding syndrome cases in Kitgum District, Uganda, by year of onset. Modified from Foltz et al. (*6*).

Figure 3. Countries in the former Onchocerciasis Control Programme in western Africa in which onchocerciasis was eliminated as a public health problem through vector control (green); countries in the African Programme for Onchocerciasis Control in which onchocerciasis control is ongoing through annual mass treatment with ivermectin (beige); and areas in Southern Sudan, northern Uganda, and southern Tanzania in which nodding syndrome has been reported (red circles).

nodding was common to all studies, and definitions used were broadly compatible with the new standards (Table 2).

General clinical descriptions identify variations in behavior and body habitus, ranging from children who appeared and behaved appropriately for age to those that were obtunded, drooling, and unable to stand or walk independently. Stunting and wasting were commonly documented, as were facial scars from burns and other injuries. It is not known whether stunting and wasting are results of the disease or predisposing factors. Dysmorphic facial features, chest wall abnormalities, dwarfism, and delayed sexual development have been noted but not consistently documented.

Focal motor abnormalities were found in a few of the 81 patients who were given complete examinations, including upper motor neuron abnormalities in 4 patients (*9*), ataxia in 1 (*7*), and involuntary movements and nystagmus in 9 (*2*). Clinical findings often associated with epileptic encephalopathy, such as altered level of consciousness, drooling, and incontinence of urine, were noted more frequently (*2*,*7*).

In contrast, all 4 investigations identified major cognitive deficits, despite the challenges of standardized testing in the rural setting in Africa. In early investigations in Sudan, 15% of patients had mental retardation (test not specified) (*2*). Of the 62 persons in Tanzania, 40% had cognitive impairment (as reported by mothers who used a simple grading system of slight, moderate, and severe impairment), half of them severe. In Uganda, a simple neurocognitive instrument administered to 78 case-patients and age-matched controls showed major cognitive impairment among case-patients (*7*). Anecdotally, cognitive impairment appears to be progressive (*2*,*7*). Evidence of psychiatric disturbances have included visual and auditory hallucinations reported in 29% and 26% of patients, respectively (*6*), along with occasional features such as shouting, screaming, and jumping up and running in circles (*2*).

Nodding episodes occur several times a week to many times per day, and episodes have been observed by investigators, recorded videographically, and documented by video–EEG (*7*). Often triggered by eating or cold weather, the head drops repeatedly toward the chest in cycles of 5–20 nods/min for several minutes. Nodding may be accompanied by automatisms or other seizure-like activity, and the child is either unaware of his or her surroundings or has a decreased ability to continue an activity (e.g., eating) or respond to questions or external stimuli.

Simultaneous recording of 2 episodes by EEG, videography, electromyography, and electrocardiography

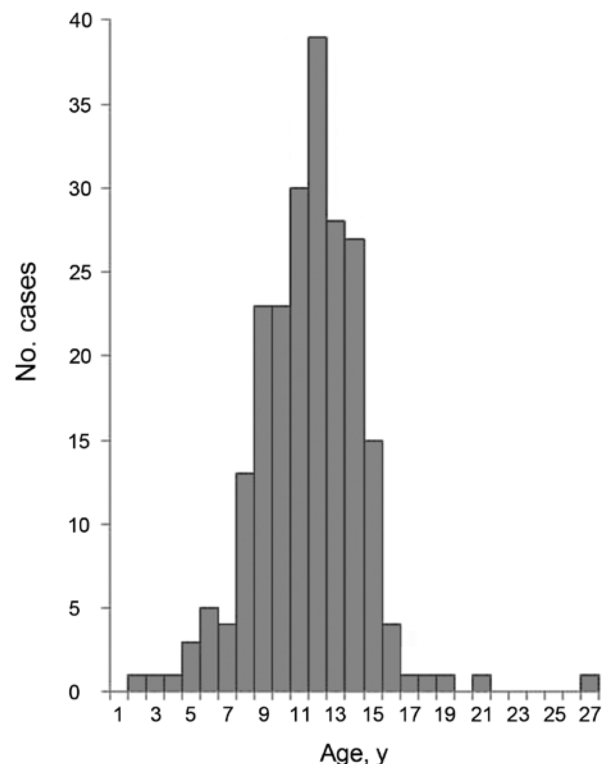

Figure 4. Age distribution of patients at onset of nodding syndrome, Kitgum District, Uganda. For nodding syndrome elsewhere, age distribution tightly clusters in persons 5–15 years of age. Modified from Foltz et al. (*6*).

Table 3. Clinical and neurodiagnostic findings of case studies of nodding syndrome*

Location, author, date, (reference)	No. patients, case definition	Clinical findings	EEG findings	CSF findings	Neuroimaging findings
Sudan, Tumwine et al., 2001 (*2*)	39 with episodes of repetitive head nodding several times a day	Neurologic examination results largely normal for 32 patients, with exception of mental retardation	39 cases evaluated; seizures recorded in 6; disease progression correlated with diffuse slowing and delta–theta activity	16 CSF specimens negative for *Onchocerca* spp. by PCR	ND
Tanzania, Winkler et al., 2008 (*9*)	62 with a repetitive short loss of neck muscle tone resulting in nodding of the head	Neurologic examination results for 12 patients largely unremarkable; 2 with upper motor neuron findings	6 of 10 with abnormal EEG results, including intermittent generalized slowing and sharp wave activity	48 CSF specimens; 3 with increased lymphocyte counts; protein and glucose levels within reference range for all	8 of 12 brain MRIs showing abnormalities, including hippocampal abnormalities (3), gliotic lesions (3), or both (2)
Uganda, Sejvar et al., 2010 (*7*)	23 with head nodding in previously normal child, with ≥1 other neurodevelopmental abnormality	Neurologic examination results for 23 patients largely unremarkable; 2 with focal findings	10 of 12 with abnormal EEG results, including generalized slowing and runs of spike activity; 2 nodding episodes recorded, demonstrating atonic seizure	16 CSF specimens; all grossly clear, with glucose and protein levels within reference ranges	4 of 5 brain MRIs showing varying degrees of cortical and cerebellar atrophy disproportionate to age; no focal/white matter lesions
South Sudan, Bunga, 2011 (unpub. data)	25 with head nodding in previously normal child, with ≥1 other neurodevelopmental abnormality	Neurologic examination for 25 nonfocal patients	ND	ND	ND

*EEG, electroencephalography; CSF, cerebrospinal fluid; ND, not done; MRI, magnetic resonance image.

documented that the nodding episodes are manifestations of atonic seizures. In these case-patients, head nodding was associated with generalized electrodecrement, followed by generalized sharply contoured rhythmic theta activity, dropping of the chin, and paraspinal electromyographic abnormalities (Figure 5) (*7*). Other EEG recordings in ≥61 patients reported or observed to have nodding syndrome have consistently identified interictal abnormalities and various electrographic seizure types (*2*,*7*,*9*). In the Sudan patient series, which included 32 patients at various stages of disease, worsening or more severe clinical disease was associated with more severe EEG findings, as shown by progressively more abnormal background activity, ultimately resulting in diffuse subcontinuous nonreacting delta–theta activity and loss of normal cerebral electrical architecture (*2*). Biphasic or triphasic periodic sharp waves of the type frequently seen in human prion diseases or metabolic disorders were not observed in any of these assessments, and there were no periodic lateralizing epileptiform discharges suggestive of encephalitis from herpesvirus or other viruses.

Assessments of CSF have been documented for ≥80 patients in Tanzania, South Sudan, and Uganda (*2*,*7*,*9*). Specimens were generally characterized as grossly clear, and all glucose and protein levels were within reference ranges for age. Among 48 patients for whom CSF cell counts were available, only 3 (6%) patients had increased leukocyte counts of 6, 8, and 28 cells/µL, and the third sample was reported as being contaminated with blood (*9*).

Brain magnetic resonance imaging has been documented for ≥17 patients in Tanzania and Uganda (*7*,*9*). For 4 of the patients in Tanzania, images were interpreted as showing normal results. Images for all 5 patients in Uganda were interpreted as showing generalized atrophy (Figure 6). Five patients from Tanzania had nonspecific signal abnormalities with hyperintensity on T2-weighted images, and 5 patients from Tanzania and 2 from Uganda had hippocampus abnormalities. None of the patients showed evidence of meningeal or parenchymal inflammation, cystic or other lesions consistent with acute disseminated encephalomyelitis, neurocysticercosis, central nervous system tuberculosis, or other focal brain infection. Pulvinar sign (*30*), cortical ribboning (*31*), or other findings suggestive of human prion disease have not been identified.

One brain from a patient with nodding syndrome in Uganda was examined pathologically at Makerere University (Kampala, Uganda) and at CDC (Atlanta, GA, USA). Because of delays in obtaining the autopsy and fixation of the brain, tissue sections were degraded and largely uninterpretable.

The response to different anti-epileptic drugs has been variably reported by parents and clinicians as occasionally but not consistently helpful. Winkler et al. characterized seizure control among 59 patients receiving therapy (primarily phenytoin and phenobarbital) as being effective compared with seizure control before treatment, but 45% still had head nodding 1–5 times/month, and 21% still had

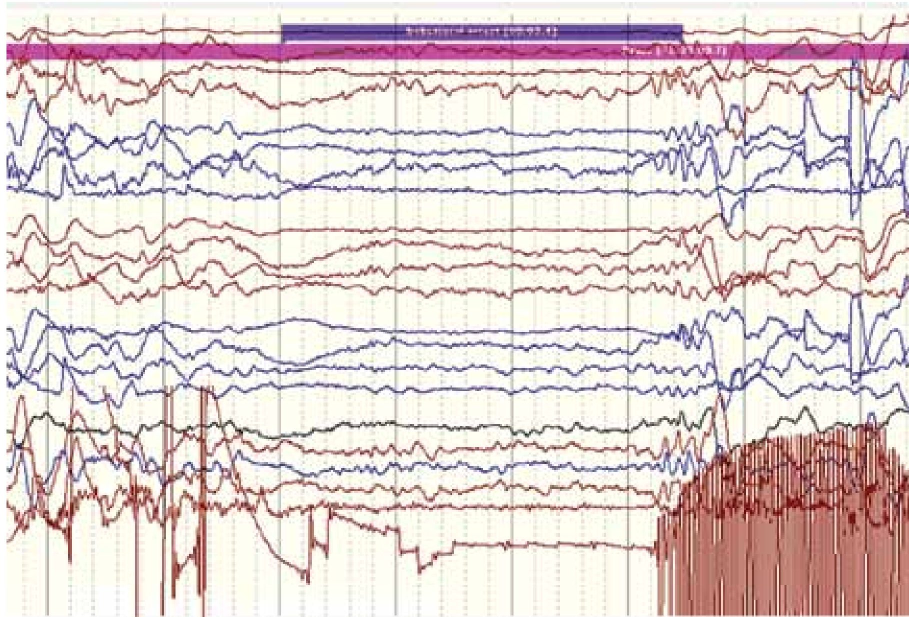

Figure 5. Ictal electroencephalographic recording of a 12-year-old boy in Uganda with nodding syndrome obtained during a typical nodding episode. Shown is a sudden electrodecremental episode with concomitant electromyographic decrease in neck muscles, followed by sharply contoured theta activity.

daily episodes (*10*). Similar impressions were reported from caretakers and parents in Uganda (*7*) and Sudan (*2*). The comparative efficacy of anti-epileptic drugs has not been assessed in controlled studies.

Risk Factors and Causes

Two general approaches for identification of the underlying cause of nodding syndrome have been taken. The first approach is laboratory testing of a series of patients for various infectious agents, toxin exposures, or nutritional deficiencies (*2*,*6*,*8*,*9*). The second approach is comparison of laboratory-based or reported exposures of such patients with a group of unaffected control children of similar ages from the same location (*2*,*6*,*8*).

Four case–control studies have been performed among 173 case-patients and 179 controls (Table 4). Dozens of exposures have been assessed by history, physical examination, or laboratory testing; only significantly associated exposures or prominent a priori hypotheses are shown in Table 4. Several exposures have been moderately more common among case-patients, and none of the findings except for results of tests for infection with *Onchocerca volvulus* nematodes has been reproducible across various investigations (Tables 5, 6).

In the early investigations from Sudan, consumption of red sorghum, consumption of baboon brain, and absence of reported history of measles were identified as being potentially associated with nodding syndrome, but assessments in the 2 studies in Uganda and the subsequent study in Sudan did not confirm these findings. Consumption of crushed roots in Uganda in 2009 was also not confirmed as a pre-illness exposure when explored with additional questions in 2010 and 2011. The association with munitions in Uganda in 2009 was found to be an association with gun raids and not chemicals, as initially hypothesized. In addition to associations with microfilariae obtained by skin snips and serum antibodies against 3 recombinant *Onchocerca* spp. nematode–specific proteins (Ov-16, Ov-FAR1, and Ov-MSA), the observation that case-patients were more likely than controls to show stunting and wasting was a consistent finding, as was a prominent deficiency of pyridoxine (vitamin B6) among case-patients and controls in Uganda and South Sudan.

Testing in these studies and observational series of case-patients in Tanzania has been extensive. Results can be grouped into those for tests for known or hypothesized

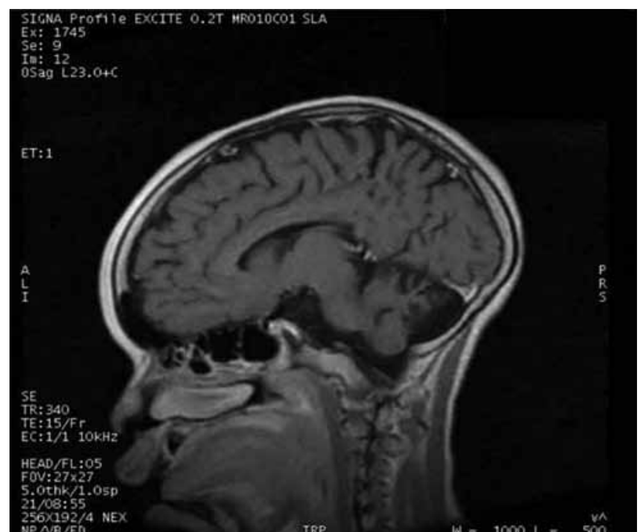

Figure 6. Magnetic resonance image of a 13-year-old boy in Uganda with nodding syndrome. Image shows prominent cortical atrophy.

causes of infectious encephalitis or postinfectious encephalopathy (Table 5), and toxic encephalopathy, nutritional neuropathologic changes, or genetic epilepsy disorders (Table 6). A molecular approach that does not require a priori hypotheses and uses broadly reactive PCR primers specific for 19 viral families and represents hundreds of potential pathogens, has shown negative results (*6*). Most of the hypothesis-directed testing has also shown negative results.

The various tests for onchocerciasis are notable exceptions to these findings. In all studies that assessed the association between onchocerciasis and nodding syndrome,

Table 4. Selected risk factors for nodding syndrome from 4 case–control studies*

Risk factor	Study (reference): Sudan, 2002; 13 case-patients, 19 controls (*2*)	Uganda, 2009; 49 case-patients, 49 controls (*6*)	Uganda, 2010; 73 case-patients, 73 controls†	South Sudan, 2011; 38 case-patients, 38 controls (*7,8*)
Infectious				
History of measles	Less common (15% cases vs. 58% controls; p = 0.03)	No difference (24% case-patients vs. 6% controls; p = 0.02‡)	NA	NA
History of malaria	NA	ND	NA	NA
History of meningitis	No difference (0% vs. 6%; p = 1.0)	NA	NA	NA
Prior treatment for onchocerciasis (ivermectin)	No difference (62% cases vs. 37% controls)	No difference (33% case-patients vs. 25% controls)	NA	NA
Consumption of animal brain (risk for prion disease)	Baboon brain consumption (46% vs. 22%; p = 0.25)	No association with brain consumption	NA	NA
Toxic/environmental				
Exposure to munitions	NA	More common in case-patients (71% vs. 51%; p = 0.06)	Exposure to gun raids more common in case-patients (54% vs. 27%)	ND
Exposure to pesticides (on seeds)	No difference	ND	NA	NA
Consumption of crushed roots	NA	More common in case-patients (39% vs. 16%; p = 0.02)	No differences in 17 root types before onset of nodding syndrome; root consumption more common after onset of nodding syndrome (22% vs. 0%)	ND
Consumption of cassava (thiocyanate toxicity)	Widely consumed, no acute toxicity reported	ND (100% consumption)	Specifically asked about bitter cassava: no difference	NA
Nutritional				
Early malnutrition	NA	NA	NA	Hunger more common for case-patients 2 y of age (24% vs. 8%; p = 0.03)
Current wasting	Weight-for-age Z scores lower in case-patients (−1.6 vs. −1.0, p = 0.09)	Low BMI for age (42% vs. 13%; p<0.01)	Low BMI for age (42% vs. 26%; p = 0.03)	Low BMI for age (16% vs. 3%; p = 0.06)
Current stunting	Height-for-age Z scores lower in case-patients (−1.5 vs. −0.8, p = 0.29)	Low height for age (60% vs. 29%; p<0.01)	Low height for age (35% vs. 20%; p = 0.05)	Low height for age (24% vs. 3%; p = 0.03)
Consumption of red sorghum	54% case-patients vs. 16% controls; p = 0.05	ND	ND	ND
Consumption of spoiled relief foods	NA	ND	NA	NA
Consumption of river fish	NA	ND	NA	ND
Consumption of rodent brain	NA	ND	NA	NA
Consumption of river water	NA	ND	NA	NA

*Selected risk factors, all positive associations and selected negative findings (see original reference for full listings). NA, not assessed; ND, no difference; BMI body mass index.
†Unpub. data.
‡Not significant after age adjustment.

there has been a trend toward more positive results for case-patients than controls, and this trend has been observed in testing for microfilaria by skin snip in the 2 studies in South Sudan (*2*,*8*) and in testing for serum antibodies against recombinant *Onchocerca* spp.–specific proteins in the 2 studies in Uganda (*6*). Skin snip positivity is strongly reduced by treatment with ivermectin (*32*), but 2 comparisons of case-patients and controls given ivermectin failed to show that case-patients were less likely to have been treated (Table 4). The assumption has been that

Table 5. Possible causes of nodding syndrome, by infectious and postinfectious findings*

Possible cause by category	Investigation (reference)	Negative findings	Positive findings
Infectious encephalitis			
Malaria	Foltz et al., Uganda (*6*)	Blood smear (98% case-patients vs. 95% controls)	None
Trypanosomiasis	Foltz et al., Uganda (*6*)	Seronegative (36 patients tested)	None
	Tumwine et al., Sudan (*2*)	Seronegative (69 patients tested)	None
Cysticercosis	Foltz et al., Uganda (*6*)	Seronegative (36 patients tested); cysts absent by MRI (5 patients tested)	None
Prion disease	Sejvar et al., Uganda (*7*)	EEG and MRI results and clinical course not compatible	None
	Winkler et al., Tanzania (*9*)	EEG and MRI results and clinical course not compatible	None
Onchocerciasis	Tumwine et al., Sudan, 2001 investigation (*2*)	None	Skin snip specimens for 93% of patients vs. 63% in controls; p<0.001
	Tumwine et al., Sudan, 2002 investigation (*2*)	None	Skin snip specimens for 93% of patients vs. 44% of controls; p<0.008
	Winkler et al., Tanzania (*9*)	48 CSF samples PCR negative for *Onchocerca volvulus* microfilariae	Microfilariae in skin correlated with lesions by MRI; p = 0.02
	Foltz et al., Uganda (6)	None	Antibody in 95% of patients vs. 49% of controls; p<0.001
	Riek et al., Sudan (*8*)	None	Skin snip in 76% of patients vs. 47% of controls; p = 0.02
Other microfilarial disease	Tumwine et al., Sudan, 2001 investigation (*2*)	None	*Mansonella perstans* nematodes in 52% of patients vs. 31% of controls; p = 0.005
	Tumwine et al,, Sudan, 2001 investigation (*2*)	Blood sample for *Loa loa* microfilariae (69 patients tested)	None
	Tumwine et al., Sudan, 2001 investigation (*2*)	Lymphatic filariasis by ICT (26 patients tested)	None
	Unpub. data, Uganda	None	2 skin snip DNA sequences similar to those of *Mansonella* spp. nematodes
Unknown pathogens	Unpub. data, Uganda	42 serum samples and 16 CSF specimens by broadly reactive PCRs for 19 virus families	None
Para/postinfectious encephalopathy			
Measles (SSPE like)	Foltz et al., Uganda (*6*)	No epidemiologic association for 16 CSF samples by PCR	None
Acute disseminated encephalomyelitis	Sejvar et al., Uganda (*7*)	Brain MRI (5 patients)	None
	Winkler et al., Tanzania (*9*)	Brain MRI (12 patients)	None
Poststreptococcal (Sydenham chorea-like)	Sejvar et al., Uganda (*7*)	No movement disorders	None
Neuronal antibodies	Unpub. data (Mayo Clinic, Rochester, MN, USA), Uganda	12 CSF samples for known neuronal antibodies	None
	Unpub. data (Emory University, Atlanta, GA, USA), Uganda	3 CSF samples for neuronal antibodies by in situ hybridization with rat brain slices and human brain homogenate	None
Hepatitis E	Foltz et al., Uganda (*6*)	Seronegative (52% case-patients vs. 58% controls)	None

*EEG, electroencephalography; MRI, magnetic resonance imaging CSF, cerebrospinal fluid; ICT, immunochromatographic test; SSPE, subacute sclerosing panencephalitis.

Table 6. Possible causes of nodding syndrome, by toxic, nutritional, and genetic factors*

Possible cause by category	Investigation (reference)	Negative findings	Positive findings
Toxic encephalopathy			
Mercury	Foltz et al., Uganda (*6*)	Urine (12 patients)	None
	Bunga. , Sudan (*8*)	Urine (20 patients)	None
Homocysteine	Foltz et al., Uganda (*6*)	Urines (23 patients)	None
Thiocyanates (cassava toxicity)	Foltz et al., Uganda (*6*)	Urinary thiocyanate levels not increased (7% patients vs. 7% controls; p = NS)	None
	Bunga, Sudan (*8*)	Urinary thiocyanate levels not increased (20% patients vs. 20% controls; p = NS)	None
Copper	Foltz et al., Uganda (*6*)	No increases in serum levels (17 patients tested)	None
Lead	Foltz et al., Uganda, 2010	No difference (all within reference ranges)	
Arsenic	Bunga, Sudan (*8*)	Urine (20 patients)	None
Nutritional neuropathology			
Cobalamin (vitamin B12)	Foltz et al., Uganda (*6*)	Normal (92% patients vs. 92% controls; p = NS)	None
	Bunga, Sudan (*8*)	Normal (97% patients vs. 100% controls; p = NS)	None
Folate	Foltz et al., Uganda (*6*)	Normal (91% patients vs. 100% controls; p = NS)	None
Pyridoxine (vitamin B6)	Foltz et al., Uganda (*6*)	None	Deficient (73% patients vs. 64% controls; p = NS)
	Bunga, Sudan (*8*)	None	Deficient (79% patients vs. 59% controls; p = 0.06)
Retinol (vitamin A)	Foltz et al., Uganda (*6*)	Normal (60% patients vs. 67% controls; p = NS)	None
Zinc	Foltz et al., Uganda (*6*)	Normal (53% patients vs. 33% controls; p = NS)	None
Selenium	Foltz et al., Uganda (*6*)	None	Deficient (all cases and controls)
Genetic epilepsy			
Deep exome sequencing	Sejvar (Washington University, St. Louis, MO, USA., unpub data) Uganda, South Sudan	No specific epilepsy genes or consistent rare variant genes (1 gene from an affected child in Sudan and 1 gene from an affected child in Uganda sequenced)	None

*NS, not significant.

microfilariae seen were those of *O. volvulus* nematodes and that antibodies are against *Onchocerca* spp. microfilariae, but these 2 assumptions might be inconsistent with some reported results. The first result is that PCRs for spinal fluid from 48 patients in Tanzania and 16 in South Sudan were negative for *O. volvulus* nematodes (*2*,*9*). The second result is that sequencing of DNA from a limited number of skin snip specimens from Uganda suggests the presence of an organism closely related to *Mansonella* spp. nematodes.

Unanswered Questions

The underlying cause of nodding syndrome is a mystery. Studies summarized in this report, taken collectively, have evaluated and eliminated dozens of reasonable hypotheses, including unknown pathogens. Documentation of pathogenesis should be helpful in narrowing the list of possibilities, but identifying the cause of this or any novel epilepsy syndrome is likely to remain a challenge.

The persistent association of nodding syndrome with onchocerciasis is puzzling. Onchocerciasis is widely distributed in areas that do not have nodding syndrome or, considering that systematic evaluations have not been undertaken, where nodding syndrome is not prevalent enough to have resulted in awareness/reporting of the syndrome. This finding suggests an unidentified cofactor or a variant strain of the organism. The possible role of onchocerciasis in epilepsy is an issue of ongoing debate (*33*–*36*), but the organism is not believed to be neuroinvasive, and negative PCR results for 64 CSF specimens from Tanzania and South Sudan further substantiates this suggestion. Recent findings of DNA sequencing of skin snip specimens from Uganda raised additional questions and may point toward a morphologically similar and antigenically cross-reactive filarial species.

Pyridoxine deficiency has been a consistent and unexpected finding among case-patients and controls tested, which is notable because of the known association between

abnormal pyridoxine metabolism and complex, intractable seizures (*37*,*38*). Pyridoxine-dependent seizure is a genetic disease that is not clinically consistent with nodding syndrome, but infants with pyridoxine-dependent seizures can be treated and cured with high daily doses of oral pyridoxine (*38*). A clinical trial is planned in Uganda to assess the role of high-dose pyridoxine, as well as conventional anti-epileptic medications, as treatment for nodding syndrome. Careful evaluation of the relationship between onchocerciasis and pyridoxine in the investigations in Uganda and South Sudan has failed to document an interaction between the 2 variables.

Nutritional toxicity remains a possible cause of nodding syndromes. Konzo, a neurologic disorder that causes permanent spastic paraparesis, shares several epidemiologic similarities to nodding syndrome, including narrow age group clustering among persons 5–15 years of age (*25*). Konzo results from consumption of improperly prepared species of cassava and is sometimes seen during times of famine and particularly among persons with dietary deficiencies of sulfur-containing amino acids. For nodding syndrome, a lack of association of cassava consumption in case–control studies and negative results of testing for urinary thiocyanate indicate that acute or ongoing cyanate exposure makes this specific etiology less likely. However, a similar but unrecognized form of nutritional toxicity remains a possible cause.

Neuronal antibodies represent 1 possible common pathway for a novel epidemic epilepsy associated with exposure to *O. volvulus* nematodes. Such a mechanism is recognized in Sydenham chorea, a distinctive movement disorder now known to result from neuronal antibodies produced against group A streptococcal antigens but cross-reacting with neuronal epitopes in basal ganglia (*39*). Why only a tiny fraction of those with a streptococcal throat infection end up with chorea remains a mystery. In recent years, the number of characterized neuronal antibodies has increased for those antibodies affecting a limited number of patients with paraneoplastic syndromes to a broader range of known antibodies and clinical manifestations (*40*). A recent description of 32 patients with autoimmune epilepsy highlighted several distinguishing features reminiscent of features of nodding syndrome, including frequent complex partial seizures refractory to anti-epileptic drugs, prominent cognitive compromise, and occasional psychiatric symptoms (*40*). Although preliminary testing for known neuronal antibodies at the Mayo Clinic (Rochester, MN, USA) (V. Lennon, unpub. data) and preliminary screening for known or unknown antibodies at Emory University (Atlanta, GA, USA) (A. Levey, unpub. data) have not identified such antibodies in specimens from patients with nodding syndrome, this possibility remains under active investigation.

Aside from questions regarding the underlying cause of nodding syndrome, many questions remain regarding the long-term course, prognosis, optimal treatment, and effective approaches for families and communities. The long-term mortality rate and how to improve survival times and rates remain purely speculative. Reports from parents and clinicians regarding effectiveness of different anti-epileptic drugs are anecdotal with rare exceptions (*10*), and a controlled trial of treatment would be invaluable. Affected families and communities will probably be coping with chronically dependent patients with nodding syndrome for many years with few resources and considerable pressure for effective management.

Acknowledgments

We thank the families and communities for ongoing support of investigations; Allan Levey and Vanda Lennon for sharing unpublished observations; Joseph Kubofcik, Martha Anker, Stella Chungong, Mickey Richer, Peter Spencer, James Tumwine, Martin Opoka, Ayana Yeneabat, Andrea Quarello, Doug Klaucke, Jeffrey Ratto, Carlos Navarro Colorado, Christina Gurnett, Lorraine Alexander, Louise Jilek-Aall, and other members of the investigative teams from South Sudan, Uganda, and Tanzania for assistance; and WHO for international leadership and coordination.

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Divergent Astrovirus Associated with Neurologic Disease in Cattle

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Using viral metagenomics of brain tissue from a young adult crossbreed steer with acute onset of neurologic disease, we sequenced the complete genome of a novel astrovirus (BoAstV-NeuroS1) that was phylogenetically related to an ovine astrovirus. In a retrospective analysis of 32 cases of bovine encephalitides of unknown etiology, 3 other infected animals were detected by using PCR and in situ hybridization for viral RNA. Viral RNA was restricted to the nervous system and detected in the cytoplasm of affected neurons within the spinal cord, brainstem, and cerebellum. Microscopically, the lesions were of widespread neuronal necrosis, microgliosis, and perivascular cuffing preferentially distributed in gray matter and most severe in the cerebellum and brainstem, with increasing intensity caudally down the spinal cord. These results suggest that infection with BoAstV-NeuroS1 is a potential cause of neurologic disease in cattle.

Astroviruses are small, nonenveloped, positive single-stranded RNA viruses with a genome of 6.4–7.3 kb. The family *Astroviridae* comprises 2 genera, *Mamastrovirus* and *Avastrovirus*, known to infect mammals and birds, respectively. Since the first description of human astrovirus (HAstV) in children with diarrhea in 1975 (*1*), a wide variety of astroviruses have been reported in multiple animals including humans, cattle, pigs, sheep, minks, dogs, cats, mice, sea lions, whales, chickens, and turkeys (*2*).

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DOI: http://dx.doi.org/10.3201/eid1909.130682

Enteric astroviruses are transmitted through the fecal–oral route, and regardless of species, most infections are asymptomatic. In humans, the prevalence of exposure is very high, and astrovirus infection is a major cause of acute enteritis in infants (*3*). Clinical disease also can affect elderly and immunocompromised persons. In these persons, the clinical course of infection is acute, with 2–4 days of watery diarrhea and, less commonly, vomiting, headache, fever, abdominal pains, and anorexia (*4*).

Astroviruses have been implicated twice in central nervous system (CNS) disease (*5*,*6*). One study demonstrated an HAstV-PS, which is distinct from the original HAstV and closely related to astrovirus HMO-C (AstV-HMO-C) and HAstV-VA1) (*7*,*8*) in the brain tissue of a 15-year-old boy with X-linked agammaglobulinemia who had encephalitis. HAstV-PS was the only virus detected, and astrocyte infection was confirmed by anticapsid antibody staining (*6*). Serologic evidence of exposure to the closely related AstV-HMO-C was found in 36% of 5–10-year-old children in the United States, which reflects a common childhood infection and indicates that the encephalitis in this child was a likely consequence of his immunodeficiency (*9*). In an outbreak of so-called "neurological shaking disease" in mink, an astrovirus (Mink AstV-SMS) was detected in the brain tissues of multiple naturally and experimentally infected animals showing neurologic signs, including shaking and ataxia (*5*).

Cattle with neurologic signs are vigilantly screened to keep the food chain free of zoonotic pathogens, such as rabies virus, *Salmonella* spp., *Listeria monocytogenes*, *Chlamydia* spp., and the prion agent of bovine spongiform encephalopathy (BSE). In particular, BSE has become a major public health concern after recognition of the association between BSE and prion-associated disease in humans. Therefore, early and rapid recognition of the cause of neurologic disease is vital to the safety of the food chain. Etiologic diagnosis of CNS disease in cattle

requires substantial effort; is costly; and usually presents a challenge because of the large number of pathogens or problems that can cause neurologic disease, including viruses, bacteria, parasites, prions, toxins, and metabolic disorders. Pathogens known to cause CNS disease in cattle include bovine herpesvirus 1 and 5 (BoHV-1 and BoHV-5), lyssavirus (rabies), ovine herpesvirus 2, *L. monocytogenes*, *Histophilus somni*, *Escherichia coli*, *Salmonella* spp., *Chlamydia* spp., *Neospora caninum*, amoebas, and prions (*10*,*11*).

Brain tissue from a yearling steer with an encephalomyelitis and ganglioneuritis of unknown origin was analyzed by using viral metagenomics, which showed a divergent astrovirus distantly related to an ovine astrovirus. By retrospective analysis, this bovine astrovirus associated with neurologic symptoms (BoAstV-NeuroS1) was detected in the brains of 3 of 32 other cattle with encephalitides of undetermined etiology. Virus was detected by RNA in situ hybridization within neurons in the brainstem, cerebellum, and/or spinal cord in all PCR-positive samples from the 4 animals in this study.

Materials and Methods

Sample Preparation and Next-Generation Sequencing

To search for potential viral etiologic agents, we performed an unbiased metagenomic analysis. Viral nucleic acids were enriched from fresh-frozen brain tissue samples (≈25 mg) by tissue homogenization, filtration, and nuclease treatment, and a library of randomly amplified PCR products from viral RNA and DNA was prepared by using a ScriptSeq version 2 RNA-Seq library preparation kit (Epicenter, Madison, WI, USA) and sequenced on the MiSeq Illumina platform (Illumina, San Diego, CA, USA) (*12*).

Bioinformatics Analysis

Paired-end reads of 250 bp generated by MiSeq were debarcoded by using vendor software from Illumina. An in-house analysis pipeline running on a 32-node Linux cluster was developed to process the data. Clonal reads were removed, and low sequencing quality tails were trimmed by using Phred quality score 10 as the threshold. Adaptors were trimmed by using the default parameters of VecScreen, which is NCBI BLASTn (*13*) with specialized parameters designed for adaptor removal. The cleaned reads were assembled de novo by using SOAPdenovo2 (*14*). The assembled contigs, along with singlets, were aligned to an in-house viral proteome database by using BLASTx. The significant hits to virus were then aligned to an in-house nonvirus–nonredundant universal proteome database by using BLASTx. Hits with more significant adjusted E-value to nonviral than to viral sequences were removed.

Genome Sequencing and Phylogenetic Analyses

The presence of astrovirus genomic sequences assembled from next-generation sequencing reads were confirmed by PCR and Sanger sequencing. By connecting gaps between sequenced viral fragments, and amplifying the 5′ and 3′ end sequences by using 5′ and 3′ rapid amplification of cDNA ends (RACE) (*15*,*16*), the complete genome of the new astrovirus was obtained. Phylogenetic analyses based on aligned amino acid sequences from full-length protease, RNA-dependent RNA polymerase (RdRp), and capsid proteins were generated by the neighbor-joining method in MEGA 4 (*17*) by using amino acid p-distances with 1,000 bootstrap replicates. Maximum parsimony and maximum likelihood methods were conducted to confirm the topology of the neighbor-joining tree.

Retrospective Search for BoAstV-NeuroS1

We selected 32 cases of histologically confirmed bovine encephalitis, each of undetermined etiology, from the archives (2003–2013) of the Veterinary Medical Teaching Hospital (Davis, CA, USA) and at the Davis, Tulare, and San Bernardino branches of the University of California, Davis–California Animal Health and Food Safety laboratory system. All animals had a histologic diagnosis of either nonsuppurative or pleocellular encephalitis, encephalomyelitis, or meningoencephalitis. Histology was reviewed, and nucleic acids were extracted from selected sections of formalin-fixed, paraffin-embedded affected brain tissue by Agencourt Formapure Kit (Beckman Coulter, Atlanta, GA, USA) (*18*).

Reverse transcription nested PCR was used to detect BoAstV-NeuroS1 in nucleic acid extracts from formalin-fixed, paraffin-embedded tissue wax scrolls. cDNA was generated with Superscript III (Invitrogen, Carlsbad, CA, USA) and random hexamer. The oligonucleotide primer sets used were as follows: AstV-sF1: 5′-ACCGCCTTTCTGATGATGTGC-3′; AstV-sR1: 5′-CTCATCAACAACCTGCCAAAT-3′; AstV-sF2: 5′-GACTCTGAGGGTCAAATAACC-3′; AstV-sR2: 5′-GCCAAATGGTTTCTCCAACAG-3′ (capsid gene region, ≈150-bp amplicon); AstV-sF3: 5′-ACCGCCTTTCTGATGATGTGC-3′; AstV-sR3: 5′-CTCATCAACAACCTGCCAAAT-3′; AstV-sF4: 5′-GACTCTGAGGGTCAAATAACC-3′; and AstV-sR4: 5′-GCCAAATGGTTTCTCCAACAG-3′ (protease gene region, ≈150-bp amplicon). The PCR conditions were 95°C for 2 min, followed by 39 cycles of 95°C for 15 sec, 55°C for 15 sec, and 72°C for 15 sec, and a final extension at 72°C for 10 min. PCR products were checked by 2% agarose gel electrophoresis, and amplicons of the appropriate size were confirmed by sequencing. PCR-positive and -negative (control) samples were concurrently used for in situ hybridization analyses.

Testing of Affected Steer

The animal was submitted to the California Animal Health and Food Safety Laboratory in Davis, California, for euthanasia and postmortem examination. PCR tests for BoHV-1, bovine viral diarrhea virus (BVDV), West Nile virus, bluetongue virus, epizootic hemorrhagic disease virus, and ovine herpesvirus 2 were negative. BVDV, BoHV-1, *L. monocytogenes*, and *Neospora* spp. were also negative by immunohistochemical examination of sections. Samples were negative for rabies virus according to fluorescent antibody test. Virus isolation, done independently at Cornell University (Ithaca, NY, USA) and at the California Health and Food Safety Laboratory (Davis, CA, USA) from a brain pool was negative. The tissue sample was placed onto bovine turbinate and swine kidney primary cell cultures. Two passages were made. At the end of the second passage, the cells were stained with a polyvalent viral antiserum, a pseudorabies antiserum, and bovine enterovirus 1–7 antiserum in an indirect immunofluorescence assay. In the sentinel animal, serologic test results for BoHV-1 were negative and for BVDV types 1 and 2 were positive at a titer of 512. *Ostertagia* spp. were detected in the abomasal contents, and *Trichostrongyle* spp. eggs and *Coccidia* oocysts were found in feces. Hepatic levels of copper (5.8 ppm) and selenium (0.13 ppm) were interpreted as being low.

Histology and In Situ Hybridization

Brains from all animals and various segments of the spinal cord from 3 animals were immersion-fixed in 10% buffered formalin, pH 7.2, for at least 48 h. Transverse sections of the brain, including cerebral cortex and corona radiata, basal nuclei, thalamus, midbrain, medulla oblongata, cerebellar peduncles, cerebellum and brainstem in all animals; the full length of the spinal cord in animals 1 and 2 (including cervical dorsal root ganglia in animal 1); and cervical cord segments only in animal 3 were processed by standard histologic techniques by using 4-µm thick sections stained with hematoxylin and eosin.

Colorimetric in situ hybridization was performed on 4-µm thick tissue sections, mounted on 3-aminopropyltriethoxylane–coated slides (Fisher Scientific, Freemont, CA, USA) by using a 28-nt oligomer probe (5′-ACATGGCTGTAAGCATTGGTGTGAAGTA-3′) complementary to the capsid region of BoAstV-NeuroS1 and 3′-labeled with digoxigenin-II-dideoxy undine (Eurofins MWG Operon (Huntsville, AL, USA). Tissue sections were deparaffinized with d-limonene (CitriSolv; Fisher Scientific) and digested by incubation with 0.25% pepsin in Tris-buffered saline (pH 2.0) at 37°C for 10 min, followed by a 5-min incubation at 105°C to stop enzymatic activity. Nucleic acid denaturation was achieved by incubation in formamide (100%) at 105°C for 5 min. Hybridization was performed at 37°C in a 10-µmol solution of the digoxigenin-labeled probe in hybridization buffer (22.5% deionized formamide, 7.5% chondroitin sulfate, 5 × saline sodium citrate, 0.25% blocking reagent, and 50 mmol phosphate buffer). Sections were incubated in a digoxigenin antibody solution (500:1 dilution) containing 2.5 mL buffer 1 (100 mM Tris, 150 mM NaCl, 0.3% Triton X-100, 1% goat serum, pH 7.5) with 5 µL of antidigoxigenin Fab fragments conjugated with alkaline phosphatase (750 U/mL) (Roche, Mannheim, Germany). Sections were then washed and developed according to manufacturer instructions before counterstaining with 1% fast-green FCF for 5 min. After counterstaining, slides were coated with aqueous mounting media (ImmunoHistoMount, Immunobioscience, Mukilteo, WA, USA) and covered with SHUR/Mount mounting medium (Triangle Biomedical Sciences, Durham, NC, USA). There was no observed hybridization in replicate tissue sections incubated with another, unrelated digoxigenin-labeled probe with similar guanine-cytosine content, and brain tissues from astrovirus-negative cattle exhibited no staining.

Electron Microscopy

Selected pieces of wax-embedded spinal cord tissue from the sentinel animal were extracted and postfixed in 2.0% glutaraldehyde and then routinely processed and embedded in epoxy resin (Eponate12 kit; Ted Pella, Redding, CA, USA). Selected thick sections were stained with toluidine blue. Ultrathin sections were examined by using a Zeiss (Göttingen, Germany) 906E transmission electron microscope.

Results

Results of Metagenomic and Phylogenetic Analysis of BoAstV-NeuroS1 in Sentinel Animal

A 168-kg crossbreed yearling steer originating from Mexico was found on a property in northern California in lateral recumbency, with opisthotonus and limbs in extensor rigidity. After deep sequencing of enriched viral particles from homogenized diseased brain tissue, we identified 170 sequence reads related to astroviruses using BLASTx (E-score <10-5), which could be assembled into 17 contigs covering ≈45% of the viral genome.

The complete genome of the new astrovirus (GenBank accession no. KF233994) was then generated by linking fragments by PCR and by using 5′ and 3′ RACE to yield a genome provisionally named bovine astrovirus NeuroS1 (BoAstV-NeuroS1).

The resulting genome was 6,471 nt long, with a GC content of 48%. As typical mamastroviruses, BoAstV-NeuroS1 had 3 putative open reading frames (ORFs), encoding the protease with ORF1a (860 aa), RdRp with ORF1b (525 aa), and capsid protein with ORF2 (757 aa). An expected ribosomal frame-shift signal was found in the ORF1a/1b

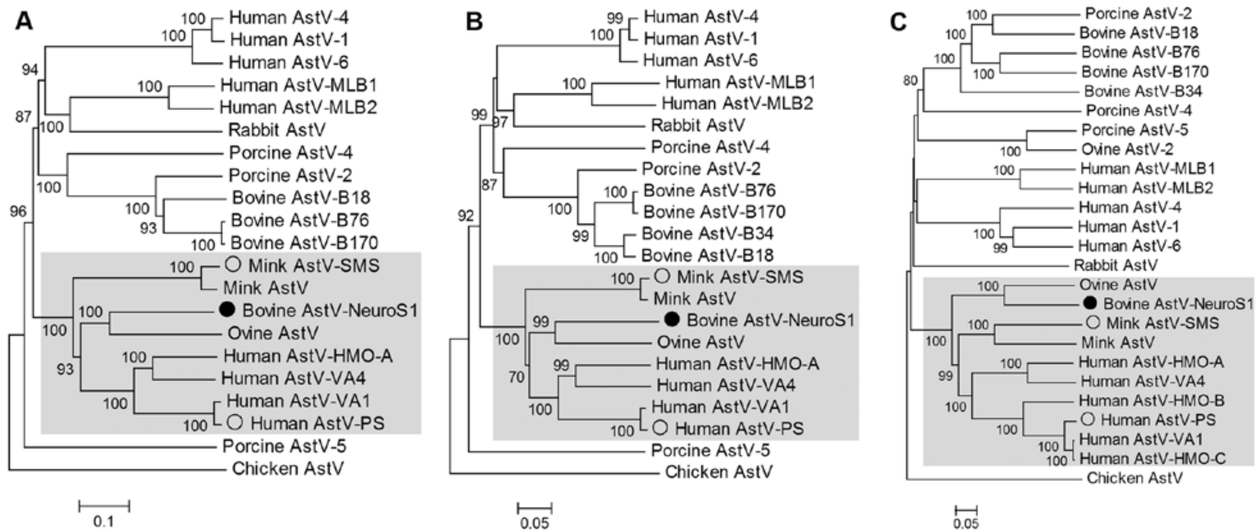

Figure 1. Phylogenetic tree based on aligned amino acid sequences of the full length of the protease (open reading frame [ORF] 1a) (A), RNA-dependent RNA polymerase (ORF1b) (B), and capsid (ORF2) region (C) of representative astrovirus (AstV) species. BoAstV-NeuroS1 labeled with filled circle. AstVs with neurotropic potential labeled by empty circles. Clade containing these 3 viruses is shaded. Scale bar indicatesestimated protein sequence phylogenetic distance. GenBank accession numbers for astrovirus used in the analysis are as follows: bovine BoAstV-NeuroS1 (KF233994), human AstV-1 (JN887820), human AstV-4 (DQ344027), human AstV-6 (HM237363), human AstV MLB1 (JQ086552), human AstV MLB2 (NC_016155), human AstV-VA1 (FJ973620), human AstV-VA4 (JX857869), human AstV-HMO-A (NC_013443), human AstV-HMO-B (GQ415661), human AstV-HMO-C (GQ415662), human AstV-PS (GQ891990), ovine AstV (NC_002469), ovine AstV-2 (JN592482), bovine AstV B18 (HQ916313), bovine AstV-B34 (HQ916315), bovine AstV-B76 (HQ916316), bovine AstV-B170 (HQ916314), porcine AstV-2 (JF713712), porcine AstV-5 (JF713711), mink AstV (AY179509), mink AstV-SMS (GU985458), rabbit AstV (JF729316), and chicken AstV (JF414802).

overlap region that consisted of the heptameric AAAAAAC sequence, followed by a potential 20-nt pseudo-knot sequence. The conserved protease motifs and RdRp motifs, such as the potential conserved proteolytic cleavage site (VHL/TNT) and the characteristic YGDD motif, were present. The amino-terminal half of the capsid protein was more conserved than the carboxy-terminal region.

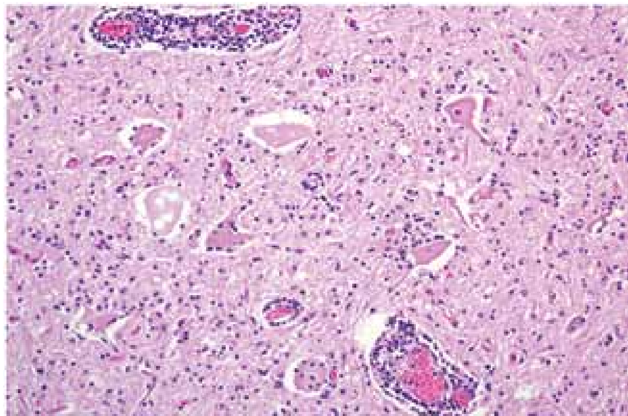

Figure 3. Spinal cord at the L5 segment of a heifer with encephalomyelitis (animal 2). Note the nonsuppurative encephalomyelitis with lymphocytic perivascular cuffs, neuronal degeneration and necrosis, spheroids from necrotic neurons, and neuronophagia with widespread microgliosis. Hematoxylin and eosin stain. Original magnification ×400.

To determine the genetic relationship between BoAstV-NeuroS1 and other astroviruses, we performed sequence alignments of the protease (ORF1a), RdRp (ORF1b), and capsid (ORF2). BoAstV-NeuroS1 shared the highest identity of 56%, 70%, and 66% aa similarity with the ORF1a, ORF1b, and ORF2 encoded proteins of ovine astrovirus, its closest relative. Phylogenetic analysis was performed, and neighbor-joining trees were generated (Figure 1). All 3 trees confirmed that BoAstV-NeuroS1 was most closely related to the ovine astrovirus prototype, which was identified in 1977 (*20*) and sequenced in 2003 (*21*), but was phylogenetically far from the recently reported ovine AsttV2 (*22*), and other known BastVs.

Retrospective Study

For 32 animals, formalin-fixed, paraffin-embedded tissues of bovine brain or spinal cord (1 or 2 tissue sections per animal) were tested for BoAstV-NeuroS1 by specific reverse transcription nested PCR, and samples from 3 (9.4%) animals (animals 2–4) were positive. Samples from these same animals also were positive by in situ hybridization. Animal 2 was a 178-kg, polled Hereford heifer from northern California that was found recumbent with intermittent seizures, unable to move the hind legs, and lacking hind limb pain withdrawal reflex. Animal 3 was a euthanized 3-year-old Angus cow that on the farm was unable to

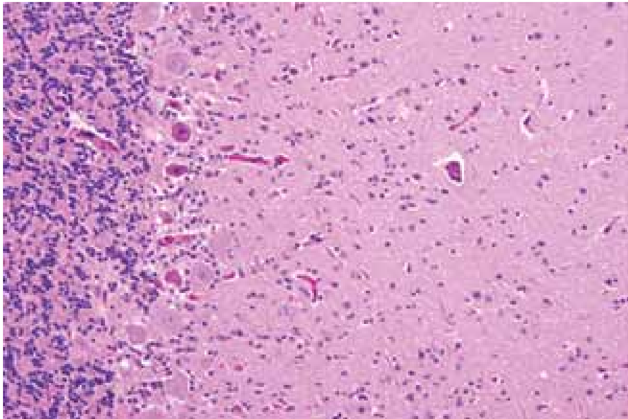

Figure 4. Cerebellum of a yearling steer with encephalomyelitis (animal 1). Note the selective extensive acute necrosis and degeneration of Purkinje cells. Numerous necrotic dendritic spheroids in the molecular layer with a cellular proliferation of Bergmann glia and of microgliosis. Hematoxylin and eosin stain. Original magnification ×400.

rise and exhibited occasional star gazing and unresponsiveness to thiamin therapy. Animal 4 was a euthanized 3-year-old Holstein cow with a history of circling and blindness. The laboratory diagnostic workup for CNS pathogens in these 3 additional animals was not consistent but included negative test results for BVDV, BoHV-1, and rabies virus. Culture for *L. monocytogenes* in animals 3 and 4 was negative; for animals 2 and 4, *Neospora caninum*, *Sarcocystis* spp., BSE, and pseudorabies histologic lesions were negative by immunohistochemistry; and immunohistochemical analysis results for West Nile virus, *Chlamydia* spp. and *Toxoplasma* spp. in animal 3 were negative.

Histology, In Situ Hybridization, and Ultrastructure

The anatomic pattern of lesion distribution was remarkably consistent and unusual in all 4 animals. The meningoencephalomyelitis was largely confined to gray matter in the brain and spinal cord, with most severe lesions in the cerebellar folia and brainstem and throughout all segments of the spinal cord gray matter. Rostrally, there was minimal involvement in the midbrain, thalamus, and basal nuclei, but the cerebral cortex and underlying corona radiata were devoid of inflammatory cell infiltrates (Figure 2, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0682-F2.htm). There was a nonsuppurative ganglioneuritis in the 1 cervical dorsal root ganglion examined.

Microscopically in all animals, the lesions were pathognomonic for a highly neurotropic viral encephalomyelitis and included moderate to marked lymphocytic cell perivascular cuffing and neuronal and ganglionic degeneration and necrosis, with microgliosis but minimal meningitis, especially in the dorsal and ventral horns of the spinal cord gray matter and multifocally in the medulla oblongata, cerebellar peduncles, and midbrain (Figure 3). Degenerating or necrotic neurons were variously swollen and hypereosinophilic and shrunken with angular borders and/or had faded, pale, or eosinophilic cytoplasm sometimes with vacuolation or central pallor. Nuclei of affected neurons remained central but were variably pyknotic, karyorrhectic, or rarely karyomegalic with absent or dispersed chromatin. Neuronophagia of these neurons by microgliosis was not a common feature and appeared delayed but was occasionally seen in individual neurons with an eosinophilic irregular granular content, especially in the gray matter of the spinal cord. Eosinophilic swollen axonal and dendritic spheroids accompanied this neuronal necrosis. The cerebellar folia also had widespread lesions, with a predilection for Purkinje cell necrosis and loss, with characteristic Bergmann glial and microglial proliferation and dendritic spheroids in the overlying layer; however, axonal torpedoes were rarely found (Figure 4). Minimal lesions occurred as far rostrally as the thalamus, and basal nuclei and were limited to mild lymphocytic and plasma cell perivascular cuffing, with scattered microgliosis and, rarely, neuronal necrosis. The cerebral cortex and underlying white matter was surprisingly free of inflammatory lesions. In addition, a dorsal root ganglion of animal 2 showed lymphocytic ganglioneuritis with occasional characteristic neuronal degeneration and necrosis (Figure 5). Throughout the length of the spinal cord in animal 2, there was a bilateral asymmetric polyneuritis restricted to the dorsal nerve roots at most segmental levels.

With in situ hybridization, many affected neurons had variably distinct, punctate to diffuse cytoplasmic staining throughout the cytoplasm or occasionally eccentrically located (Figure 6). In the cerebellar folia, degenerative and necrotic Purkinje cells with their associated necrotic

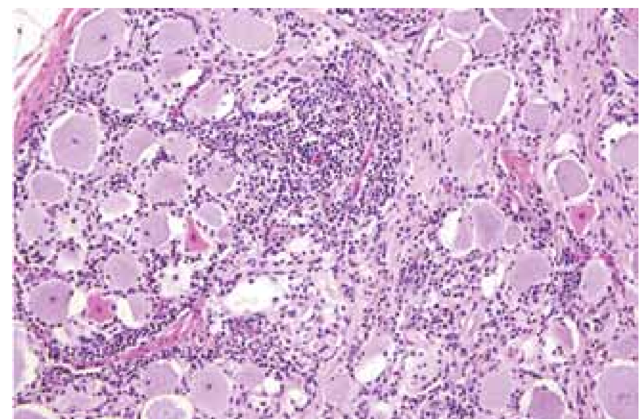

Figure 5. Dorsal root ganglion of a heifer with encephalomyelitis (animal 2). Multifocal marked interstitial lymphocyte, macrophage, and plasma cell infiltrates with multifocal neuronal degeneration and necrosis can be seen. Hematoxylin and eosin stain. Original magnification ×400.

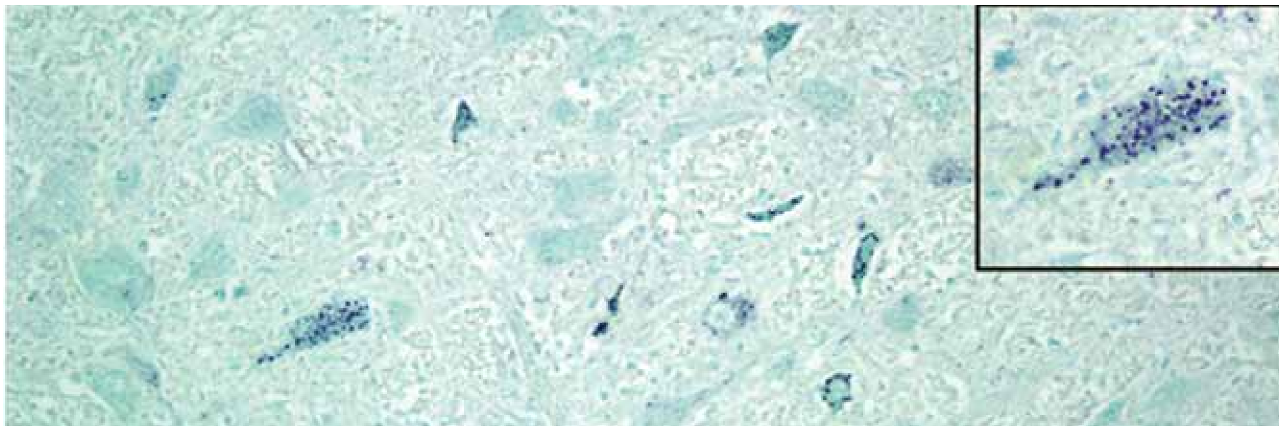

Figure 6. Medulla oblongata of a heifer with encephalomyelitis (animal 2). Punctate cytoplasmic staining (green) in multiple neurons within a nucleus can be seen; inset shows a higher magnification of a positive brainstem neuron. In situ hybridization for viral RNA. Original magnification ×400.

dentritic spheroids in the molecular layer were positive for BoAstV-NeuroS1 (Figure 7). Ultrastructural analysis of the spinal cord from the sentinel steer showed paracrystalline, stacked arrays of empty viral-like particles within the cytoplasm of neurons (Figure 8). The diameter of individual particles was ≈27.5 nm.

Discussion

We report the genomic characterization of a novel astrovirus (BoAstV-NeuroS1) in the brain tissue of 4 cattle from ranches in California with a clinical neurologic disorder characterized histologically as a neurotropic meningoencephalomyelitis and ganglioneuritis. The virus was initially identified by viral metagenomics, and its presence was subsequently confirmed retrospectively in the CNS of 3 other cattle that had bovine encephalomyelitis of unknown etiology by PCR and in situ hybridization for viral RNA. Also, ultrastructure studies in 1 animal demonstrated intracytoplasmic particles in degenerating neurons tentatively considered morphologically compatible with an astrovirus. Our attempts to recover this virus in tissue culture have not been successful, and no concurrent intestinal infection was detected.

Other astroviruses have been identified and associated with neurologic signs in an immunodeficient child and in minks with a shaking syndrome in farm outbreaks reported in Denmark, Sweden, and Finland (*5, 6*). BoAstV-NeuroS1 infection shares neuropathologic features with the disease in mink. Both diseases have a seemingly identical anatomic distribution and type of lesions, and both occur with neurologic symptoms generally as isolated cases in mixed-breed animals.

BoAstV-NeuroS1 is now the third separate astrovirus species detected in brain tissue that has been associated with neurologic disease. It may be relevant that these 3 astrovirus species associated with neurologic symptoms (HAstV-PS, mink AstV-SMS, and BoAstV-NeuroS1) fall into the same genetic clade of astroviruses, together

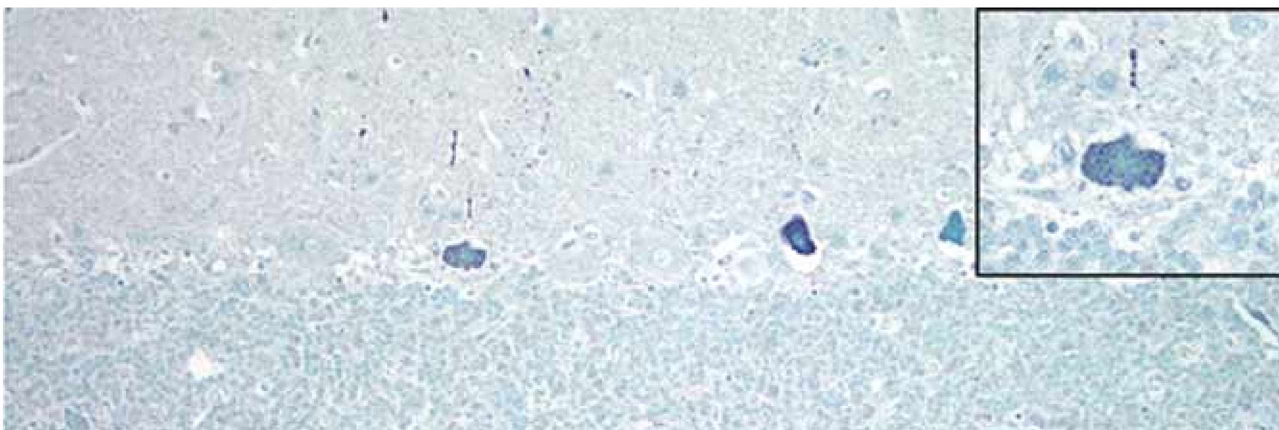

Figure 7. Cerebellum of a yearling steer with encephalomyelitis (animal 1). Punctate to diffuse positive (green) staining of Purkinje cells cytoplasm and dendritic processes can be seen; inset shows a higher magnification of a positive Purkinje cell. In situ hybridization for viral RNA. Original magnification ×400.

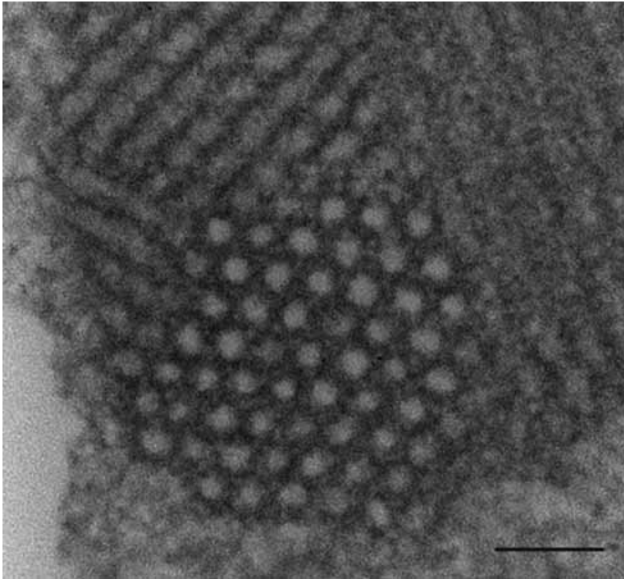

Figure 8. Transmission electron microscopic image of necrotic neuron in the lumbar region of a heifer with encephalomyelitis. Intracytoplasmic paracrystalline array of 27–28-nm diameter viral-like particles. Scale bar indicates 100 nm.

with viruses of other species so far not associated with disease (Figure 1).

Bovine neurologic diseases can be caused by bacteria, parasites, viruses, toxins, or nutritional disturbances (*10*,*11*,*23*). Common infectious agents causing neurologic disease in cattle are regionally variable but include *Clostridium botulinum*, *Clostridium tetani*, *L. monocytogenes, Histophilus somni, Babesia bovis,* BSE prion, and BoHV-1 and -5. Infection with BoAstV-NeuroS1 is a new addition to the differential diagnosis of neurologic disorders in cattle, and constituted ≈9% of the undiagnosed cases of encephalitis in our retrospective study. This finding probably underestimates the prevalence of BoAstV-NeuroS1 disease because detection in this retrospective study was limited to formalin-fixed, paraffin-embedded tissues from a single region of the brain. Our preliminary microscopic and in situ hybridization results suggest that the best target for virus detection would be the spinal cord. Rapid diagnosis of astrovirus RNA by PCR or in situ hybridization in the brain or spinal cord of cattle with neurologic signs may enable more rapid exclusion of infection with the BSE prion. The involvement of the cerebellum is distinctive and rare and has been reported only with the still enigmatic European sporadic bovine encephalitis. However, the neurotropic lesions in that disease also intensively involve the hippocampus. Arthropod-borne diseases, such as louping-ill in sheep and Russian tick-borne encephalitis in dogs and horses, share the features of neurotropism, Purkinje cell necrosis, and a similar anatomic distribution with BoAstV-NeuroS1 infection.

Further research will be required to determine whether development of the neurologic signs seen here required other factors, including co-infections and/or a genetic or acquired immunodeficiency. PCR testing and genomic analysis of bovine fecal isolates also may provide information about the incidence and duration of virus shedding, which–as for other asymptomatic intestinal astrovirus infections–is expected to be short. In our study, in situ hybridization was negative on intestinal sections of the affected cattle. Seroprevalence studies in healthy cattle of different ages could measure any prior exposure to BoAstV-NeuroS1 and determine how frequently such asymptomatic infections occur.

Electron microscopy support was provided by the California Animal Health and Food Safety Laboratory.

The work was supported by the Blood Systems Research Institute and National Institutes of Health R01 HL105770 to E.D. The Bernice Barbour Foundation and the University of California, Davis, Center for Companion Animal Health supported the research of P.P.

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Antigenic and Molecular Characterization of Avian Influenza A(H9N2) Viruses, Bangladesh

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Human infection with avian influenza A(H9N2) virus was identified in Bangladesh in 2011. Surveillance for influenza viruses in apparently healthy poultry in live-bird markets in Bangladesh during 2008–2011 showed that subtype H9N2 viruses are isolated year-round, whereas highly pathogenic subtype H5N1 viruses are co-isolated with subtype H9N2 primarily during the winter months. Phylogenetic analysis of the subtype H9N2 viruses showed that they are reassortants possessing 3 gene segments related to subtype H7N3; the remaining gene segments were from the subtype H9N2 G1 clade. We detected no reassortment with subtype H5N1 viruses. Serologic analyses of subtype H9N2 viruses from chickens revealed antigenic conservation, whereas analyses of viruses from quail showed antigenic drift. Molecular analysis showed that multiple mammalian-specific mutations have become fixed in the subtype H9N2 viruses, including changes in the hemagglutinin, matrix, and polymerase proteins. Our results indicate that these viruses could mutate to be transmissible from birds to mammals, including humans.

Initially infecting poultry, avian influenza A(H9N2) viruses have been sporadically identified in pigs and humans, which suggests that some of these viruses have adapted to bind mammalian host receptors or have acquired mutations that increase mammalian receptor specificity (*1–3*). Human infection with avian influenza A(H9N2) virus was initially identified in Hong Kong and China in 1999 (*4*); in 2011, infection with this subtype was reported for a patient in Bangladesh (*5*). Detection of these viruses in humans outside of China highlights the necessity and urgency for comprehensive surveillance because of the viruses' expanding host range.

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DOI: http://dx.doi.org/10.3201/eid1909.130336

Phylogenetically, avian influenza A(H9N2) viruses can be grouped into 3 distinct sublineages represented by their prototype strains: A/Qa/HK/G1/97 (G1-like), A/Dk/HK/Y280/97 (Y280-like), and A/Ck/Korea/38349-p96323/96 (Korean-like) (*1*,*6*,*7*). Genetic and antigenic analyses of subtype H9N2 isolates from the past 2 decades have shown that these viruses are gradually evolving from the Eurasian lineage into several distinct sublineages and are becoming established in domestic poultry (*7–18*). Phylogenetic analyses of subtype H9N2 viruses isolated in China and the Middle East have shown that these viruses have undergone reassortment with other subtypes to generate multiple novel genotypes consisting of gene segments from different lineages (*7*,*11*,*13*,*19*,*20*).

Worldwide, Bangladesh is among countries with the highest numbers of reported outbreaks of highly pathogenic avian influenza (HPAI) (H5N1) (*21*). Since an initial outbreak in February 2007, Bangladesh has reported 550 outbreaks of infection with HPAI (H5N1) virus (493 at commercial farms and 57 among backyard poultry) (*22–24*). In Bangladesh, live-bird markets are the most common outlets for purchase of poultry and poultry meat; an estimated 95% of poultry meat and eggs sold in the country are sold at these markets (*25*). Previous surveillance conducted at live-bird markets in Bangladesh found that avian influenza virus (AIV) is prevalent (23%); the low pathogenicity H9N2 subtype predominated, but other subtypes were

isolated, including H5N1, H1N2, H1N3, H3N6, H4N2, and H10N7 (*26*). However, this surveillance report did not include information about the molecular properties of circulating subtype H9N2 viruses.

We reviewed surveillance data and conducted molecular and genetic analyses of influenza A(H9N2) viruses circulating among poultry in Bangladesh. Our study had 3 primary goals: 1) characterize the antigenic and molecular properties of subtype H9N2 isolates; 2) define genetic and phylogenetic relationships between the genes identified in these viruses and those of other AIVs; and 3) determine whether these viruses have acquired genomic changes that could facilitate transmission from avian to mammalian hosts.

Materials and Methods

Sample Collection and Screening

Surveillance for influenza viruses in poultry began in Bangladesh in November 2008. Each month, trained personnel collected 200–600 samples from apparently healthy domestic live birds (chickens, quail, pigeons, ducks, and turkeys) at retail markets, a pet market, chicken layer and duck farms, and wild birds (50–200 samples from each location). Samples consisted of oropharyngeal, cloacal, and environmental (fecal matter and water samples from cages and fecal digesters) swab specimens.

During November 2008–August 2011, a total of 17,438 samples (3,078 oropharyngeal, 3,377 cloacal, 10,983 environmental) were collected (Table 1). Screening for AIVs was performed as described (*26*). Briefly, all samples were subjected to real-time reverse transcription PCR by using influenza A–specific primer and probes; samples with positive results were injected into egg to confirm the presence or absence of virus. A total of 734 AIV (H9N2) isolates were extracted from the 17,438 samples collected (Table 1).

Virus Isolation and Propagation

To determine the genetic and evolutionary diversity of the AIV (H9N2) viruses circulating in Bangladesh, we selected 44 of the 734 isolates for further analysis (Table 2); these isolates were representative of the location, time, species, and sample types from which they were isolated. With the exception of 1 isolate from a chicken on a farm, all isolates examined were from chicken, duck, and quail samples collected at retail markets. The viruses were propagated in 10-day-old embryonated chicken eggs, and initial subtyping was done by sequencing the hemagglutinin (HA) and neuraminidase (NA) genes, as described (*26*).

Hemagglutination Inhibition Assay

Polyclonal serum samples were obtained from ferrets that had been inoculated with 1 of the following: prototype viruses from different influenza (H9N2) lineages; influenza (H9N2) isolates from chickens or quail collected in Bangladesh; or a prototype human isolate provided by the US Centers for Disease Control and Prevention. We then conducted hemagglutination inhibition (HI) assays for these serum samples by using 0.5% chicken erythrocytes, as described (*27*).

Phylogenetic Analysis and Molecular Characterization

Viral RNA extraction and reverse transcription PCR were performed as described (*28*,*29*). DNA sequencing

Table 1. Sources and subtypes of avian influenza viruses isolated in Bangladesh, 2008–2011

Site	Primary species from which samples were obtained*	Primary species from which sample was influenza positive	Total no. samples	Subtypes: H1N2, H1N3, H3N6, H3N8, H4N2, H10N7	Subtypes: H5N1	Subtypes: H9N2
Live bird market-1	Quail	Quail	821		20	37
Live bird market-2	Chickens, ducks, pigeons	Chickens Duck	1,691	12	13	85 2
Live bird market-3	Chickens	Chickens Ducks	1,877	7	38	360 2
Live bird markets-4	Chickens	Chickens	46			12
Live bird markets-5	Chickens	Chickens Duck	2,677	14	20	205 2
Pet market	Pet and wild birds	Quail	1,859	3	1	20
Farm-1	Ducks	Ducks	100	24		
Farm-2	Chickens	Chickens	3,945	2		9
Farm-3	Chickens		40			
Farm-4	Ducks		100			
Farm-5	Ducks		100			
Farm-6	Ducks		901			
Wild birds, environmental-1	Wild birds		836			
Wild birds, environmental -2	Wild birds		471			
Wild ducks, environmental-1	Water		1,724			
Wild ducks, environmental-2	Ducks		250			
Total			17,438	62	92	734

*Other species that were sampled at some locations included turkeys, wild birds, and exotic birds.

using specific primers was completed in the Hartwell Center at St. Jude Children's Research Hospital (Memphis, TN, USA). Samples were analyzed by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Invitrogen, Carlsbad, CA, USA) on 3730XL DNA analyzers (Applied Biosystems, Foster City, CA, USA), according to the manufacturers' recommendations. DNA sequences for all genes were edited, compiled, assembled, and analyzed by using SeqMan in Lasergene 8 (DNASTAR, Madison, WI, USA). Nucleotide sequences were compared with sequences available in GenBank. Multiple nucleotide sequence alignment and the alignment of the deduced amino acids of all of the gene segments were conducted by using ClustalW in BioEdit 7.09.0 (www.mbio.ncsu.edu/bioedit/bioedit.html). The phylogeny of each gene was investigated within the maximum-likelihood framework by using PhyML under the general time reversible substitution model with gamma rate heterogeneity (*30*). The robustness of the grouping was assessed by using 100 bootstrapping replicates (*30*). MegAlign in Lasergene 8 (DNASTAR) was used to determine the percentage of nucleotide sequence similarities, and the NetNGlyc 1.0 Server (*31*) was used to predict the glycosylation sites in the HA and NA genes. The sequences we obtained (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/9/13-0336-Techapp1.pdf) were deposited into GenBank under accession nos. KC757782–KC758115.

Table 2. Avian influenza A(H9N2) virus isolates from animals in live bird markets, Bangladesh, 2008–2011*

Isolate	Host common name	Sample type	Mixtures†
A/Env/BD/907/2009 (quail)	Quail	F	NDV
A/Env/BD/1041/2009 (duck)	Duck	F	H5
A/Dk/BD/1231/2009	Duck	OP	H5
A/Ck/BD/2075/2009	Chicken	OP	H5
A/Pigeon/BD/4303/2009	Pigeon	OP	NDV
A/Env/BD/5144/2009 (quail)	Quail	F	H5
A/Ck/BD/5209/2009	Chicken	OP	NDV
A/Env/BD/5745/2010 (duck)	Duck	F	H5
A/Env/BD/8202/2010 (chicken)	Chicken	F	
A/Ck/BD/8411/2010	Chicken	OP	
A/Ck/BD/8413/2010	Chicken	OP	
A/Ck/BD/8415/2010	Chicken	OP	
A/Env/BD/8463/2010 (chicken)	Chicken	W	
A/Env/BD/8465/2010 (chicken)	Chicken	W	
A/Ck/BD/8725/2010	Chicken	OP	
A/Ck/BD/8731/2010	Chicken	OP	
A/Ck/BD/8996/2010	Chicken	C	NDV
A/Ck/BD/9029/2010	Chicken	OP	NDV
A/Env/BD/9306/2010 (parrot)	Parrot	F	NDV
A/Ck/BD/9334/2010	Chicken	OP	
A/Env/BD/9350/2010 (chicken)	Chicken	W	NDV
A/Env/BD/9457/2010 (chicken)	Chicken	F	
A/Env/BD/10234/2011 (chicken)	Chicken	W	
A/Env/BD/10306/2011 (quail)	Quail	F	NDV
A/Env/BD/10307/2011 (quail)	Quail	F	NDV
A/Env/BD/10313/2011 (quail)	Quail	F	
A/Env/BD/10316/2011	Quail	F	
A/Ck/BD/10401/2011	Chicken	OP	
A/Ck/BD/10411/2011	Chicken	OP	
A/Ck/BD/10450/2011	Chicken	C	NDV
A/Ck/BD/10897/2011	Chicken	OP	
A/Ck/BD/11154/2011	Chicken	C	NDV
A/Env/BD/11173/2011 (chicken)	Chicken	F	
A/Ck/BD/11309/2011	Chicken	OP	
A/Ck/BD/11315/2011	Chicken	OP	
A/Env/BD/11597/2011 (chicken)	Chicken	W	
A/Env/BD/12068/2011 (pigeon)	Pigeon	F	NDV
A/Env/BD/12077/2011 (turkey)	Turkey	F	NDV
A/Env/BD/12093/2011 (quail)	Quail	F	
A/Env/BD/12103/2011 (quail)	Quail	F	
A/Env/BD/12116/2011 (quail)	Quail	F	
A/Env/BD/12119/2011 (quail)	Quail	F	
A/Ck/BD/13916/2011	Chicken	OP	H5
A/Ck/BD/13962/2011	Chicken	OP	H5

*F, fecal; NDV, Newcastle disease virus; OP, oropharyngeal; W, water; C, cloacal.
†Some hosts infected with influenza (H9N2) viruses were co-infected with H5N1 or NDV; thus, mixtures of isolates were obtained from some birds. Species in parentheses indicate that the samples were collected from the cages where these particular birds were caged.

Results

Prevalence of Avian Influenza in Bangladesh

During November 2008–August 2011, a total of 17,438 samples were collected from live-bird markets, farms, and wild and environmental sources (*26*). In the markets and farms, samples were predominantly collected from chicken, quail, and ducks. The environmental samples were collected from cages in the markets in which only 1 species was housed (e.g., only chickens or only ducks). The wild bird samples were from a lake that was a feeding station for wild migratory ducks.

The predominant subtypes identified in these samples were low pathogenicity H9N2 (n = 734) and highly pathogenic H5N1 (n = 92) (Table 1). Other influenza subtypes identified, at low percentages and only during 2008–2009, were H1N2, H1N3, H3N6, H3N8, H4N2, and H10N7 (Table 1). None of the wild-bird samples tested and only 35 (0.87%) of 4,045 farm samples had positive test results for avian influenza (Table 1). The subtype H9N2 and H5N1 viruses were isolated from domestic poultry, mainly chickens and quail that were sampled in live-bird or pet markets (Table 1).

The number of subtype H5N1 viruses isolated from field samples increased during the surveillance period (Figure 1, panel B). The numbers of other influenza subtypes identified were low during 2009–2010; during 2010–2011, subtypes H9N2 and H5N1 were most commonly isolated.

AIVs and Newcastle disease virus (NDV) are the 2 most common viruses that infect poultry in Bangladesh; therefore, all of the influenza-positive samples that were subtyped and used in this study were screened again for H9N2, H5N1, and NDV to determine whether a single host species was co-infected with AIV and NDV. In a substantial proportion of the isolates screened, we identified mixtures of either subtype H9N2 and NDV or subtypes H9N2 and H5N1 (Table 2). All of the samples containing a mixture of viruses were obtained from apparently healthy birds. The highest incidence of influenza (H5N1) virus isolation was during the winter months (January–March), whereas subtype H9N2 was isolated year-round (Figure 1, panel A)

Antigenic Properties

Antigenic analysis showed that most chicken H9N2 viruses circulating in Bangladesh were antigenically homogenous and similar to the human H9N2 virus (A/BD/0994/11) isolated from Bangladesh (Table 3, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0336-T3.htm). Antigenic variation was seen in subtype H9N2 viruses isolated from quail; specifically, A/Env/BD/907/2009 (H9N2) (quail) was distinguishable from other contemporary quail isolates when postinfection ferret serum of

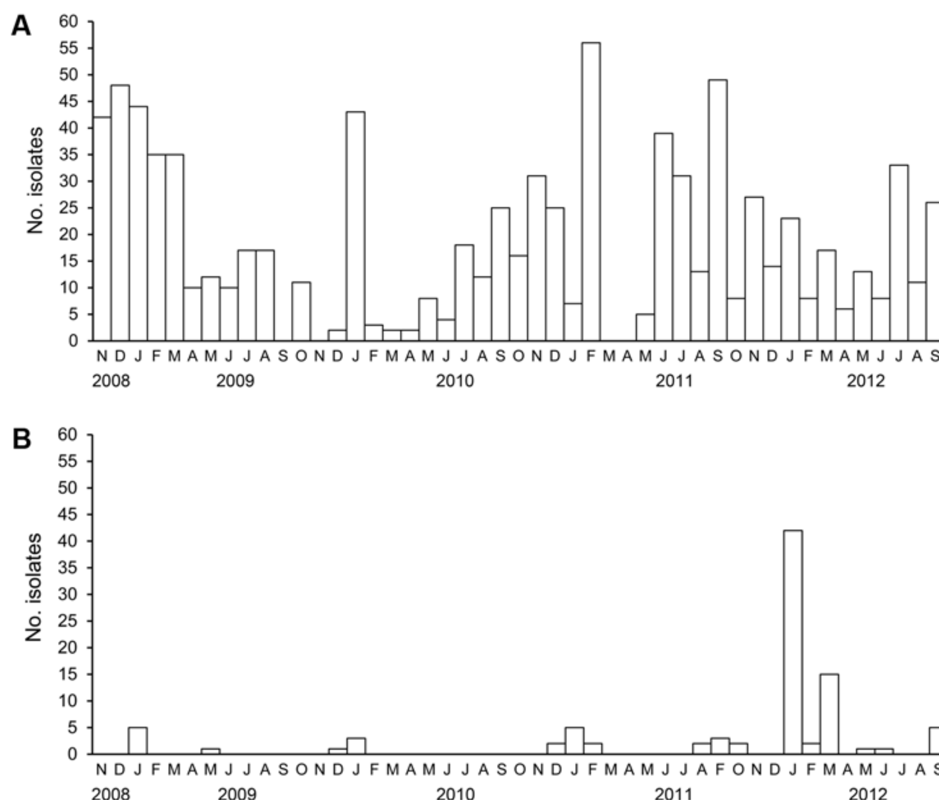

Figure 1. Influenza virus surveillance in Bangladesh, November 2008–September 2012, showing the total number of avian influenza viruses isolated per month. A) Low pathogenicity subtype H9N2 viruses; B) highly pathogenic subtype H5N1 viruses.

A/Env/BD/907/2009 (H9N2) (quail) and chicken H9N2 viruses was used (Table 3, Appendix). The chicken H9N2 viruses had moderate cross-reaction to antiserum of A/Env/BD/907/2009 (H9N2) (quail), indicating slight antigenic variation from subtype H9N2 viruses isolated from quail (Table 3, Appendix). The low level of cross-reactivity seen across the quail viruses and between the quail and chicken viruses indicates that the HAs of the subtype H9N2 viruses maintained in quail were antigenically distinct from those of subtype H9N2 viruses maintained in chickens. In addition to the quail isolate, A/Env/BD/12068/2011 (H9N2) (pigeon) reacted with 2 serum samples, the polyclonal serum against A/Env/BD/907/2009 (H9N2) (quail) and that against A/Ck/Pk/(NARC-2434)/06 (H9N2). The HI titers of those reactions were 320 and 160, respectively.

These antigenic analyses show that antigenic variation occurs among influenza (H9N2) viruses isolated from different host species. The AIV (H9N2) viruses from Bangladesh cross-reacted well to antiserum against G1 clade H9N2 viruses that were isolated in Bangladesh, Dubai, or Pakistan; however, they showed low reactivity to antiserum against A/Qa/HK/G1/97 (prototype G1), indicating that the viruses from Bangladesh were related to but very distinct from the G1 clade isolates. These results indicate antigenic diversity within the G1 clade.

Phylogenetic Relationships

The phylogenetic relationships of all 8 genes of the 44 representative AIV (H9N2) viruses were analyzed on the basis of their nucleotide sequences and by comparing them with nucleotide sequences of subtype H9N2 viruses belonging to the main lineages from Asia (*14*). All sequences from Bangladesh except 1 formed a monophyletic cluster. Phylogenetic analyses showed that all the subtype H9N2 viruses isolated from Bangladesh are part of the lineage represented by A/Qa/HK/G1/97 (G1), which is the most dominant lineage worldwide. The HA, NA, nucleoprotein (NP), matrix (M), and polymerase PB2 genes of the viruses from Bangladesh originated from the prototype G1 virus A/Qa/HK/G1/97 (H9N2). The nonstructural (NS) and polymerase PA and PB1 genes were closely related to those in the subtype H7N3 isolate from Pakistan (*14*).

Phylogenetically, the HA and NA genes of most of the subtype H9N2 viruses from Bangladesh tightly clustered with those of viruses from India and Pakistan (Figure 2; online Technical Appendix Figure, wwwnc.cdc.gov/EID/article/19/9/13-0336-Techapp1.pdf). These viruses had >95% sequence homology, grouped within the G1 lineage, and shared a common ancestor with A/Qa/Hk/G1/97. One duck isolate, A/Env/BD/1041/09 (H9N2) (duck), clustered with subtype H9N2 viruses from Korea and shared a common ancestor with A/Dk/HK/Y439/97 (Figure 2). This isolate also showed only 85% nt similarity with the other viruses from Bangladesh.

Three internal genes (NP, M, and PB2) of the subtype H9N2 viruses from Bangladesh showed a high level of nucleotide homology with the G1-like lineage. All 3 genes were phylogenetically closely related to viruses from India, Pakistan, or the Middle East (online Technical

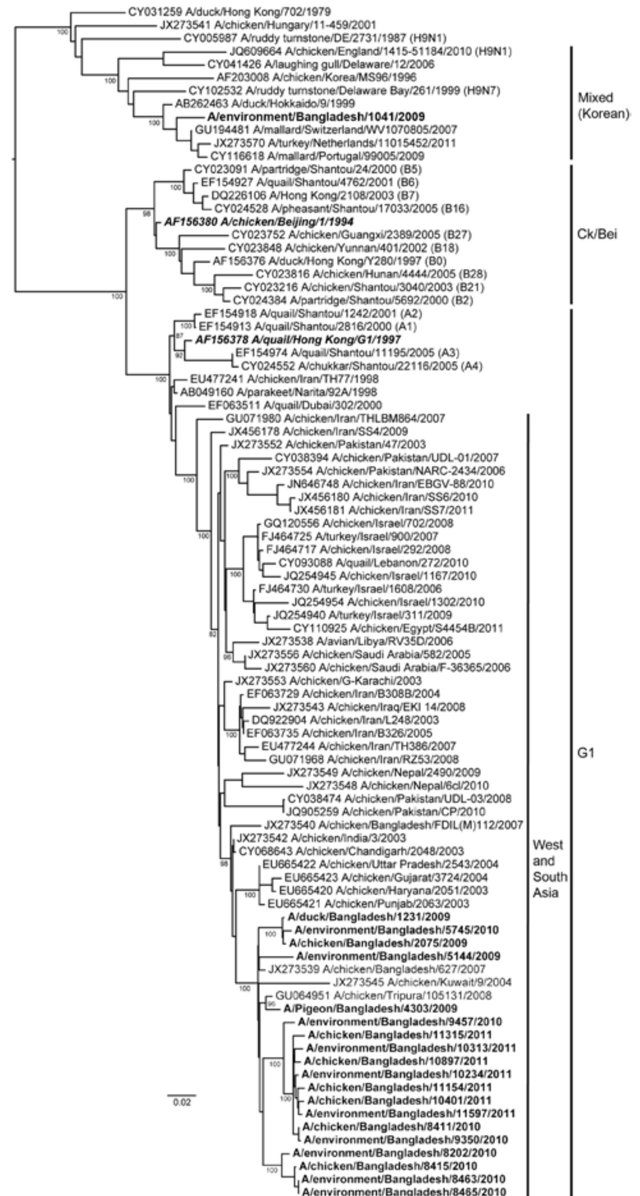

Figure 2. Phylogenetic relationships of hemagglutinin genes of avian influenza (H9N2) viruses (**boldface**) isolated in Bangladesh. Full-length DNA sequencing, starting from the first codon, was used. The phylogenetic trees were generated by PhyML (*30*) within the maximum-likelihood framework. Numbers above the branches indicate bootstrap values; only values >60 are shown. ***Boldface italics*** indicate prototype subtype H9N2 viruses from the Ck/Bei and G1 clades. Scale bar indicates distance between sequence pairs.

Appendix Figure). The duck isolate A/Env/BD/1041/09 (H9N2) (duck) showed similar characteristics in the HA and NA trees, where it shared a common ancestor with a lineage from Korea. The other 3 internal genes (NS, PB1, and PA) of all the subtype H9N2 viruses from Bangladesh grouped into a single cluster, sharing high sequence homology with A/Ck/Karachi/NARC-100/04 (H7N3) (Figure 3; online Technical Appendix Figure). These genes formed a distinct clade adjacent to the G1 and Ck/Bei lineages. Although subtype H5N1 viruses were isolated separately and in a mixture with subtype H9N2 viruses, phylogenetic analysis showed no reassortment between the isolates characterized in this study and the subtype H5N1 strains.

Molecular Characteristics

To identify the possible determinants of transmission of AIV (H9N2) from birds to humans, we aligned the amino acid sequences of all genes of the subtype H9N2 viruses from Bangladesh and compared them with those of representative subtype H9N2 viruses from different clades (online Technical Appendix Tables 2, 3). When compared with the prototype G1 virus, the viruses from Bangladesh showed that they have evolved to acquire mammalian host–specific mutations throughout the genome (online Technical Appendix Tables 2, 3). Comparing the amino acid sequences of all genes of the subtype H9N2 viruses from Bangladesh with the prototype subtype H9N2 viruses showed that certain amino acid substitutions throughout the viral genome have become fixed as the viruses have evolved (Figure 4). The receptor-binding site (RBS) of the virus HA influences the generation of human viruses from avian precursors (*1*). Within the RBS, 42 (95.5%) of 44 isolates had leucine (L) at position 226 (H3 numbering), whereas the other 2 viruses had glutamine (Q) at the same position. Of the 2 isolates with 226Q, 1 was a duck virus isolated in early 2009 and 1 was a quail virus isolated in 2011. All viruses with 226L were isolated during 2009–2011 and were found in all of the poultry species sampled. Amino acids at positions 183, 189, 190, and 226 (H3 numbering) are located within the RBS of the HA protein, and the combination of these 4 residues is essential for respiratory droplet transmission of a subtype H9N2 or H3N2 reassortants in ferrets (*6*). Of the 44 viruses analyzed, 42 had 2 (183H and 226L) of the 4 (183H, 189H, 190E, 226L) substitutions. Variations in the number of potential glycosylation sites within the HA are thought to be associated with the adaptation of duck viruses to poultry (*7*). Seven potential glycosylation sites (29, 105,141, 298, 305, 492, and 551) were found in the HAs of 43 (97.7%) of the 44 isolates; the N-X-T/S motif (in which X may be any amino acid except proline) and 2 potential glycosylation sites were lost at positions 208 and 218 (online Technical Appendix Tables 2, 3). In the NA proteins of all of the analyzed viruses, we found no R292K substitution, which is associated with resistance to the neuraminidase inhibitor oseltamivir.

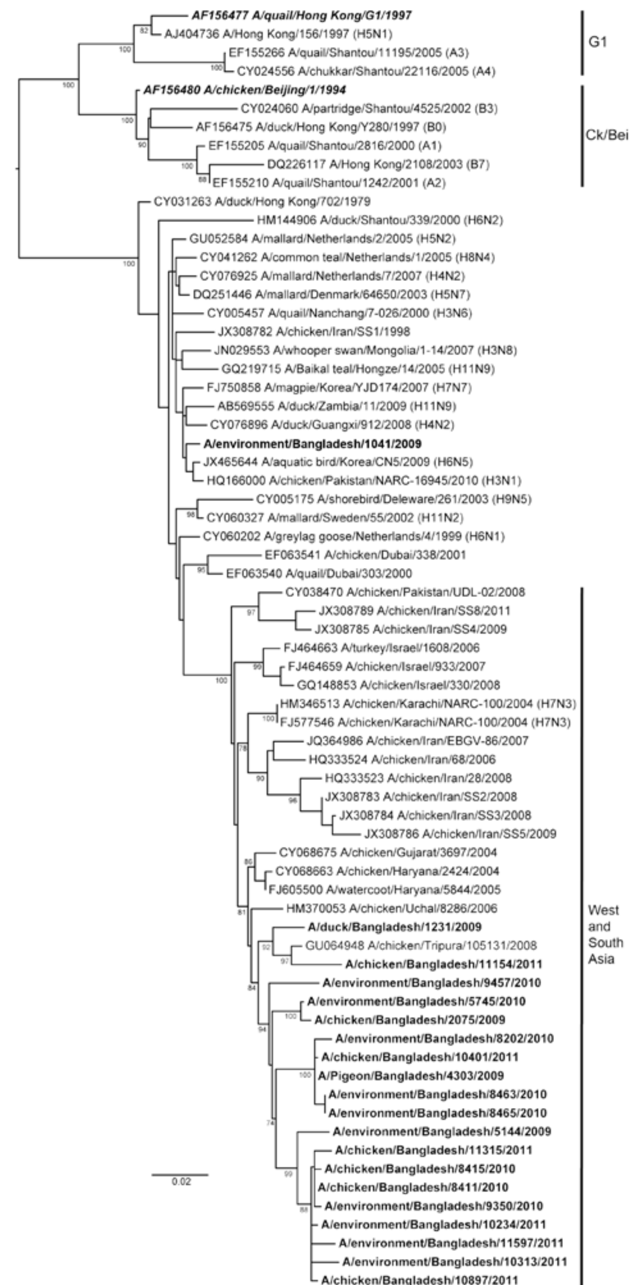

Figure 3. Phylogenetic relationships of nonstructural protein genes of avian influenza (H9N2) viruses (**boldface**) isolated in Bangladesh. Full-length DNA sequencing, starting from the first codon, was used. The phylogenetic trees were generated by PhyML (*30*) within the maximum-likelihood framework. Numbers above the branches indicate bootstrap values; only values >60 are shown. ***Boldface italics*** indicate prototype subtype H9N2 viruses from the Ck/Bei and G1 clades. Scale bar indicates distance between sequence pairs.

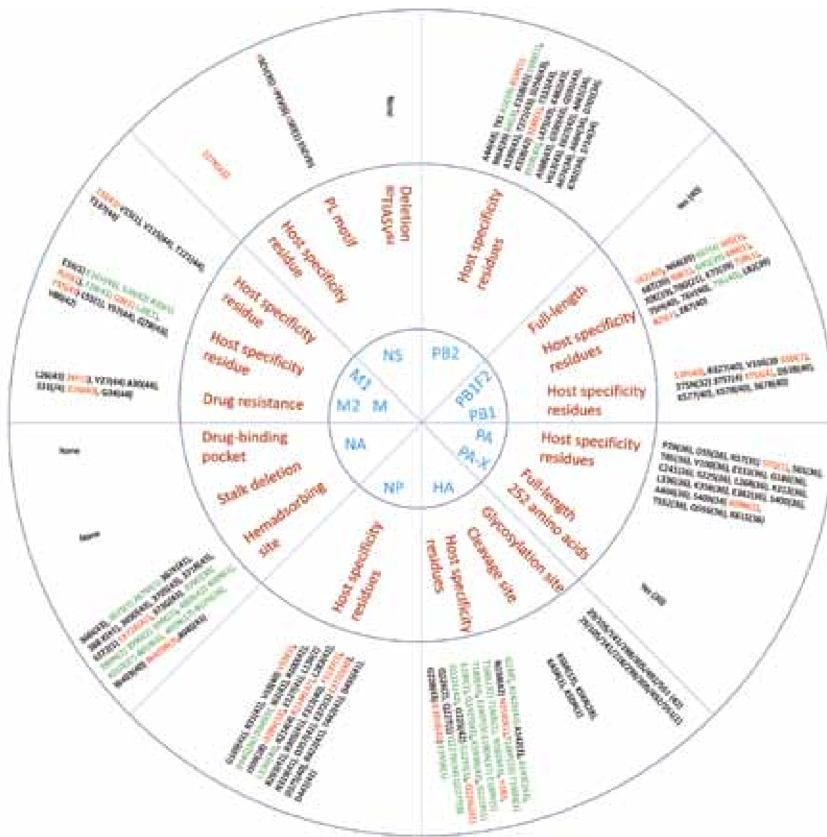

Figure 4. Host range and pathogenicity determinants in avian influenza (H9N2) viruses isolated from different poultry species in Bangladesh during 2008–2011. Numbers in parentheses indicate number of viruses containing specific amino acid residues of the 44 virus isolates analyzed. Blue indicates the 11 genes that were assessed; red indicates the residues that are critical for influenza pathogenesis, enhanced replication in mammalian hosts, or those that are identical to residues present in human influenza viruses; green indicates unique substitutions in the viruses. HA, hemagglutinin; NA, neuraminidase; M, matrix; NS, nonstructural; NP, nucleoprotein; PA and PB, polymerase genes.

Sequence analysis of the internal gene products also identified mammalian host–specific markers (*11*,*14*) that were fixed in the M protein (M1 V15I, 43 isolates; M2 L55F, 44 isolates) and NP (*11*,*14*) (R214K, 37 isolates; E372D, 40 isolates; PB1-L13P, 40 isolates) (Figure 4). In the NS protein, all isolates were full length and possessed the PDZ-(PL) C-terminal motif (*11*,*14*). The NS1 protein also harbored the mammalian-specific glutamate (E) to lysine (K) substitution at position 227 in 43 of the 44 isolates tested (Figure 4).

Our comparison of the deduced amino acid sequences of the subtype H9N2 viruses from Bangladesh revealed that, in positions that were previously identified as important for host specificity, unique substitutions whose functions are unknown have become fixed. In the RBS of the HA protein, the Q227I substitution was in 35 of 44 isolates. This substitution was previously identified in subtype H9N2 viruses from Pakistan and the Middle East but was not found in any of the reference strains. The other unique substitutions were in the M2 protein (E16V and I28F, 43 isolates) and the PB1-F2 protein (R79L, 40 isolates) (Figure 4). Alignment of the M2 proteins showed that 41 of the 44 isolates had a substitution at position 31 (S31N), suggesting resistance to M2-blocker antiviral drugs such as amantadine.

The amino acid sequence of the HA1/HA2-connecting peptide of HA is a major determinant of pathogenicity in terrestrial poultry, and the pathogenicity of highly pathogenic subtype H5N1 and H7N3 viruses is influenced by the presence of a polybasic cleavage site in the connecting peptide (*32*). The amino acid sequences of the cleavage site of the HA1/HA2 junction in the subtype H9N2 viruses from Bangladesh possessed 4 cleavage motifs (Figure 4). Of the 44 isolates we tested, 28 had the KSKR/GLF motif (identified in all recent poultry isolates and a 2011 quail isolate); 14 isolates had the KSSR/GLF motif (identified in chickens, ducks, and older quail isolates); and 1 duck and 1 pigeon isolate had the ASDR/GLF and KASR/GLF motifs, respectively. Thus, the altered amino acid sequences in the connecting peptide of viruses we analyzed were distinct from the RSSR/GLF motif found in the connecting peptides of prototype subtype H9N2 viruses. This finding is clear evidence of genetic variation within isolates from different poultry species.

Discussion

We isolated a large number of low pathogenicity avian influenza A(H9N2) viruses and an increasing number of highly pathogenic avian influenza A(H5N1) viruses from apparently healthy birds in Bangladesh. During 2008–2011, subtype H9N2 viruses were isolated from multiple poultry species (predominantly chickens and quail) throughout the year, without seasonal prevalence; subtype H5N1 viruses

were predominantly isolated from quail during the winter of 2011. Phylogenetic analyses revealed that the subtype H9N2 viruses are of the G1 clade; antigenically, the G1 clade consists of 2 branches distinguished by host species, but the Bangladesh viruses were divergent from the prototype G1 virus. Our comparison of the deduced amino acid sequence showed that the subtype H9N2 viruses have acquired mammalian host–specific mutations in their surface glycoproteins and internal genes.

We isolated subtype H9N2 and H5N1 viruses individually and in viral mixtures; the latter may result from co-infection of the same host species with multiple influenza subtypes. In our surveillance, we isolated influenza subtypes other these than mostly during 2008–2009, with very few other subtypes isolated after 2009. This finding suggests that subtypes H9N2 and H5N1 predominate in live-bird markets.

Phylogenetic analyses showed that, with 1 exception, the isolates we analyzed were homogenous and shared the common ancestor A/Qa/HK/G1/97 (H9N2). G1 was the only lineage identified. The viruses we analyzed shared close nucleotide homology with those of G1 lineage isolated from birds in Pakistan or India, which suggests that the Bangladesh viruses may have their origin in those countries (or vice versa). We speculate that, during the early 2000s, subtype H9N2 viruses circulating in Pakistan were introduced into Bangladesh through regional poultry or pet trade and then established themselves in domestic poultry. Although subtype H9N2 and H5N1 viruses were co-isolated, phylogenetic analysis did not identify any reassortment between the subtypes. However, the subtype H9N2 viruses still maintained the internal genes that were part of the subtype H5N1 reassortant previously implicated in human infection (*7*).

Results from our analyses support our hypothesis that the subtype H9N2 isolates from Bangladesh are related to those from Pakistan and have evolved into 2 distinct subpopulations based on host species. The chicken viruses we analyzed were antigenically homogenous and are related to human viruses but distinct from quail viruses. Furthermore, small changes in the quail viruses should be monitored closely because quail were the host species implicated during the emergence of the subtype H9N2 virus that jumped to humans in 1999 (*7*). In addition, quail were suggested to be the contributing host species of the H9N2/H5N1 reassortant of the subtype H5N1 virus that infected humans in 1997 (*4*)

Our analyses also shows that subtype H9N2 viruses circulating in Bangladesh show a trend toward accumulating molecular markers that favor interspecies transmission. Distinct mutations throughout the viral genomes have been implicated in the adaptation of viruses to a mammalian host (Table 4, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0336-T4.htm; and Table 5, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0336-T5.htm). In almost all of the Bangladesh viruses, the RBS of HA has the Q226L substitution, which has been implicated in human virus–like receptor specificity and is critical for replication and direct transmission of these viruses in ferrets (*33,34*).

Although the viruses we isolated have low pathogenicity and do not possess a polybasic cleavage site, they carry a motif similar to cleavage sites seen in the highly pathogenic H5N1 and H7N3 viruses. This result suggests that subtype H9N2 viruses have the potential to acquire a polybasic site and become highly pathogenic. In the HA of nearly all the isolates that we analyzed, 2 potential glycosylation sites were lost; this process was also seen in the adaptation of duck viruses to poultry (*32,35*). Because the duck isolate A/Env/BD/1041/2009 retained its glycosylation site, we hypothesize that the introduction of the Y439 lineage via ducks was unsuccessful because the G1 lineage was already dominant in the local poultry species.

In most of the viruses we analyzed, we observed residues in the avian–human signature positions becoming fixed (HA 226L and 183H, M1 V15I, M2 L55F, NS E227K, NP R214K, E372D, PB1-L13P); in certain isolates, the mutations were fixed by unique substitutions whose functions are unknown (HA Q227I, M2 E16V, I28F, PB1-F2 R79L). In addition, these viruses have acquired substitutions similar to those seen in highly pathogenic subtype H5N1 and H7N3 and pandemic subtype H1N1 and H3N2 viruses (*36*). Noticeably, in the NS gene's PDZ motif, most subtype H9N2 isolates carried the KSEV motif seen in the 1918 pandemic subtype H1N1 virus (*11*,*37*). In the PA genes of 2 subtype H9N2 viruses, we identified R57Q and S409N substitutions, both of which were in the 1968 pandemic subtype H3N2 virus. These observations confirm that the Bangladesh subtype H9N2 viruses have accumulated molecular markers that influence host specificity and pathogenesis.

In conclusion, our results show that the Bangladesh subtype H9N2 viruses are genetically similar to, but distinct from, the A/Qa/HK/G1/97 isolate, which has been previously implicated in human infection (*7*). However, the Bangladesh viruses have accumulated molecular characteristics required for infecting humans and are antigenically similar to the human subtype H9N2 virus isolated from a patient in Bangladesh. These viruses have a tendency to reassort with highly pathogenic subtype H5N1 (*7*) and H7 viruses (*38,39*), which is particularly concerning in light of the emergence of avian influenza A(H7N9) virus in China that contains 6 gene segments from H9N2 influenza viruses and causes lethal infection in humans (*40*). The emergence of this reassortant virus emphasizes the potential for, and the danger of, transmission of subtype H9N2 viruses to humans.

Acknowledgments

We thank Jerry Parker and Richard Elia for maintaining the influenza database at St. Jude Children's Research Hospital, James Knowles for providing administrative assistance, and Angela McArthur for scientific editing. We also thank the Centers for Disease Control and Prevention for providing the human influenza A(H9N2) virus from Bangladesh.

This work was supported by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services (contract nos. N01-AI30071 and HHSN272200900007C); and the American Lebanese Syrian Associated Charities.

Dr Shanmuganthan is a postdoctoral fellow working in Dr Webster's laboratory at St. Jude Children's Research Hospital. His primary research interest is the epidemiology, control, and prevention of zoonotic diseases.

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Protection by Face Masks against Influenza A(H1N1)pdm09 Virus on Trans-Pacific Passenger Aircraft, 2009

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In response to several influenza A(H1N1)pdm09 infections that developed in passengers after they traveled on the same 2 flights from New York, New York, USA, to Hong Kong, China, to Fuzhou, China, we assessed transmission of influenza A(H1N1)pdm09 virus on these flights. We defined a case of infection as onset of fever and respiratory symptoms and detection of virus by PCR in a passenger or crew member of either flight. Illness developed only in passengers who traveled on the New York to Hong Kong flight. We compared exposures of 9 case-passengers with those of 32 asymptomatic control-passengers. None of the 9 case-passengers, compared with 47% (15/32) of control-passengers, wore a face mask for the entire flight (odds ratio 0, 95% CI 0–0.71). The source case-passenger was not identified. Wearing a face mask was a protective factor against influenza infection. We recommend a more comprehensive intervention study to accurately estimate this effect.

After influenza A(H1N1)pdm09 virus was identified in April 2009 (*1*), it spread rapidly, largely through air travel by infected passengers (*2*). On May 2, 2009, China implemented intensive screening of arriving air passengers by using thermal cameras to detect fever and a short questionnaire about existing respiratory symptoms and fever; passengers were advised to seek medical consultation if fever or respiratory symptoms developed ≤7 days of arrival (*3,4*). Nasopharyngeal swab specimens collected from all arriving febrile passengers were tested for virus at the nearest provincial, city, or county Centers for Disease Control (CDC) laboratory by using real-time reverse transcription PCR (RT-PCR) (*5*). If any of these results were positive results, all passengers on the same flight were quarantined.

On May 11, 2009, this system detected the first confirmed influenza A(H1N1)pdm09 infection in mainland China in a US traveler (*6*). As of May 29, the system detected 21 other imported infections in passengers arriving on international flights. On May 29, the first locally acquired influenza A(H1N1)pdm09 infection was detected.

On May 30, acute onset of fever (38.3°C), cough, sore throat, and headache developed in a 22-year-old man. He sought treatment at a clinic in Fuzhou, China, where medical staff learned that he recently arrived from New York, New York, USA (hereafter referred to as New York) and reported a suspected case of influenza A(H1N1)pdm09 infection to the county CDC. On May 31, duplicate nasopharyngeal swabs specimens from the patient were positive for influenza A(H1N1)pdm09 virus at Fuzhou CDC and Fujian Provincial CDC.

On May 27 at 10:40 AM (all times are Bejing local time), the patient had departed New York on a flight to Hong Kong, China. After flying for 5 hours and 50 min, the plane made a scheduled stopover in Vancouver, British Columbia, Canada. All passengers remained on board during the stopover, which lasted 1 hour and 15 min (4:30 PM–5:45 PM). Air-handling systems were fully operational. The aircraft left Vancouver and flew for 13 hours and 15 min and arrived in Hong Kong at 7:00 AM on May 28. In Hong Kong, 63 passengers transferred to a Hong Kong to

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DOI: http://dx.doi.org/10.3201/eid1909.121765

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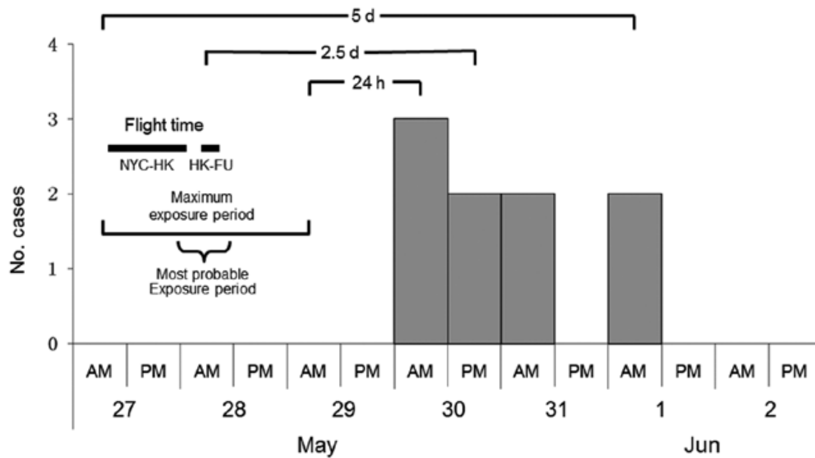

Figure 1. Time of disease onset for persons infected with influenza A(H1N1)pdm09 virus on an international flight from New York, New York (NYC), to Hong Kong (HK) and Fujian Province (FU), China, May 2009. The most probable exposure period was calculated by subtracting median incubation time for influenza A(H1N1)pdm09 (2.5 days) from the time interval containing median onset of a case (PM, May 30). Beginning of the maximum exposure period was calculated by subtracting the maximum incubation period (5 days) from the midpoint of the interval containing onset of the most recent case (AM, June 1). End of the maximum exposure period was calculated by subtracting the minimum incubation period (24 h) from the midpoint of the interval containing onset of first case (AM, May 30).

Fuzhou flight, which departed Hong Kong at 8:50 AM and arrived at in Fuzhou City Airport at 10:30 AM (flight time 1 hour and 40 min.).

The aforementioned patient had no fever or respiratory symptoms when screened on arrival in Fuzhou. The Fujian Provincial CDC, concerned that other passengers on the Hong Kong to Fuzhou flight might be infected, traced and quarantined (involuntary social distancing) the arriving passengers and crew members in their own homes, designated hotels, or hospitals. According to Chinese Ministry of Health guidelines (*7*), social contacts of this confirmed case-patient were traced and quarantined. These passengers, crew members, and contacts were monitored for 7 days for fever and respiratory illness; nasopharyngeal swab specimens were obtained from symptomatic persons. This effort identified 7 additional case-passengers on the Hong Kong to Fuzhou flight in whom symptoms developed during May 30–June 1 and had influenza A(H1N1)pdm09 infection confirmed by RT-PCR. All 8 case-passengers had arrived in Hong Kong on the same New York to Hong Kong flight. The China CDC and Fujian Provincial CDC initiated an outbreak investigation to assess possible transmission of influenza A(H1N1) pdm09 virus on those flights and better understand risks for influenza spread in confined settings.

Methods

Case Definition

We defined a suspected case of influenza A(H1N1) pdm09 infection as an acute, febrile respiratory illness with

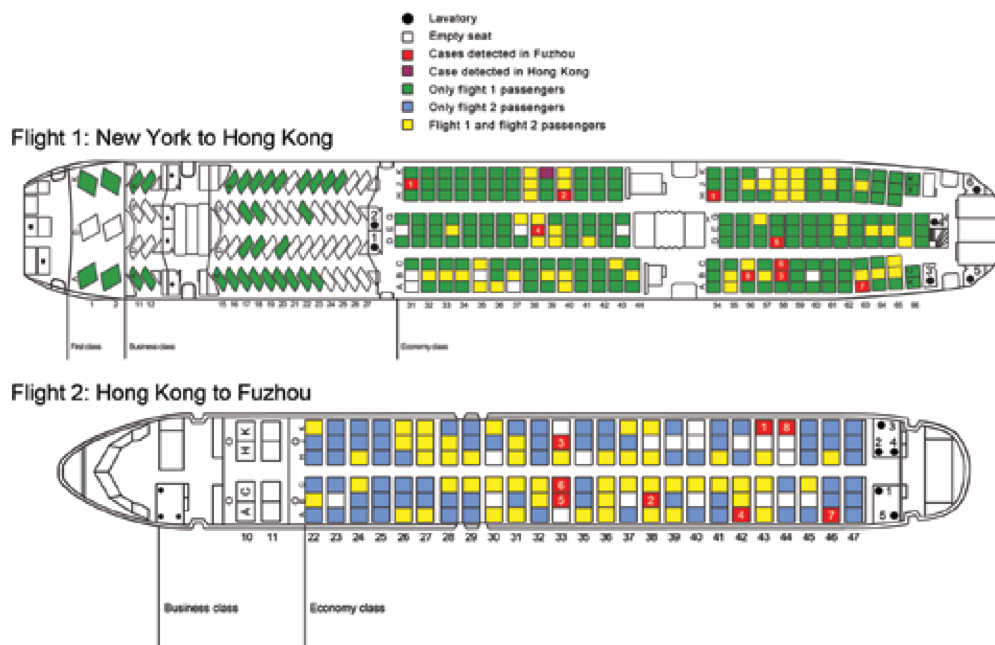

Figure 2. Schematic diagrams of the plane for the flight from New York, New York, to Hong Kong, China (Flight 1), and the plane for the flight from Hong Kong to Fuzhou, China, (Flight 2), May 2009. Case-passenger 1 on the flight from New York to Hong Kong changed his seat in Vancouver, British Columbia, Canada.

onset during May 21–June 4, 2009, among passengers or crew members on the New York to Hong Kong flight on May 27 or the Hong Kong to Fuzhou flight on May 28. A confirmed case was a suspected case with laboratory evidence of influenza A(H1N1)pdm09 infection by PCR testing of respiratory specimens (*5*). We defined influenza-like illness (ILI) as acute onset of fever (≥37.5°C) and cough or sore throat.

Retrospective Investigation

From the Fuzhou airport quarantine post, we obtained a list of passengers who arrived in Fuzhou on the Hong Kong to Fuzhou flight. All passengers had been quarantined for 7 days at home or in designated hotels or hospitals. Body temperatures were measured daily; if fever (≥37.5°C) or respiratory symptoms developed in passengers, a nasopharyngeal swab specimen was obtained and tested for influenza A(H1N1)pdm09 by using RT-PCR. Health professionals at the Centre for Health Protection, Department of Health, Hong Kong, attempted to contact all passengers on the New York to Hong Kong flight who had disembarked in Hong Kong. These professionals obtained information from these passengers regarding onset of fever and respiratory symptoms, medical care, antiviral drugs, and underlying medical conditions. We were unable to contact passengers who transferred to connecting flights from Hong Kong to other destinations in China or Southeast Asia.

To approximate the most probable exposure period for this apparent point-source outbreak (Figure 1), we subtracted the median incubation period for influenza A(H1N1)pdm09 (2.5 days) (*8–11*) from the 12-hour interval for onset of the median case (PM, May 30) (*12*). To approximate the beginning of the maximum exposure period, we subtracted the maximum incubation period (5 days) from the midpoint of the interval for onset of the most recent case (AM, June 1). Similarly, for the end of the maximum exposure period, we subtracted the minimum incubation period (24 hours) from the midpoint of the interval containing onset of the first case (AM, May 30). We compared attack rates by flight and examined aircraft seating charts for spatial distribution of case-passengers and their mutual proximity.

Case–Control Study

To assess risk factors for transmission of influenza A(H1N1)pdm09 on the New York to Hong Kong flight, we conducted a case–control study. We compared exposure history and other risk factors of 9 confirmed case-passengers with those of 32 control-passengers in the economy-class cabin. We attempted to contact 55 noninfected passengers who disembarked in Fuzhou and 18 noninfected passengers who disembarked in Hong Kong, and we interviewed all persons >5 years of age who agreed to be interviewed. Crew members and business-class passengers were excluded. A total of 32 noninfected passengers provided complete information and served as controls. Of these 32 control-passengers, 28 boarded in New York; 27 disembarked in Fuzhou and 1 disembarked in Hong Kong; and 4 boarded in Vancouver and disembarked in Hong Kong.

We conducted face-to-face interviews with case- and control-passengers bound for Fuzhou at hospitals or hotel rooms where they were quarantined. For passengers quarantined at home or who disembarked in Hong Kong (including 1 case-passenger in Hong Kong), interviews were conducted by telephone. Using a standard questionnaire, we interviewed case- and control-passengers on factors potentially affecting the likelihood of influenza A(H1N1)pdm09 virus infection during the 7 days before and during the flight. These factors included contact with ILI patients ≤1 week before the flight, moving around the airplane during the flight, lavatory use, handwashing, face mask use (wearing a face mask, for how long, and when they wore it and did not wear it), and talking with other passengers.

Laboratory Testing

Respiratory specimens (nasal, throat, and nasopharyngeal swab specimens and nasopharyngeal aspirates) were collected from suspected case-passengers and persons being quarantined in whom fever or respiratory symptoms developed. We detected influenza A(H1N1)pdm09 virus nucleic acid by using RT-PCR and standard PCR with virus-specific primers according to standard protocols (*5,13,14*) at biosafety level 2 laboratories at the Fuzhou CDC, the Fujian CDC, and the Public Health Laboratory Centre at the Hong Kong Department of Health.

Statistical Analysis

We used Fisher exact test to compare frequencies between case and control groups and StatXact 8 (*15*) to calculate exact odds ratios (ORs), 95% CIs, and p values. All statistical tests were 2-sided and had a power of $\alpha = 0.05$.

Results

Outbreak Description

Of 144 persons (136 passengers and 8 crew) on the Hong Kong to Fuzhou flight, follow-up and quarantine measures were completed for 140; 8 (5.7%) had confirmed influenza A(H1N1)pdm09 infections; all 8 had ILI. Four additional febrile passengers did not have respiratory symptoms and were negative for influenza A(H1N1)pdm09 virus. In addition, 3 (7.5%) of 40 social contacts of case-passengers had ILI; 2 had confirmed influenza A(H1N1)pdm09 infections. All 8 confirmed case-passengers with influenza A(H1N1)pdm09 infections were among 63 passengers who had transferred from the New York to Hong Kong flight (attack rate

13%), compared with none among 73 other passengers who boarded in Hong Kong (attack rate 0) (p<0.01, by Fisher exact test) or among the 8 crew members. The investigation focused on the New York to Hong Kong flight. All 9 (8 in Fuzhou and 1 in Hong Kong) case-passengers had departed on the New York to Hong Kong flight at 10:40 AM on May 27 (total flight time 20 hours and 20 min).

A total of 260 passengers and 14 crew members were on the New York to Hong Kong flight. After arrival in Hong Kong, 63 passengers transferred to the Hong Kong to Fuzhou flight, 91 passengers disembarked at Hong Kong, and 106 passengers transferred to flights bound for other cities in China or Southeast Asia. The Centre for Health Protection at the Hong Kong Department of Health traced 19 of 91 passengers who disembarked in Hong Kong. One (5.3%) had ILI and a confirmed influenza A(H1N1)pdm09 infection. The attack rate for the 63 Fuzhou passengers and 19 Hong Kong passengers who could be evaluated was 11% (9/82). Among 72 passengers who disembarked in Hong Kong but could not be contacted, some probably continued traveling in China by bus, ferry, train, and car. We were unable to trace the 106 passengers who disembarked in Hong Kong and flew to other destinations.

All 9 infected passengers had mild, self-limiting ILI characterized by fever (100%) and cough (78%) or sore throat (44%). Onset of fever or respiratory illness occurred during May 30–June 1 (3 days) (median onset time during the second 12 hours of May 30), suggesting a point source (Figure 1). Using the 2.5-day median incubation periods for influenza A(H1N1)pdm09, the most probable exposure period was from midnight to noon on May 28, which coincides with the final 6 hours of the New York to Hong Kong flight, waiting in the Hong Kong airport, and during the Hong Kong to Fuzhou flight. The maximum estimated exposure period for this point-source outbreak was from 12 hours before departure from New York to 12 hours after arrival in Fuzhou. Case-passengers sat throughout economy-class cabins on the New York to Hong Kong flight (Figure 2). Age range of the 9 infected passengers (5 male passengers) was 6–46 years (median 20 years).

All 144 passengers and crew members on the Hong Kong to Fuzhou flight and the 91 passengers and crew members on the New York to Hong Kong flight were screened for fever and respiratory symptoms at arrival at Fuzhou Airport. The other 106 passengers who flew to other cities in China or Southeast Asia were not screened in Hong Kong. One passenger on arrival in Fuzhou had fever (37.5°C) and a stuffy nose, but duplicate nasopharyngeal specimens were negative for influenza A(H1N1)pdm09 virus, and repeat temperature checks showed no fever (37.2°C). Three days later, ILI abruptly developed in this passenger (temperature 38.5°C), and infection with influenza A(H1N1)pdm09 virus was confirmed. This passenger and 2 contacts in New York (father and a co-worker) had nasal congestion without fever or ILI since May 16. No other case-passenger recalled recent respiratory illness before or during the flights or contact with any person with respiratory illness during the week before departure or with another passenger who had respiratory illness during either flight or after arriving in Fuzhou. During the 5 days before onset, 1 person had taken another flight and 1 had visited a tourism site (Chinatown) in New York.

Case–Control Investigation

Children were underrepresented in the control group, but age and sex of these children did not differ (Table). From New York to Vancouver, 11% (1/9) case-passengers wore a face mask compared with 57% (16/28) of control-passengers (OR 0.094, 95% CI 0.002–0.91). From Vancouver to Hong Kong, no case-passengers wore a face mask compared with 47% (15/32) of control-passengers (OR 0, 95% CI 0–0.71). For the New York to Hong Kong flight, no case-passengers wore a face mask compared with 47% (15/32) of control-passengers (OR 0, 95% CI 0–0.71). Among control-passengers who used face masks, 4 did not use them during the New York to Vancouver trip, and 3 did not use them during the Vancouver to Hong Kong trip. Exposure to any lavatories or specific lavatories, talking with other passengers, moving around the aircraft, and reported hand hygiene during the New York to Hong Kong flight were not associated with being a case-passenger (Table). Reported handwashing was highly homogeneous among case- and control-passengers and was performed exclusively at each visit to the lavatory and by using the wet towel provided before meals. No one in the case and control groups had contacted with patients with ILI ≤1 week before the flight.

Discussion

During this outbreak, influenza A(H1N1)pdm09 virus appeared to have been transmitted on a New York to Hong Kong flight. No other common time–place exposure could account for the point-source pattern. The most probable exposure period was during the New York to Hong Kong flight, in the Hong Kong airport, or during the Hong Kong to Fuzhou flight. Lack of cases in passengers or crew members on the Hong Kong to Fuzhou flight who were not on the New York to Hong Kong flight and the case in the Hong Kong resident suggested that exposure was not on the Hong Kong to Fuzhou flight or after landing in Fuzhou. Our results do not support exposure in New York before arrival at the airport, except that the estimated exposure period included the final 12 hours in New York. Exposure at common points in the airport in New York (e.g., at the check-in counter or security checkpoints) would have been brief and thus unlikely to lead to a high attack rate.

Table. Case–control analysis of potential risk or protective factors for 9 case-passengers infected with influenza A(H1N1)pdm09 virus and 32 control-passengers on a flight from New York, New York, to Hong Kong, China, May 2009*

Risk or protective factor	No response, no. (%)† Case	No response, no. (%)† Control	Response, no. (%) Case	Response, no. (%) Control	OR (95% CI)‡	2-tailed p value‡
Age, y					NA	0.06§
<20	0	0	4 (44)	4 (12)	NA	NA
20–40	0	0	4 (44)	15 (47)	NA	NA
>40	0	0	1 (11)	13 (41)	NA	NA
Male sex	0	0	5 (56)	15 (47)	1.42 (0.25–8.29)	0.06
Chinese ethnicity	0	0	8 (89)	32 (100)	0 (0–10.97)	0.22
Flight from New York to Vancouver						
Wearing mask	0	0	1 (11)	16 (57)	0.094 (0.002–0.910)	0.037
Using lavatory 3	0	3 (11)	2 (22)	7 (28)	0.73 (0.061–5.40)	1.0
Using lavatory 5	1 (11)	3 (11)	2 (25)	4 (16)	1.75 (0.13–16)	0.91
Using lavatory 3 or 5	1 (11)	1 (3.6)	3 (38)	11 (41)	0.87 (0.11–5.70)	1.0
Using lavatory 3, 4, 5, or 6	0	1 (3.6)	6 (67)	14 (52)	1.86 (0.31–14.00)	0.71
Flight from Vancouver to Hong Kong						
Wearing mask	0	0	0	15 (47)	0 (0–0.71)	0.018
Using lavatory 3	0	2 (6)	5 (56)	7 (23)	4.1 (0.65–22)	0.16
Using lavatory 5	1 (13)	2 (6)	1 (13)	5 (17)	0.71 (0.013–8.200)	1.0
Using lavatory 3 or 5	1 (13)	0	5 (63)	12 (38)	2.8 (0.44–21.00)	0.38
Using lavatory 3, 4, 5, or 6	0	0	7 (78)	20 (63)	2.1 (0.32–24.00)	0.67
Flight from New York to Hong Kong						
Wearing mask	0	0	0	15 (47)	0 (0–0.71)	0.018
Talking with other passengers	0	0	2 (22)	6 (19)	1.2 (0.1–9.2)	1.0
Moving around airplane	0	0	1 (11)	3 (9.4)	1.4 (0.023–20.000)	1.0
Contact with patients with ILI	0	0	0	0	NE	NE
Hand sanitation¶						
Washing hands when using lavatory	0	0	9 (100)	32 (100)	NE	NE
Cleaning hands before eating	0	0	8 (89)	29 (91)	0.83 (0.06–49.00)	0.55
Among 9 case- and 17 control-passengers who did not wear masks during flight from Vancouver to Hong Kong						
Using lavatory 3	0	1 (6)	5 (56)	6 (38)	2.1 (0.297–15.000)	0.65
Using lavatory 3 or 5	1 (13)	0	5 (63)	9 (53)	1.5 (0.20–13.00)	0.99
Using lavatory 3, 4, 5, or 6	0	0	7 (78)	13 (76)	1.1 (0.12–15.00)	1.0

*Of the 32 control-passengers on the flight from New York to Hong Kong, 28 boarded in New York and 4 boarded in Vancouver, British Columbia, Canada. OR, odds ratio; NA, not applicable; ILI, influenza-like illness; NE, not estimated.
†No. case-passengers or control-passengers who did not answer the question.
‡By Fisher Exact test and StatXact 8 (*15*).
§Difference among 3 age groups.
¶Always washed hands when using lavatory and always used wet towel before eating.

Furthermore, passengers did not wear masks at these points, and we would not have shown their protective effect. Before arrival at the airport, case-passengers were not together at the same place at the same time to account for the point-source pattern. For the 4 nonstop flights/day from New York airports to China during May 29–June 2, there were 4 confirmed influenza A(H1N1)pdm09 infections, which is equivalent to 0.2 infections/flight. Exposure in New York led to a prevalence of infection among passengers similar to the prevalence of influenza A(H1N1)pdm09 during the same week among the general population of New York. However, published surveillance estimates in the United States indicated that the 348 confirmed influenza A(H1N1)pdm09 virus infections reported in New York that week would be equivalent to a prevalence of 0.31%, which is similar to the previous estimate of <1 case among the passengers on the New York to Hong Kong flight (*16*).

This outbreak highlights the role of air travel in spread of influenza infections (*17–20*). All 9 infected passengers during the incubation period passed through airport fever and symptom screening, indicating that transmission on flights can escape detection. Also, 106 passengers on the New York to Hong Kong flight flew to other destinations and passed through different quarantine posts. In addition, an unknown number of the 91 passengers who traveled to Hong Kong continued into China by bus, ferry, train, and car through different quarantine posts. By the time we recognized the link to the New York to Hong Kong flight, passengers had dispersed and could not be traced. We estimate that 106 economy-class passengers, for whom risk for infection was 11%, traveled onward, potentially leading to dissemination of 12 infections to multiple sites.

The case-passengers were seated in 2 separate cabins of economy class. Previous investigations showed that increased risk for influenza in aircraft clustered within 2 rows in front of and behind a passenger with ILI (*18–21*). The source case-patient(s) might have been among the 106 transit passengers who were not screened in Hong Kong and who flew to other destinations and could not be traced. Without the source case-patient(s) being identified, we cannot explain the dispersed distribution, but we can offer some possibilities. There might have been ≥2 unrelated source

case-passengers on the flight seated in each of the economy class cabins. A crew member serving economy class might have been infectious. However, all 14 crew members showed negative results when screened in Hong Kong. A common and frequently visited area such as a lavatory or food-service area might have been heavily contaminated with nasopharyngeal droplets from an infectious passenger. However, we did not find an association with lavatory use or general frequency of moving around the aircraft.

Airborne transmission in the airplane might be possible. Experiments and simulations show that particles <2 µm in diameter could be distributed widely, albeit at a low concentration, from a single source throughout an aircraft cabin (*22*). Influenza outbreaks in a train and an aircraft cabin with nonoperating air conditioning showed wide distribution of secondary cases, suggestive of airborne transmission (*23,24*). Infection from a fellow passenger should also have resulted in clustering from the much longer and closer exposure to respiratory droplet and aerosols during the 20-hour exposure during the flight.

Observational studies in hospitals, households, and community settings have shown a range of protective effects of face mask use against confirmed influenza, ILI, or respiratory infection (range 0%–74% reduction) (*25–34*). Several factors might explain the stronger effect observed in this outbreak. Exposure was for <24 hours in a confined space with limited activity of exposed persons. The other studies all involved days to months of exposure in the community or hospitals with free movement outside the immediate setting where face masks were used. Compliance with face mask use was probably greater among travelers on a single flight who were concerned about unpredictable health effects of the new virus. In 2 household studies, contacts were already exposed before the face mask was first worn (*26,29*). Only 2 of 7 other studies detected protection against confirmed influenza infection (*29,30*).

Extensive surveillance data for the United States showed that even at the peak of seasonal influenza transmission, <35% of persons with ILI had confirmed influenza (*35*). Other viruses causing ILI and having higher ratios of droplet transmission will lessen the observed epidemiologic effect of measures that protect against aerosol transmission. Face masks also have an unintended effect of reducing frequency of touching the mouth and nose and self-infection from contaminated hands. Accordingly, their protective effect, although suggestive, is not conclusive for airborne transmission of inhaled or inspired aerosols. Because long-distance air travel is a major route of dissemination of influenza virus (*17,18,36*), our findings regarding the effect of face mask use on flights should be evaluated further and considered for decreasing spread of influenza virus.

Hand hygiene has been recommended for preventing influenza transmission (*37*). In this outbreak, reported handwashing after lavatory use was universal and hand cleaning before meals was nearly universal for all passengers. Thus, we were unable to examine any effect of hand hygiene. However, hand hygiene would not have altered the effect of face mask use.

Direct experimentation and computer simulations indicate that N95 face masks should reduce the risk for airborne transmission of influenza virus by aerosols containing droplet nuclei (diameter <2 µm) in aircraft cabins by 90% (*38–40*). Less efficient face masks (e.g., surgical or medical) also decrease exposure to aerosols of droplet nuclei to a lesser (8–12 fold) degree than N95 masks (*36*), and they provide protection against larger droplets. We did not determine the type of mask worn by the passengers; presumably, individually acquired masks represented a mixture of N95 and other less efficient masks. Our findings are based on a small number of influenza infections, and an actual effectiveness of 90% is well within the confidence level of our estimate. The source case-person(s) of influenza virus on the flight might have taken a cough suppressant and might not have been actively coughing. If influenza virus had been expelled by normal breathing only, protection by an N95 mask for a 4-hour flight could approach 100% (*40*). Finally, infection from larger inspired or inoculated droplets from an infected person who actively circulated throughout the economy cabins could also explain the observed protection afforded by less-efficient mask types.

This investigation had several limitations. We lacked seating and illness information for 68% of the economy-class passengers on the New York to Hong Kong flight, among whom was probably the source case-passenger. The missing source case-passenger is also a gap in the evidence that transmission occurred on the flight. We were unable to determine the outcome of passengers and crew who disembarked in Vancouver and whether transmission occurred during 1 or both legs of the flight. Types of face masks used were unknown. With only 9 cases in 25% of the passengers, our case–control study had poor sensitivity.

In summary, this outbreak probably resulted from a common source exposure to influenza A(H1N1)pdm09 virus on the New York to Hong Kong flight. Wearing a face mask was associated with a decreased risk for influenza acquisition during this long-duration flight. Border entry screening did not detect case-passengers during the influenza incubation period. We recommend a more comprehensive intervention study to accurately estimate the protective effect of face masks for preventing influenza virus transmission on long-distance flights.

Acknowledgments

We thank participating hospitals, local health departments, the Fujian Provincial Center for Disease Control and Prevention,

and the Centre for Health Protection, Department of Health, Hong Kong, for assistance with coordinating data collection.

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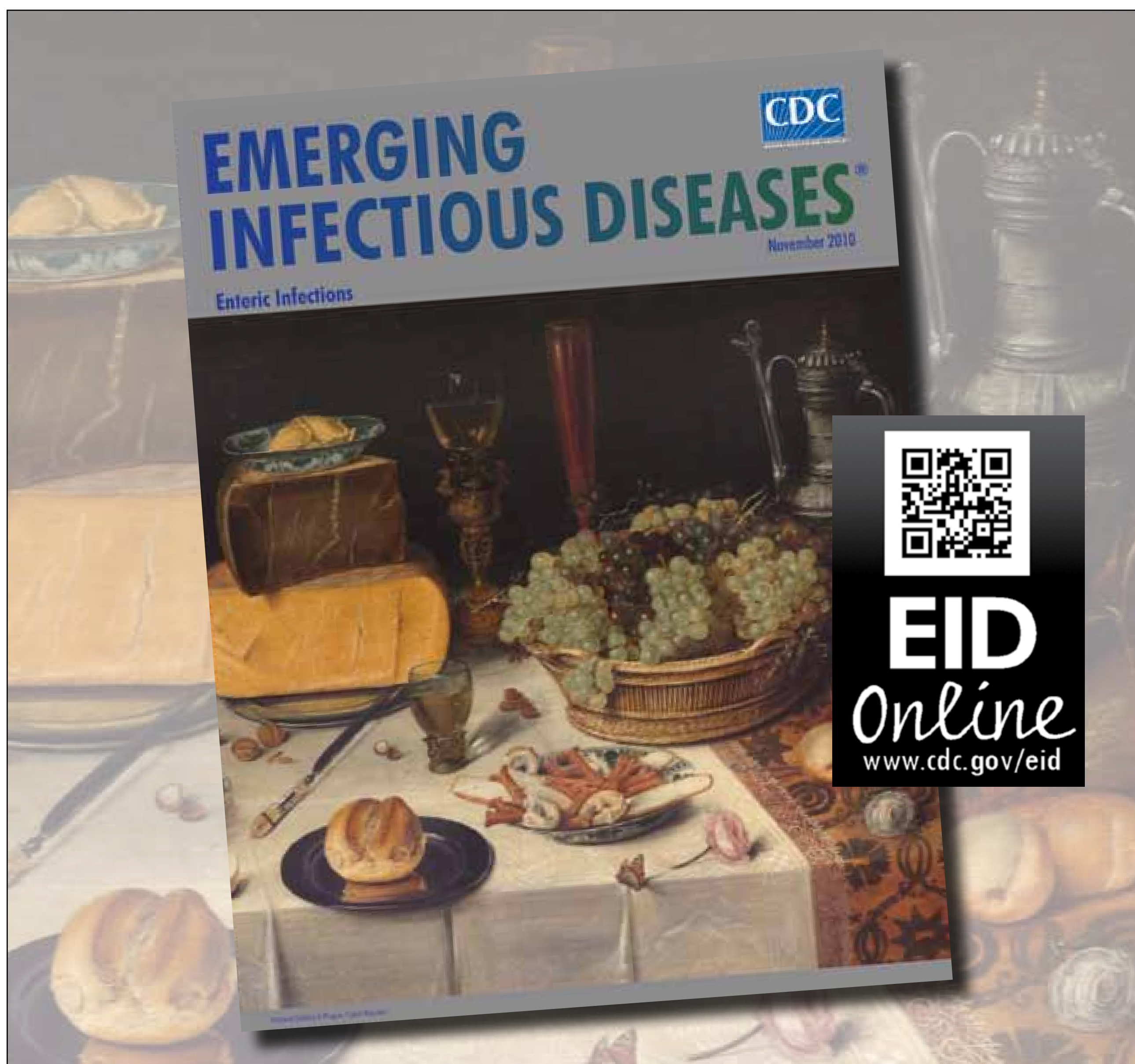

Mumps Postexposure Prophylaxis with a Third Dose of Measles-Mumps-Rubella Vaccine, Orange County, New York, USA

Amy Parker Fiebelkorn, Jacqueline Lawler, Aaron T. Curns, Christina Brandeburg, and Gregory S. Wallace

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Release date: AUGUST 22, 2013; Expiration date: AUGUST 22, 2014

Learning Objectives

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

- Assess means to prevent mumps
- Distinguish recommendations regarding the use of the measles-mumps-rubella (MMR) vaccine as postexposure prophylaxis
- Analyze the presentation of mumps among patients in the current study
- Assess the effectiveness of the MMR vaccine as postexposure prophylaxis during an outbreak of mumps.

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Carol E. Snarey, MA, Technical Writer/Editor, *Emerging Infectious Diseases. Disclosure: Carol E. Snarey, MA, has disclosed no relevant financial relationships.*

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DOI: http://dx.doi.org/10.3201/eid1909.130299

Although the measles-mumps-rubella (MMR) vaccine is not recommended for mumps postexposure prophylaxis (PEP), data on its effectiveness are limited. During the 2009–2010 mumps outbreak in the northeastern United States, we assessed effectiveness of PEP with a third dose of MMR vaccine among contacts in Orthodox

Jewish households who were given a third dose within 5 days of mumps onset in the household's index patient. During the first incubation period after onset in the index patient, mumps attack rates were compared between persons who received a third MMR dose and 2-dose–vaccinated persons who had not. Twenty-eight (11.7%) of 239 eligible household members received a third MMR dose as PEP. Mumps attack rates were 0% among third-dose recipients versus 5.2% among 2-dose recipients without PEP (p = 0.57). Although a third MMR dose administered as PEP did not have a significant effect, it may offer some benefits in specific outbreak contexts.

Mumps is an acute, viral illness that classically is manifested as parotitis and can cause severe complications, including encephalitis (*1*), deafness (*2*,*3*), and orchitis (*4*). In 1977, the Advisory Committee on Immunization Practices (ACIP) recommended 1 mumps vaccine dose for routine childhood vaccination, and in 1989, the committee recommended that 2 doses of measles-mumps-rubella (MMR) vaccine be given to school-aged children and select high-risk groups for improved measles control (*5*). ACIP does not recommend administering MMR vaccine during mumps outbreaks as postexposure prophylaxis (i.e., vaccine administered during a brief window after exposure to prevent mumps infection) (*5*). Antibody response to the mumps component of MMR vaccine is generally believed to develop too late to provide effective prophylaxis after a person has been exposed to mumps (*6*,*7*), but data are insufficient for assessing a possible prophylactic effect.

During 2009–2010, a large mumps outbreak affected 3,502 persons in the Orthodox Jewish community in the northeastern United States. Students, from elementary school through college, had 2,370 (67.7%) cases; of these case-patients, 85% had received the recommended 2 doses of MMR vaccine (*8*). Yeshivas (i.e., private, traditional Jewish schools with extended school days) and households characterized by large families, typical in the Orthodox Jewish community, were the primary settings for mumps transmission (*8*). The objective of this study was to assess secondary mumps attack rates among Orthodox Jewish household contacts in Orange County, New York, who received postexposure prophylaxis (PEP) with a third dose of MMR vaccine within 5 days of mumps introduction into a household by a family member, and compare them with secondary mumps attack rates of household contacts with 2 previous MMR doses who did not receive PEP.

Methods

Study Population

The study population was a geographically and socially clustered community of ≈20,000 persons, primarily Orthodox Jews, in Orange County, New York. A common feature of this community was its high household contact rates because of large family size (average, 6 members) and shared bedrooms (*9*). Most members of this community followed ACIP vaccination recommendations; 2-dose MMR vaccine coverage among school-aged children in the community was 94.3%, which was higher than the national average (*8*,*10*,*11*).

Case Definitions and PEP Definition

Mumps cases were classified according to the case definition for mumps of the Council of State and Territorial Epidemiologists in 2008 (*12*). Household members were considered secondary case-patients if mumps onset occurred 12–25 days (1 incubation period) after parotitis onset in the household's index case-patient. Household members were considered to be co-primary case-patients if mumps onset occurred within 11 days after onset in the household index patient. PEP was defined as a dose of MMR vaccine given to a household contact within the first 5 days of another household member's onset of provider-diagnosed mumps parotitis. Any dose administered earlier than this was not considered a PEP dose.

Study Design and Eligibility Criteria

Suspected cases of mumps within the affected community were reported to the local health department, and parents were encouraged to contact one of the community's 2 primary medical providers. The provider invited the parent to bring the case-patient and all other household members to the clinic and instructed the parent regarding routine measures required by the practice to prevent patients with respiratory illnesses from causing the other patients to be exposed to the virus. At the initial visit, the provider assessed the case-patient, and if a diagnosis of mumps was confirmed, the provider determined whether other household members had a history of mumps and their vaccination status. If the family visited the healthcare provider during the study period, February 24–April 24, 2010, then household members who met the eligibility criteria (i.e., had received 2 documented doses of MMR vaccine, had no contraindications for vaccination, had no history of mumps, and 5 days had not yet elapsed since onset of parotitis in the household index case-patient) were offered a third dose of MMR vaccine. Household members who were not up to date with their routine vaccinations were offered a first or second dose of MMR vaccine as PEP. Adult household members whose vaccination history was not documented were eligible to receive a dose.

Eligible family members who did not receive PEP either chose not to be vaccinated or lived in a household in which mumps had been diagnosed in a case-patient earlier in the outbreak, and it was too late for family members to

receive PEP. Household members were not eligible for PEP and were excluded from the analysis if they had received a recent MMR vaccine dose within the past 60 days (i.e., either at their health care provider's office or by participating in a recent school-based third-dose MMR intervention study [*10*]), if they had a history of mumps, if they were too young to be vaccinated (i.e., <1 year of age), or if they were a co-primary or index case-patient. Members of households who chose not to be vaccinated and members of households of mumps case-patients identified earlier in the outbreak who were not offered a third MMR dose were used as a comparison group.

Because the use of a third dose of MMR vaccine is not recommended by ACIP for PEP, a protocol was submitted and approved by the US Centers for Disease Control and Prevention and New York State Institutional Review Boards. Participants provided written consent or assent.

Baseline and Follow-up Surveys

Baseline surveys captured demographic characteristics, MMR vaccination history, and mumps history of household members. Follow-up surveys were completed at least 60 days after the date of parotitis onset for the household mumps index case-patient; information gathered included any MMR doses received by family members since the baseline interview and whether mumps developed in any household members.

Vaccination Status Verification

Vaccination status of study participants was assessed. Health care provider records were reviewed to verify this information.

Data Analysis

All data were analyzed with SAS 9.3 (SAS Institute Inc., Cary, NC, USA). For each household, we added the number of family members eligible for PEP with MMR vaccine and the number who received PEP. χ^2 and Wilcoxon rank sum tests were used to compare 1) demographic characteristics and intervals since last MMR dose among index case-patients and 2) household members who received a third dose of MMR vaccine as PEP with persons who had 2 previous doses and did not receive PEP. Secondary mumps attack rates during the first incubation period after mumps onset in the index case-patient were calculated.

Results

Characteristics of the Index Case-Patients

Of the 49 index case-patients, 25 were male (51.0%) (Table 1). The median age was 9 years (range 1–39 years). Thirty-two (65.3%) had received 2 doses of MMR vaccine. Eleven (22.4%) index case-patients were unvaccinated or had unknown MMR vaccination status. Among the 38 (77.6%) who reported receiving ≥1 doses of MMR vaccine, the median interval since their last dose was 47 months (range 3–170 months).

PEP

In 49 households, there were 365 household members, of whom 239 (65.5%) were eligible to receive PEP and 126 (34.5%) were deemed ineligible and excluded from further analysis of mumps risk factors. Those excluded were the following: 59 household members who had received a recent dose of MMR vaccine within 60 days before the intervention, 49 who were the household index patients, 15 who were <1 year of age, 2 who became co-primary case-patients, and 1 woman with a history of having had mumps in 1979 (Figure).

Forty-four (18.4%) of the 239 eligible household members received a postexposure dose of MMR vaccine; 28 (11.7%) received a third MMR vaccine dose, 6 (2.5%) received a second MMR vaccine dose, 2 (0.8%) received a first MMR vaccine dose, and 8 (3.3%) adults with unknown vaccination status received a dose. The age groups of household members who received a third dose of MMR vaccine as postexposure prophylaxis included 10 (27.8%) of 36 children aged 4–6 years, 17 (24.3%) of 70 children 7–17 years of age, and 1 (1.1%) of 88 adults ≥18 years. Of the 16 other household members who received PEP with MMR vaccine, 2 children 1 year of age received a first dose, 6 children 1–17 years of age received a second dose, and 8 adults with unknown MMR vaccination status received a dose (Table 2).

Postexposure vaccinations were not administered to 195 (81.6%) eligible household members. Of eligible

Table 1. Characteristics of index case-patients with mumps, Orange County, New York, 2009–2010*

Characteristic	No. (%)
Age. y	
Median (range)	9 (1–39)
0–3	3 (6.1)
4–6	8 (16.3)
7–17	27 (55.1)
≥18	11 (22.4)
Sex	
F	24 (49.0)
M	25 (51.0)
No. MMR doses	
0	11 (22.4)
1	5 (10.2)
2	32 (65.3)
3	1 (2.0)†
≥1	38 (77.6)
Median interval (range) since last dose, mo	47 (3–170)

*MMR, measles-mumps-rubella vaccine; values are in no. (%) unless otherwise indicated.

†Person received a third MMR dose in January 2010 prior to parotitis onset in February 2010. This person did not receive the third dose as part of the study.

persons who did not receive postexposure vaccine, 77 had previously received 2 doses (of whom 21 were 4–6 years of age, 50 were 7–17 years, and 6 were ≥18 years), 40 had previously received 1 dose (of whom 33 were 1–3 years, 3 were 4–6 years, 2 were 7–17 years, and 2 were ≥18 years), and 78 had unknown vaccination status (of whom 6 were 1–3 years of age, 71 were ≥18 years, and 1 was <18 years, but the exact age was not available).

Secondary Case-Patients

Of the 9 household secondary cases that occurred during the first incubation period after the index patient's mumps onset, 3 (33.3%) were in male patients. Only 1 (11.1%) case-patient received the MMR vaccine as PEP. He was a 27-year-old father with unknown vaccination status. Eight (88.9%) persons who did not receive PEP became infected with mumps during the first incubation period after their exposure (2 were from the same household).

The median age of the 8 secondary case-patients who did not receive PEP was 18.5 years (range 6–39 years). The ages and vaccination status of these 8 included the following: 1 child 6 years of age who had a history of 2 doses of MMR vaccine, 3 children 7–17 years of age who had a history of 2 doses of MMR vaccine, and 4 adults with unknown vaccination status. All household members ≥18 years of age who were infected were parents of index case-patients. The interval between the last MMR vaccine dose and reported mumps onset was 18 days for the father who received PEP and from 2 to 6 years for the 4 case-patients with known vaccination status who did not receive PEP.

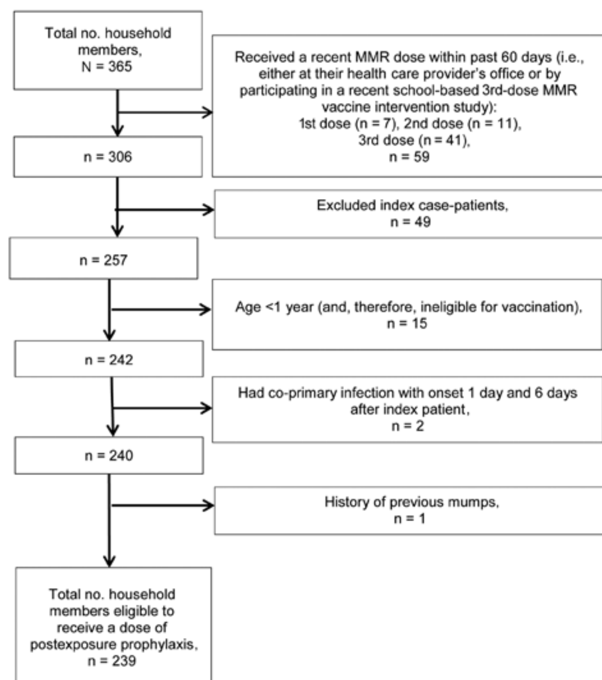

Figure. Exclusion criteria for household members eligible to receive a dose of measles-mumps-rubella (MMR) vaccine as postexposure prophylaxis, Orange County, New York, USA, 2009–2010.

Mumps also developed in 2 persons within the first 11 days of the onset of the index case; these patients were considered co-primary. No secondary cases developed during the first incubation period after the index patient's mumps onset among remaining family members at risk for mumps in the 2 households with co-primary case-patients (5 members were at risk in each household).

The interval between receipt of the last dose of MMR vaccine and mumps onset among the index case-patients did not differ between households with a secondary case-patient and those without (median interval 3 years; both groups, p = 1.0). Additionally, the ages of the index case-patients did not significantly differ between households with a secondary case-patient and those without (median ages 7.5 years and 9 years, respectively; p = 0.21).

Persons Who Received a Third Dose of MMR Vaccine versus Those with 2 Doses

None of the 28 family members who received a third dose of MMR vaccine as PEP became infected with mumps virus in contrast with 4 (5.2%) of the 77 who had previously received 2 doses but did not receive PEP (Table 3). The difference in secondary attack rates between the 2 groups was not statistically significant (p = 0.57). Two of the 2-dose case-patients were male; the sex-specific attack rates were 6.9% for male patients and 4.3% for female patients (p = 0.62). The median age of those receiving a third dose was 8 years (range 5–20 years) and also 8 years (range 4–20 years) among those eligible who did not. The median number of years since the last MMR dose (before the PEP dose) was 10 years (range 2–39 years) among those who received a third dose, compared with 11 years (range 0–39 years) among those eligible who did not (p = 0.47).

Discussion

Although the attack rate among persons who received a third dose of MMR vaccine as PEP was 0%, compared with a 5.2% attack rate for those with 2 doses who did not receive PEP, the difference was not statistically significant. Nonetheless, MMR vaccine administered as PEP might offer some benefits. If the exposure did not result in infection, the vaccine should boost antibody titers high enough to induce protection against subsequent infection (*13*,*14*). Such boosting of antibody titers would be useful during an outbreak in which the virus continues to circulate and future exposures are likely. If infection does occur, the postexposure vaccine dose may lead to milder clinical manifestations, lower complication rates, and shorter duration of virus shedding (*15*).

Table 2. Classification by age group of eligible family members who did or did not receive a dose of MMR vaccine as postexposure prophylaxis, Orange County, New York, USA, 2009–2010*

Age, y	No. received 3rd MMR dose	No. received 2nd MMR dose	No. received 1st MMR dose	No. that received any dose (dose unknown)†	No. that did not receive any dose	Total no. eligible
1–3	0	3	2	0	39	44
4–6	10	2	0	0	24	36
7–17	17	1	0	0	52	70
≥18	1	0	0	8	79	88
Unknown	0	0	0	0	1	1
Total (%)	28 (12)	6 (3)	2 (1)	8 (3)	195 (82)	239

*MMR, measles-mumps-rubella vaccine.
†MMR vaccine doses were administered as postexposure prophylaxis.

Although a third dose of MMR vaccine has been previously administered for outbreak control (*10*,*16*), to our knowledge, a third dose of MMR vaccine has never previously been administered in a study to assess its effectiveness as PEP. In 1986, a first dose of MMR vaccine was given as PEP in a Tennessee public high school to 53 of 178 students with no presumptive evidence of immunity. During the Tennessee outbreak, in 15 (28.3%) of 53 students who received a first dose of MMR vaccine as PEP, mumps developed between 1 and 21 days (1 incubation period) after they visited the clinic compared with mumps developing in 51 (40.8%) of 125 nonvaccinated students who did not receive PEP (6).

In addition to the outbreak in the northeastern United States, other large mumps outbreaks have occurred among highly vaccinated US populations in recent years. In 2006, a total of 6,584 reported cases occurred, primarily in college students in the midwestern United States. Standard control measures (e.g., isolation and vaccine catch-up campaigns) were implemented for outbreak control (*17*) with modest effectiveness. The outbreak did not subside until summer break when the students left their college campuses. During 2009-2010, a total of 505 mumps cases were reported in the US Territory of Guam, primarily among school-aged children 9–14 years of age, 96% of whom had received 2 doses of MMR vaccine. In addition to application of standard control measures, a third dose of MMR vaccine was administered to the most affected age group for outbreak control, not as PEP. The effectiveness of the intervention was inconclusive (*16*). Outbreaks have also been reported in other industrialized countries among populations in which the proportion who received 2 doses of vaccine was high (*18*–*21*).

Two MMR vaccine doses provide 66%–95% effectiveness against mumps (*22*,*23*), and the 2-dose policy has reduced mumps incidence by >99% compared with incidence during the prevaccine era (*24*). Nonetheless, mumps outbreaks in well-vaccinated populations continue to occur, posing challenges for outbreak control. Current public health measures for preventing the spread of mumps during outbreaks, including isolation, quarantine, contact tracing, and increasing vaccine coverage have had limited effect (*17*,*25*). When schools follow public health guidance and send infected students home for 5 days, the intervention may be too late. Mumps can spread from symptomatic persons before parotitis onset. Mumps can also spread from persons who have asymptomatic infections, which can be as high as 15%–27% of infected persons (*4*,*26*). In addition, isolating patients and quarantining contacts may be ineffective when infected persons live in large households with many other susceptible persons. Finally, raising vaccine coverage is also difficult in contexts where 2-dose vaccine coverage is already high, because current policy does not recommend a routine third MMR vaccine dose (*5*).

In the 2 households with co-primary cases in this study, no additional cases occurred during the first incubation period. This finding suggests that those households were not more infectious than households with only 1 index patient.

This study was subject to limitations, however. Household members may have been exposed to mumps by a contact outside the home. Although our methods might have been more robust if we could have randomly selected household contacts to receive PEP, because of ethical considerations, it was necessary to offer PEP to all eligible household contacts. Some household members had received the third dose during a school intervention a couple of months before this study. In addition, some members received either a first, second, or third dose during the outbreak but not as part of the study. Although these persons were excluded from the analysis because their doses were not administered as PEP, these doses outside the study may have limited the effect of the study doses because additional family members were protected. This could have lowered mumps attack rates in the households by reducing the number of susceptible persons. When the risk for mumps among persons potentially susceptible was assessed, the limited sample size and low attack rates resulted in large confidence intervals. Finally, the power of the study to detect a significant difference was extremely low because of the small number of study households, the relatively late implementation of the study during the outbreak, and the low number of mumps cases that occurred in the study population.

Table 3. Demographic characteristics, median number of months since second MMR vaccine dose, and number of mumps case-patients among household members, Orange County, New York, USA, 2009–2010*

Characteristic	Received 3rd MMR dose as PEP, n = 28	Had 2 previous MMR vaccine doses, received no PEP, n = 77	p value
Sex			
M	16 (57.1)	29 (37.7)	0.19
F	12 (42.9)	47 (61.0)	
Unknown	0	1 (1.3)	
Age, y			
4–6	10 (35.7)	21 (27.3)	0.58
7–17	17 (60.7)	50 (64.9)	
≥18	1 (3.6)	6 (7.8)	
Median no. months since 2nd MMR dose, IQR	120 (62–177)	139 (62–210)	0.47
Minimum–maximum no. months	32–468	10–468	
Mumps onset, attack rate†	0	4 (5.2)	0.57

*MMR, measles-mumps-rubella vaccine; PEP, postexposure prophylaxis; IQR, interquartile range; values are no. (%) unless otherwise indicated.
†Onset of mumps occurred 12–25 days after onset of mumps in index case-patient.

Although 2 MMR doses are sufficient for preventing mumps in most settings, administering a third MMR dose may be worthwhile in specific outbreak contexts, even if it does not offer protection as PEP. Our findings support the need for additional evaluations in which third doses of MMR vaccine are used as PEP in outbreaks among populations with high 2-dose vaccination coverage. Future studies on administering any dose of MMR vaccine for mumps PEP during mumps outbreaks are also warranted.

Acknowledgments

We thank Alan Werzberger, Beth Post, Ezras Choilim, the community, and the patients for their cooperation and participation in this study.

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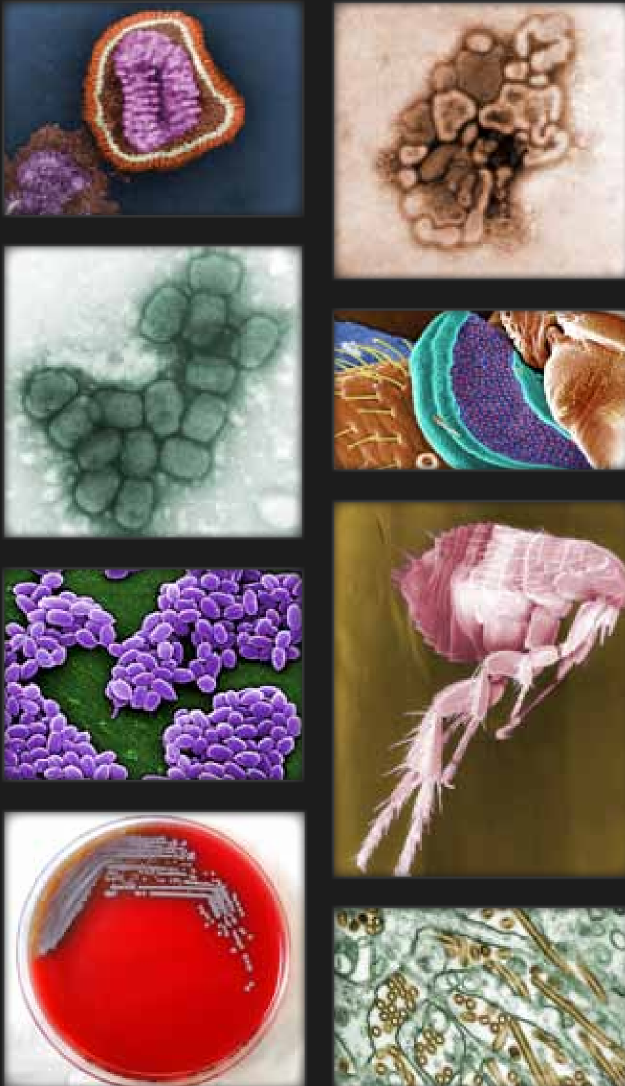

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Continued Evolution of West Nile Virus, Houston, Texas, USA, 2002–2012

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We investigated the genetics and evolution of West Nile virus (WNV) since initial detection in the United States in 1999 on the basis of continual surveillance studies in the Houston, Texas, USA, metropolitan area (Harris County) as a surrogate model for WNV evolution on a national scale. Full-length genomic sequencing of 14 novel 2010–2012 WNV isolates collected from resident birds in Harris County demonstrates emergence of 4 independent genetic groups distinct from historical strains circulating in the greater Houston region since 2002. Phylogenetic and geospatial analyses of the 2012 WNV isolates indicate closer genetic relationship with 2003–2006 Harris County isolates than more recent 2007–2011 isolates. Inferred monophyletic relationships of these groups with several 2006–2009 northeastern US isolates supports potential introduction of a novel WNV strain in Texas since 2010. These results emphasize the need to maintain WNV surveillance activities to better understand WNV transmission dynamics in the United States.

The emergence of West Nile virus (WNV) in the Western Hemisphere in 1999 poses an ongoing public health threat in North America as the most common cause of epidemic encephalitis in the United States (*1*). WNV transmission is maintained in an enzootic cycle between mosquitoes and birds; equids, humans, other mammals, and some bird species act as dead-end hosts (*2*). Human infections are asymptomatic in 80% of cases, and West Nile fever develops in ≈20% of infected patients, which progresses to neuroinvasive disease in <1% (*3*).

After introduction of WNV in the United States in 1999 (*4*), local transmission of the original New York genotype (NY99) in resident *Culex* spp. mosquito and wild bird populations fueled the geographic expansion of WNV from the northeastern region across the continental United States, north into Canada, and south into Central and South America (*5*–*7*). Subsequent introduction into Texas in 2002 resulted in 105 confirmed human infections, high mortality rates among local corvids, and a 31.2% seroconversion rate among resident birds of Harris County, Texas (Houston metropolitan area) alone (*8*). Uninterrupted surveillance of the related St. Louis encephalitis virus in local *Culex* spp. mosquito populations by the Harris County Mosquito Control Division since 1964 provided an ideal infrastructure for the expanded detection of WNV activity in the mosquito vector and the wild bird reservoir on a major bird migratory pathway. Routine collections of WNV-positive birds and mosquito pools to date have provided an outstanding opportunity to investigate WNV diversity and evolution on a fine geographic scale comparable to similar surveillance foci in the midwestern and New England regions of the United States (*9*–*11*). However, because of its geographic location, Harris County represents a different ecosystem, namely a warm year-round climate with unique resident mosquito and avian species.

Phylogenetic examination of 2002–2004 Harris County isolates confirmed rapid displacement of the NY99 genotype with the novel North American genotype (NA/WN02) in 2002 (*12*). Fine-scale geospatial genetic comparisons of these isolates provided further evidence of increased WNV genetic diversification in the greater Houston region relative to the homogenous distribution of the now extinct NY99 genotype (*12*,*13*). Subsequently, McMullen et al. identified the emergence of the southwestern genotype (SW/WN03) in the southwestern United States in 2003 and positive selection for the encoded NS4A-A85T and NS5-K314R amino acid substitutions in the WNV nonstructural (NS) proteins (*14*). To date, the NA/WN02 and SW/WN03 genotypes still appear to co-circulate.

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DOI: http://dx.doi.org/10.3201/eid1909.130377

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Endemic transmission of WNV in the United States since 2006 has shown a dramatic decrease in the confirmed incidence of clinical WNV disease; <1,100 annual human cases were reported during 2008–2011 (*15*). Despite identification of regional heterogeneous WNV populations, a relative stasis in WNV evolution has been observed in Harris County, consistent with the logistic molecular clock model and a decreasing viral growth rate proposed on a national scale (*16*–*18*). Notably, the current 2012 WNV transmission season demonstrates major divergence from this status quo; >5,600 human infections have been reported nationwide (*15*). Incidence of clinical WNV disease in the Texas outbreak alone accounted for >33% of the cases in the United States (1,868 cases, including 844 reports of neuroinvasive disease and 89 deaths) and >994 confirmed cases in the greater Dallas/Fort Worth, Texas, metropolitan area and 101 cases in Harris County (*15*,*19*). These changes reflect final US and Texas and WNV cases for 2012 reported by the Centers for Disease Control and Prevention (Atlanta, GA, USA) (www.cdc.gov/media/releases/2013/a0513-west-nile.html) after submission of this report after review. Therefore, studies concerning the continued evolution of WNV in the central and southern United States remain vital for elucidating the role of dynamic genetic heterogeneity and accumulation of novel mutations in the transmission dynamics and incidence of clinical WNV disease.

We report consensus sequence analyses of 17 novel full-length 2010 (n = 1), 2011 (n = 1), and 2012 (n = 15) WNV isolates collected from WNV-positive birds and *Culex* spp. mosquito pools in Harris County Texas (n = 14) and the greater Dallas/Fort Worth region (n = 3). Inclusion of these new isolates with 28 additional 2002–2009 Harris County WNV isolates in phylogenetic and geospatial analyses of the greater Houston region provides an ideal model for investigating the role of ongoing WNV evolution relative to environmental and clinical incidence reported over the past decade. Furthermore, isolates from the recent WNV epidemic demonstrate closer phylogenetic relationships with original 2002–2003 Harris County isolates, inconsistent with phylogenetic trends observed until 2011 and supporting evidence for the recent introduction of a novel WNV strain(s) in Texas from another geographic region.

Materials and Methods

Virus Isolates

The World Reference Center for Emerging Viruses and Arboviruses at the University of Texas Medical Branch in Galveston, Texas, supplied the WNV isolates characterized herein provided by the Harris County Mosquito Control Division and Texas Department of State Health Services (Table 1). The fourteen 2010–2012 Harris County isolates were cultured from brain tissue of collected dead WNV-positive birds in Vero cells at the University of Texas Medical Branch in Galveston. Secondary passage of each isolate in Vero cells provided working stocks that were stored at –80°C. These same procedures were used for the 3 Dallas/Fort Worth mosquito isolates.

Genomic Sequencing

Extraction of viral RNA, reverse transcription PCR, and sequencing was conducted according to established protocols (*12*,*14*). Resulting sequences were aligned and edited relative to the prototype NY99-flamingo382-99 strain (AF196835) by using ContigExpress in the VectorNTI program (Invitrogen, Carlsbad, CA, USA) (*4*). Sequences were assembled in BioEdit version 7.0.9.0 (*20*) with 358 full-length North American WNV isolates published in GenBank (as of November 2012) and the IS-98 STD (AF481864) Israeli isolate (*21*). The encoded 10,299 nt open reading frames (ORFs) for all 372 WNV isolates were aligned by using the MUSCLE algorithm (*22*); the ORF was used because some viruses published in GenBank include the ORF alone and not the entire genome. Two additional alignments included the ORF of all 42 2002–2012 Harris County WNV isolates with and without the three 2012 Dallas/Fort Worth isolates and the prototype NY99-flamingo382-99 and IS-98 STD reference strains. Screening for potential site-specific positive selection was performed by using the Datamonkey server (*23*,*24*) and the single-likelihood ancestor counting, fixed effect likelihood (FEL), and internal branches FEL methods (*25*,*26*). Positive selection was defined as $d_N > d_S$ and a p value <0.05 in >1 method.

Phylogenetic Analyses

Neighbor-joining (NJ) phylogenetic analyses of all alignments were processed in Seaview version 4.3.0 by using the Hasegawa–Kishino–Yano 85 substitution model and 10,000 bootstrap replicates (*27*). Maximum-likelihood (ML) analyses used RAxML-HPC Blackbox version 7.3.2 (*28*,*29*) on the CIPRES Science Gateway version 3.1 server (*30*) with the generalized time reversible substitution model with invariable sites, a gamma distribution, and 1,000 bootstrap replicates. Bayesian-inferred coalescent phylogenies were produced in BEAST version 1.6.2 (http://beast.bio.ed.ac.uk) by using the generalized time reversible substitution model with invariable sites and a gamma distribution with applied taxa dates, an uncorrelated lognormal relaxed clock model, and Bayesian Skyline prior constraints (*31*). Resulting BEAST.log and TRE files were down-sampled from triplicate 50,000,000 state runs by using LogCombiner version 1.7.4 and validation in Tracer version 1.5 (*31*). Inferred phylogenetic trees were edited in FigTree

version 1.3.1 (www.mybiosoftware.com/phylogenetic-analysis/2407). In all NJ and ML tree topologies, the IS-98 STD isolate was used as a common phylogenetic outgroup.

Statistical Analyses

The Fischer exact test and post hoc analyses were performed at α = 0.05 (IBM SPSS statistics version 20; IBM, Armonk, NY, USA) to test the association between year of collection and determined phylogenetic groupings. Adjusted standardized residuals (*z* scores) at α = 0.05 were compared against the critical *z* value (± 1.96) with a Bonferroni correction for multiple comparisons.

Results

WNV Collection

A total of 14 WNV isolates from Harris County (Table 1) were examined. Two isolates were collected

Table 1. West Nile virus isolates described in sequence and phylogenetic analyses, Harris County , Texas, USA,1998–2012*

Strain	Map code†	Location	Zip code‡	Source§	Collection year	GenBank accession no.
IS-98 STD	–	Eilat, Israel	NA	White stork	1998	AF481864
NY99-flamingo382-99	–	New York, NY, USA	NA	Chilean flamingo	1999	AF196835
Kuritz [TVP 8553]	–	Beaumont, TX, USA	NA	Human	2002	AY289214
TX114	B1-1	Harris Co., TX, USA	77043	Blue jay	2002	GU827998
TX 2002 1	–	Harris Co., TX, USA	NA	Human	2002	DQ164198
TX 2002 2	–	Harris Co., TX, USA	NA	Human	2002	DQ164205
TX1153	B2-1	Harris Co., TX, USA	77077	Mourning dove	2003	AY712945
TX1171	B3-1	Harris Co., TX, USA	77030	Blue jay	2003	AY712946
TX1175	B4-3	Harris Co., TX, USA	77346	Blue jay	2003	GU828000
TX1461	–	Harris Co., TX, USA	NA	Avian	2003	AY712947
TX 2003	–	Harris Co., TX, USA	NA	Human	2003	DQ164199
v4095	M10-2	Harris Co., TX, USA	77093	*Culex quinquefasciatus*	2003	GU828002
v4369	M11-2	Harris Co., TX, USA	77039	*Cx. quinquefasciatus*	2003	AY712948
v4380	M12-2	Harris Co., TX, USA	77093	*Cx. quinquefasciatus*	2003	GU828001
M12214	M1-5	Harris Co., TX, USA	77020	*Cx. quinquefasciatus*	2005	JF415914
TX5058	B5	Harris Co., TX, USA	77057	Blue jay	2005	JF415929
M6019	M2-6	Harris Co., TX, USA	77026	*Cx. quinquefasciatus*	2006	JF415930
TX5810	B6-6	Harris Co., TX, USA	77345	Common grackle	2006	JF415915
TX6276	B7-10	Harris Co., TX, USA	77373	Northern mockingbird	2006	JF415916
M19433	M3-5	Harris Co., TX, USA	77020	*Cx. quinquefasciatus*	2007	JF415919
TX6647	B8-5	Harris Co., TX, USA	77084	Blue jay	2007	JF415917
TX6747	B9	Harris Co., TX, USA	77346	Blue jay	2007	JF415918
TX7191	B10	Harris Co., TX, USA	77005	Blue jay	2007	JF415920
TX7558	B11-5	Harris Co., TX, USA	77375	Blue jay	2008	JF415921
M20122	M4-4	Harris Co., TX, USA	77026	*Aedes albopictus*	2009	JF415928
M20140	M5-4	Harris Co., TX, USA	77021	*Ae. albopictus*	2009	JF415926
M20141	M6-4	Harris Co., TX, USA	77021	*Cx. quinquefasciatus*	2009	JF415927
M37012	M7-4	Harris Co., TX, USA	77021	*Cx. quinquefasciatus*	2009	JF415922
M37906	M8-4	Harris Co., TX, USA	77021	*Cx. quinquefasciatus*	2009	JF415923
M38488	M9-4	Harris Co., TX, USA	77004	*Ae. albopictus*	2009	JF415925
TX7827	B12-7	Harris Co., TX, USA	77060	Blue jay	2009	JF415924
TX8092	**B13-7**	**Harris Co., TX, USA**	**77084**	**House sparrow**	**2010**	**KC333374**
TX8349	**B14-5**	**Harris Co., TX, USA**	**77016**	**House sparrow**	**2011**	**KC333375**
TX8546	**B15-9**	**Harris Co., TX, USA**	**77065**	**Blue jay**	**2012**	**KC333376**
TX8551	**B16-10**	**Harris Co., TX, USA**	**77449**	**Blue jay**	**2012**	**KC333377**
TX8559	**B17-9**	**Harris Co., TX, USA**	**77506**	**Blue jay**	**2012**	**KC333378**
TX8560	**B18-8**	**Harris Co., TX, USA**	**77062**	**Blue jay**	**2012**	**KC333379**
TX8562	**B19-10**	**Harris Co., TX, USA**	**77450**	**Blue jay**	**2012**	**KC333380**
TX8567	**B20-9**	**Harris Co., TX, USA**	**77065**	**Blue jay**	**2012**	**KC333381**
TX8571	**B21-8**	**Harris Co., TX, USA**	**77059**	**Blue Jay**	**2012**	**KC333382**
TX8572	**B22**	**Harris Co., TX, USA**	**77080**	**Blue jay**	**2012**	**KC333383**
TX8589	**B23-10**	**Harris Co., TX, USA**	**77049**	**Loggerhead shrike**	**2012**	**KC333384**
TX8590	**B24-10**	**Harris Co., TX, USA**	**77339**	**Blue jay**	**2012**	**KC333385**
TX8599	**B25-8**	**Harris Co., TX, USA**	**77058**	**Blue jay**	**2012**	**KC333386**
TX8604	**B26**	**Harris Co., TX, USA**	**77021**	**House sparrow**	**2012**	**KC333387**
TX AR12-1486	**–**	**Denton Co., TX, USA**	**NA**	***Cx. quinquefasciatus***	**2012**	**KC711057**
TX AR12-1648	**–**	**Collin Co., TX, USA**	**NA**	***Cx. quinquefasciatus***	**2012**	**KC711058**
TX AR12-10674	**–**	**Collin Co., TX, USA**	**NA**	***Cx. restuans***	**2012**	**KC711059**

*Strains sequenced in this study are indicated in **boldface**. Co., county.
†Map code designations include B (bird) and M (mosquito) for isolates collected in Harris County, TX, with phylogenetic relationships (groups 1–10) indicated after the hyphen (see Figure 1). –, isolates collected outside Harris County, Texas, that have no known geographic collection information.
‡Zip codes indicated for region of isolate collection within Harris County, TX (see Figure 1). NA, not available.
§Mosquito (*Culex* spp.) isolates were collected from mosquito pools.

from house sparrows (*Passer domesticus*) in 2010 and 2011, and 12 isolates were obtained from WNV-positive birds in 2012: 10 from blue jays (*Cyanocitta cristata*) and 1 each from a loggerhead shrike (*Lanius ludovicianus*) and a house sparrow.

Phylogenetic and geospatial analyses characterized herein compared all 14 WNV isolates with 28 other published Harris County isolates collected during 2002–2009 from mosquito pools (n = 12), birds (n = 13), or humans (n = 3) (*12*,*14*,*16*). Genomic sequences were determined for WNV isolates from each year except in 2004: 2002 (n = 3), 2003 (n = 8), 2005 (n = 2), 2006 (n = 3), 2007 (n = 4), 2008 (n = 1), and 2009 (n = 7). Sample coverage was restricted on the basis of sequence and sample availability. The Kuritz (also known as TVP8533; GenBank accession no. AY289214) isolate (Southeast Coastal Texas genotype) was included as a common Texas outgroup, and the TX114 (Bird114; GU827998) isolate served as the prototypic member of the NA/WN02 genotype (*12*,*32*).

Divergent WNV Evolution

Nucleotide Changes

Comparison of the 14 novel Harris County isolates with the NY99 (NY99-flamingo382-99) prototype strain identified 45–79 nt differences (0.41%–0.72%) per 11,029-nt genome (*4*). Each isolate encodes 8 of the 13 nt changes characteristic of the NA/WN02 genotype (*12*). In addition, novel U→C transitions at positions 7015 in the NS4B gene and 8811 in the NS5 gene were conserved in 11 of the 12 Harris County isolates from 2012 (except TX8604).

Amino Acid Substitutions

These 14 isolates differed at 48 unique residues in the encoded 3,433-aa polyprotein relative to NY99 and had 2–10 (0.06%–0.29%) substitutions per isolate. Each isolate encoded the E-V159A substitution characteristic of the NA/WN02 genotype (*12*); however, the signature NS4A-A85T SW/WN03 genotype substitution (*14*) was identified only in the 2011 TX8349 isolate. Furthermore, 11 of the 48 deduced amino acid substitutions were conserved in >1 isolate. Single-likelihood ancestor counting, FEL, and internal branches FEL method analysis identified potential positive selection of the NS2A-H119Y substitution in the 2012 TX8546 isolate.

Phylogenetic and Geospatial Analysis

Phylogenetic reconstruction of ancestral topologies among all 42 published 2002–2012 Harris County WNV isolates used NJ, ML, and relaxed clock Bayesian coalescent methods. Consistent tree topologies rooted to the common IS-98 STD isolate outgroup were produced with all 3 methods. Inclusion of 2010–2012 isolates retained the phylogenetic clustering of the 2002–2009 Harris County isolates within the 6 monophyletic groups (groups 1-6) proposed by McMullen et al. (*14*) (Figure 1). Conserved nucleotide and amino acid divergence among the fourteen 2010–2012 isolates indicates emergence of 4 novel monophyletic clusters of avian isolates (groups 7–10) (Table 2). Furthermore, groups 7–10 demonstrate a significant ($p \leq 0.044$) relationship between year of collection and identified monophyletic lineage with more 2012 isolates clustering within groups 8–10.

Group 7 (0.57% nt divergence from NY99) includes the 2009 TX7827 and 2010 TX8092 isolates. Group 8 consists of the 2003 TX1461 (Bird1461) outgroup and three 2012 isolates: TX8560, TX8571, and TX8599 (0.01%–0.04%). Group 9 (0.40%–0.45%) includes three 2012 isolates: TX8546, TX8559, and TX8567. Group 10 includes the 2006 TX6276 isolate and 2012 isolates from this study:

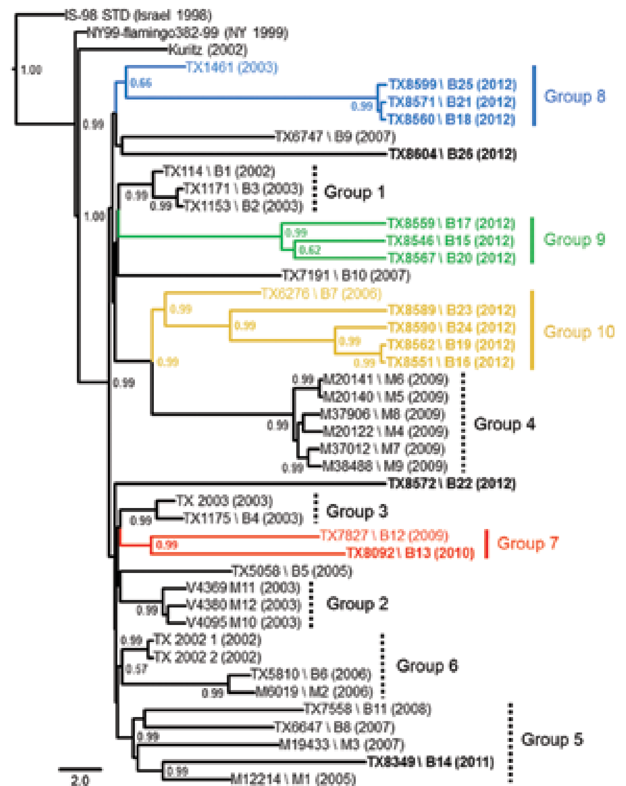

Figure 1. Bayesian-inferred, 50% majority-rule, coalescent phylogenetic tree of published, full-length West Nile virus isolates, Harris County, Texas, USA, 2002–2012. Novel 2010–2012 Harris County isolates cluster into 4 distinct monophyletic groups designated group 7 (red), group 8 (blue), group 9 (green), and group 10 (yellow). Strain names link geographic map code (e.g., B1, B2, M1, M2) with year of collection annotated in parentheses. Isolates sequenced in this study are indicated in **boldface**. Posterior probabilities ≥0.90 are indicated along branches to provide statistical support for inferred topologies. Scale bar indicates divergence time in years.

Table 2. Conserved amino acid substitutions in West Nile virus isolates, Harris County, Texas, USA, 2002–2012*

			Gene																	
			C	prM	E		NS1	NS2A				NS2B	NS3		NS4A	NS4B			NS5	
	Strain†	Year	104	156	**159**	460	236	52	90	95	188	120	180	334	**85**	14	24	240	49	**314**
G	NY99	1999	K	V	**V**	I	I	T	M	L	R	V	E	S	**A**	S	K	I	V	**K**
1	TX114	2002	·	·	**A**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
	TX1153	2003	·	I	**A**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
2	v4369	2003	·	·	**A**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
3	TX1175	2003	·	·	**A**	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·
4	M37906	2009	·	·	**A**	L	·	·	·	·	·	·	·	·	·	·	·	M	·	·
	M38488	2009	·	·	**A**	L	·	·	·	·	·	·	·	·	·	·	·	M	·	·
	M20140	2009	·	·	**A**	L	·	·	·	·	·	·	·	·	·	·	·	M	·	·
5	TX7558	2008	·	·	**A**	·	·	·	·	·	·	·	·	·	**T**	·	·	·	·	·
	TX6647	2007	·	·	**A**	·	·	·	V	·	·	·	·	·	**T**	·	·	·	·	·
	TX8349	2011	·	·	**A**	·	·	·	·	F	·	·	·	·	**T**	·	·	·	·	·
	M19433	2007	·	·	**A**	·	·	·	·	·	·	·	·	·	**T**	·	·	M	·	**R**
6	TX 2002 1	2002	·	·	**A**	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·
	TX5810	2006	·	·	**A**	·	·	·	·	·	·	·	·	·	·	·	·	M	·	·
7	TX7827	2009	·	I	**A**	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·
	TX8092	2010	·	·	**A**	·	·	·	V	·	·	·	·	·	·	·	·	·	·	·
8	TX1461	2003	·	·	**A**	·	·	·	·	·	·	·	D	·	·	·	·	·	·	·
	TX8599	2012	·	·	**A**	·	·	·	·	·	·	·	D	·	·	·	·	·	·	·
9	TX8559	2012	·	·	**A**	·	·	I	·	F	·	·	·	T	·	I	·	·	·	·
10	TX6276	2006	·	·	**A**	M	·	·	·	·	·	·	·	·	·	·	·	M	·	·
	TX8589	2012	·	·	**A**	·	·	·	·	·	K	·	·	·	·	·	·	M	·	·
	TX8590	2012	R	·	**A**	·	V	·	·	·	K	I	·	·	·	·	·	·	I	·
	TX8551	2012	R	·	**A**	·	V	·	·	·	K	I	·	·	·	·	R	·	I	·

*G, group; C, capsid; prM, premembrane; E, envelope; NS, nonstructural. Amino acid changes are relative to the prototype NY99 strain AF196836. Substitutions characteristic of defined NA/WN02 and SW/WN03 genotypes are indicated in **boldface**. Dots indicate no change from the NY99 isolate.
†Indicated substitutions conserved in >1 representative isolate for each of the proposed phylogenetic groups (see Figure 1).

TX8551, TX8562, TX8589, and TX8590 (0.00%–0.61%). Overall, the 37 Harris County isolates in groups 1–10 differ at 80 nt positions and 27 had substitutions shared in >1 isolate (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0377-Techapp1.xlsx). Five substitutions (prM-V156I, NS2A-M90V, NS2A-L95F, NS4B-I240M, and NS4B-E249G) were conserved in >1 phylogenetic group. Furthermore, the signature NS4A-A85T SW/WN03 genotype substitution was present only in the 2011 TX8349 and other group 5 isolates. The TX8572 and TX8604 isolates cluster as outliers (>0.44% nt divergence) to the proposed phylogenetic groups and show increased divergence (1.13% nt and 0.38% aa) between these 2 isolates.

Superimposition of 38 of the 42 Harris County isolates (4 isolates were excluded because of unknown collection location) on a vector-borne WNV incidence map of Harris County, based on known collection information in the 268 mosquito control operational areas, highlights the shift in WNV circulation patterns across the greater Houston region over the past decade (Figure 2). Mosquito pool isolates demonstrate robust genetic homogeneity and limited geographic distribution (Figure 2) in 9 of the 268 operational areas, suggestive of WNV overseasoning in resident mosquito populations. In particular, isolates M1 (2005) and M3 (2007) cluster within group 5, indicating transmission of the same virus strain in Houston across multiple years. In contrast, avian-derived isolates illustrate widespread incidence of similar genetic signatures in 2002–2011 WNV isolates, as highlighted in dispersal of several 2005–2011 group 5 SW/WN03 genotype isolates. However, group 7–10 isolates demonstrate comparable geographic distribution but limited monophyletic support with group 1–6 isolates. Furthermore, group 8 isolates remain geographically restricted compared to more pervasive group 10 isolates. Overall, phylogenetic and geospatial analysis of novel 2012 WNV isolates indicate a closer genetic relationship with 2003–2006 Harris County isolates than with more recent 2007–2011 WNV isolates.

Novel Introduction Event in 2012 WNV Outbreak in Texas

Inclusion of the fourteen 2010–2012 Harris County isolates in an additional phylogenetic analysis with 358 published North American WNV isolates enabled us to evaluate the potential influence of active WNV transmission in North America on the recent WNV evolution dynamics observed in Harris County. NJ, ML, and Bayesian relaxed clock methods produced consistent overall tree topologies with retention of the published NY99, NA/WN02 (*12*), and SW/WN03 (*14*) genotypes (Figure 3, panel A). Groups 7–10 showed conserved clustering within the NA/WN02 genotype and robust (≥0.90 posterior probabilities) monophyletic support for shared lineage with several 2006–2009 New York and Connecticut isolates (Figure 3, panels B–F). Furthermore, the 2010 TX8092 isolate demonstrated consistent monophyletic clustering within the NA/WN02 genotype with the 2009 TX7827 and additional 2008 New York (WNV-1/US/BID-v4622/2008) isolates

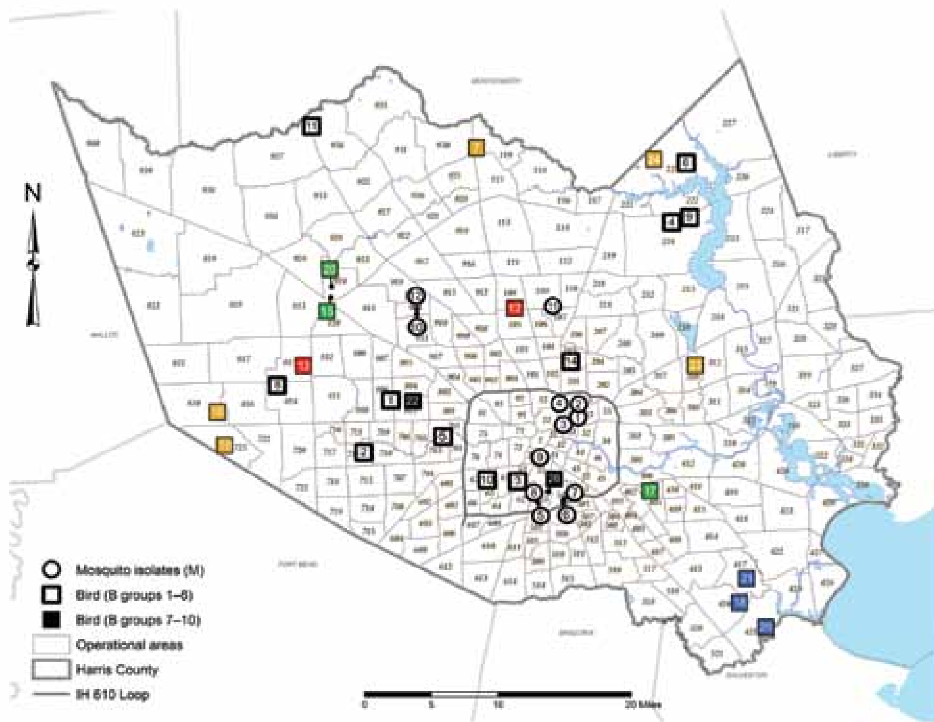

Figure 2. Incidence of vector-borne West Nile Virus (WNV) in Harris County, Texas, USA, 2002–2012, showing cumulative distribution of confirmed avian and mosquito (*Culex* and *Aedes* spp.) WNV isolates. Small numbers indicate reference codes for each of the 268 mosquito control operational areas. Black open symbols indicate 2002–2009 group 1–6 mosquito (circles) and bird (squares) isolates collected in mosquito control operational areas. Colored solid symbols indicate 2002–2012 Harris County isolates that cluster within monophyletic group 7 (red), group 8 (blue), group 9 (green), or group 10 (gold). Black solid symbols indicate nonclustering 2012 TX8572 and TX8604 isolates within groups 1–10. Not shown are TX 2002 1, TX 2002 2, TX 2003, and TX1461. IH, interstate highway.

(Figure 3, panel G). In contrast, the novel 2011 TX8349 Harris County isolate retained topologic distribution within the SW/WN03 genotype. Outside the identified group 7–10 monophyletic clusters, all inferred basal node topologies exhibited poor statistical support (≤0.70 posterior probabilities) within the NA/WN02 genotype.

Principal support for the identified monophyletic lineages is based on limited nucleotide divergence (<0.65%) and retention of unique substitutions between the 2010 and 2012 Harris County isolates and those from the northeastern United States compared with published isolates from Texas and the southwestern United States. In particular, the group 9 monophyletic lineage (Figure 3, panel E) encodes several conserved substitutions: NS2A-T52I, NS2A-L95F, NS3-S334T, and NS4B-S14I with a single NS2A-H119Y substitution shared between the 2008 New York (WNV-1/US/BID/v4097/2008) outgroup and the 2012 TX8546 isolate. Each isolate also encodes the characteristic E-V159A substitution with the conserved absence of the NS4A-A85T and NS5-K314R substitutions supporting monophyletic distribution and ancestral lineage within the NA/WN02 versus NY99 and SW/WN03 genotypes.

Harris County Paradigm—Model for WNV Evolution

To evaluate application of the proposed Harris County paradigm outside the greater Houston metropolitan region, we collected 3 WNV isolates from *Culex* spp. mosquito pools in the recent 2012 Dallas/Fort Worth, Texas WNV outbreak (Table 1) were sequenced and included in a Bayesian phylogenetic analysis with all 42 2002–2012 Harris County isolates. Both Collin County isolates (TX AR12-1648 and TX AR12-10674) exhibited limited nucleotide divergence (≤0.30%) and robust monophyletic clustering (≥0.98 posterior probabilities) with several group 9 and 10 isolates in the Harris County paradigm (Figure 4). In addition, the TX AR12-1468 Denton County isolate demonstrated shared lineage with the nonclustering TX8572 isolate. Overall, our results support potential application of the Harris County paradigm as a relevant model for WNV evolution in Texas as a whole and, possibly, on a national scale.

Discussion

Surveillance of WNV transmission in Harris County, Texas, provides an excellent model for elucidating the dynamics of endemic and epidemic WNV evolution on a fine geographic scale. The southeastern coastal region of Texas serves as a temporary roosting site on a major flyway for migratory birds in transit between more temperate and tropical regions of the Americas. Consequently, in addition to resident bird populations, this region hosts more avian species (and possible WNV reservoir hosts) than anywhere else in the United States. Prior applications of the proposed Harris County paradigm confirmed emergence of NA/WN02 (*12*) and SW/WN03 (*14*) genotypes

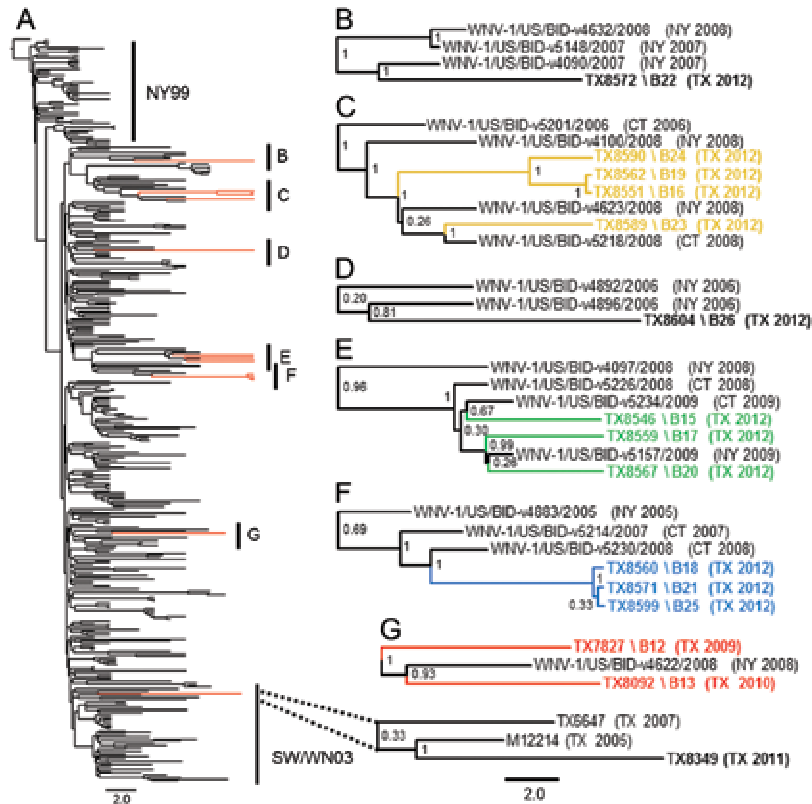

Figure 3. Evolution of West Nile virus (WNV) in North America, 1999–2012. A) Bayesian coalescent tree of all published North American WNV isolates. The NY99 (NY99) and southwestern (SW/WN03) genotypes flank the North American (NA/WN02) genotype containing inferred monophyletic lineages B–G of the novel 2010–2012 Harris County, Texas, WNV isolates. Red indicates WNV isolates sequenced in this study. B) TX8572 2012 Harris County isolate; D) TX8604 2012 Harris County isolate. C and E–G) Proposed monophyletic groups 7–10 described in Figure 1: C) group 10 (yellow); E) group 9 (green); F) group 8 (blue); G) group 7 (red). Isolates sequenced in this study are indicated in **boldface**. Dotted lines indicate distribution of the 2011 TX8349 isolate in the SW/WN03 genotype. Posterior probabilities (range 0.00–1.00) are indicated along branches to provide statistical support for inferred topologies. Scale bars indicate divergence time in years.

from the United States in 2002 and 2003, respectively, as a surrogate model for WNV evolution on a national scale. Our analyses support emergence of 4 novel monophyletic groups of 2010–2012 Harris County isolates (groups 7–10) distinct from group 1–6 phylogenetic clusters identified (Figure 1) (*14*). Furthermore, these isolates exhibit closer ancestral lineage with 2002–2003 Harris County isolates compared with more recent 2007–2009 strains. The single 2011 TX8349 isolate clusters with several 2005–2008 isolates within the group 5 SW/WN03 genotype with conserved expression of the signature NS4A-A85T substitution.

Restriction of conserved substitutions to individual monophyletic groups (Table 2) indicates increased genetic heterogeneity among circulating 2010–2012 populations than with earlier 2002–2009 Harris County WNV populations. Geographic reconstruction of the 42 WNV isolates on a map of Harris County based on mosquito control operational areas (Figure 2) supports a heterogeneous transmission model with limited correlation between fine-scale geographic dispersion and sequence divergence over time (*9*,*10*). However, regional phylogenetic foci among group 2, 4, and 5 mosquito pools and group 8 avian isolates supports potential homogeneous and trans-seasonal WNV transmission in localized vector populations as documented in other studies (*11*,*13*,*33*).

Before the 2010–2012 phylogenetic analyses, a relative stasis in WNV evolution had been observed in Harris County (*16*) and the northeastern United States (*11*,*13*,*17*,*33*) after confirmed emergence of the NA/WN02 and SW/WN03 genotypes. Fine-scale geographic phylogenetic analyses in suburban Chicago and Illinois have identified maintenance and active evolution of heterogeneous WNV populations in local mosquito and avian vectors from the initial introduction into the United States in 1999 until 2008 but not more recently (*9*,*10*,*18*). Applied genomic and phylogenetic comparisons highlight the major divergence of the fourteen 2010–2012 Harris County isolates from historical dynamics of local WNV evolution and emergence of 3 distinct monophyletic lineages (groups 8–10) in 2012 (Figure 1). However, the 2010 and 2012 Harris County isolates exhibit major sequence divergence (>0.42%) relative to group 1–6 isolates despite shared geographic distribution and environmental conditions. In addition, co-circulation of these novel genetic signatures was confirmed in 3 *Culex* spp. mosquito pool isolates obtained from the 2012 WNV outbreak in the greater Dallas/Fort Worth region. On the basis of these observations, introduction of a novel or existing strain from the United States into the circulating greater Houston WNV populations, and possibly Texas as a whole, since 2010 offers an alternative explanation for

these divergent genetic signatures. However, our results do not exclude possible emergence of related existent viral populations as dominant regional strains in the recent epidemic WNV transmission season.

To test these hypotheses, we included fourteen 2010–2012 Harris County isolates in a comprehensive Bayesian phylogenetic analysis with all 358 published North American WNV isolates. Shared lineage of group 7–10 isolates with several 2006–2009 strains form the northeastern United States within the NA/WN02 genotype was identified with robust monophyletic support (≥0.90 posterior probabilities) (Figure 3, panels B–G). Principal evidence for these monophyletic lineages is highlighted on the basis of limited divergence (≤0.65%) and conserved expression of unique substitutions relative to observed genetic diversity (0.8%–1.0%) between published group 1–6 strains from the southwestern United States (*14*).

Our results support inferred lineage of 2010 and 2012 Harris County isolates from ancestral strains in the northeastern United States, consistent with a single or multiple introduction event(s) during 2010–2012 in the greater Houston region. However, poor statistical confidence (≤0.70 posterior probabilities) for all inferred basal node topologies within the NA/WN02 genotype limits direct comparison of independent evolution between Harris County isolates in different monophyletic lineages. Furthermore, consistent phylogenetic grouping of the 2011 TX8349 isolate within the SW/WN03 genotype indicates co-circulation of this genotype in Harris County until the 2012 transmission season. However, limited clustering of sequenced 2010–2012 Harris County isolates within the SW/WN03 genotype may be an artifact of inherent bias in sample collection.

Sampling bias is a recognized constraint in phylogenetic and paired geospatial analyses. Unfortunately, the predominance of WNV surveillance remains restricted to limited regional foci. Of the 372 full-length WNV isolates in our analyses, 31.5% (n = 117) were collected in Connecticut, 22.0% (n = 82) in New York, 16.4% (n = 61) in Texas, and 8.1% (n = 30) in Illinois during 1999–2012. In contrast, a single isolate has been characterized for each of the midwestern/central states of Michigan, North Dakota, and South Dakota, all of which reported a major increase in the number of clinical WNV cases (n≥89) in the 2012 epidemic season not seen since 2007 (*15*). Increased WNV surveillance in mosquito and avian vector populations across the entire United States is needed to provide critical, unbiased insight into the underlying dynamics of WNV evolution and transmission in US host populations.

The Harris County Public Health and Environmental Services Mosquito Control Division, which was founded in response to a St. Louis encephalitis virus outbreak in 1964, provides a model infrastructure for WNV surveillance in resident mosquito and avian populations in the

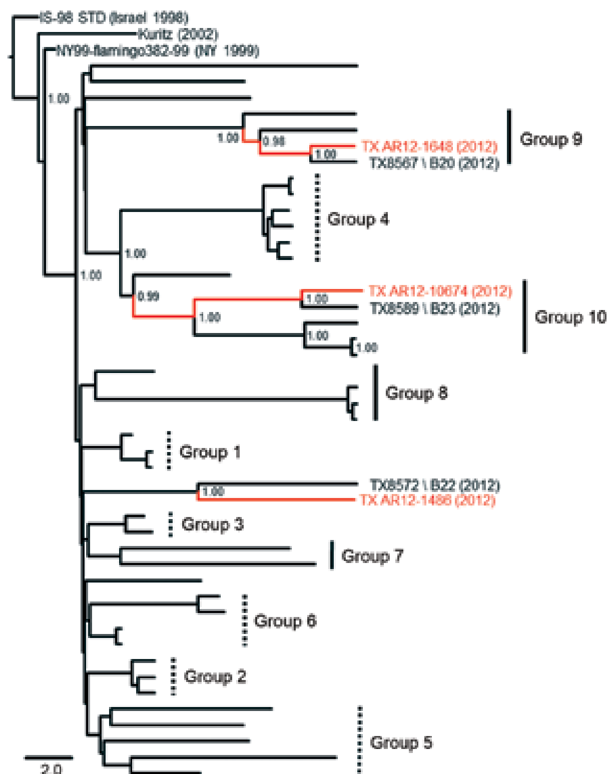

Figure 4. Phylogenetic support for expanded application of the proposed Harris County, Texas, USA, paradigm as a model for West Nile virus (WNV) evolution during the Dallas/Fort Worth, Texas, outbreak, 2012. Bayesian coalescent tree depicts shared monophyletic lineage of the novel Collin County WNV isolates in group 9 (TX AR12-1648) and group 10 (TX AR12-10674) with the TX AR12-1486 Denton County, Texas, isolate clustering with the TX8572 2012 Harris County isolate. Red indicates novel Collin County and Denton County isolates sequenced in this study. Posterior probabilities ≥0.90 are indicated along the branches to provide statistical support for inferred topologies. Scale bar indicates divergence time in years.

greater Houston region (*8*). Uninterrupted collection and processing of WNV-positive bird and mosquito pools since the 2002 introduction of WNV into Harris County has provided a conduit for the scientific investigation of real-time disease outbreaks with direct translation of findings towards optimized vector-borne disease control and prevention. Incorporation of this paradigm in public health directives across the United States would provide a proactive approach towards detection and response to clinical outbreak scenarios of endemic and exotic pathogens in the United States.

In conclusion, retrospective analysis of WNV evolution in Harris County over the past decade indicates a recent shift in the genetic and phylogenetic signature of circulating WNV populations, designated the Harris County paradigm. Further evidence supports introduction of a

strain into Texas from the northeastern United States since 2010. Continued WNV surveillance is needed to confirm the effect of this genetic shift in the transmission dynamics and incidence of clinical WNV disease in the greater Houston and surrounding US regions.

Acknowledgments

We thank the Texas Department of State Health Services Laboratory Arbovirus Team for generously providing WNV samples from the Dallas/Fort Worth region, and Amy Schuh for providing helpful discussions and statistical insight in preparation of this manuscript.

This study was supported in part by National Institutes of Health grant AI 067847 to A.D.T.B. and contract HHSN272201000040I/HHSN27200004/D04 to R.B.T. B.R.M. and A.R.M. were supported by National Institutes of Health T32 training grant AI 007526 from the National Institute of Allergy and Infectious Diseases.

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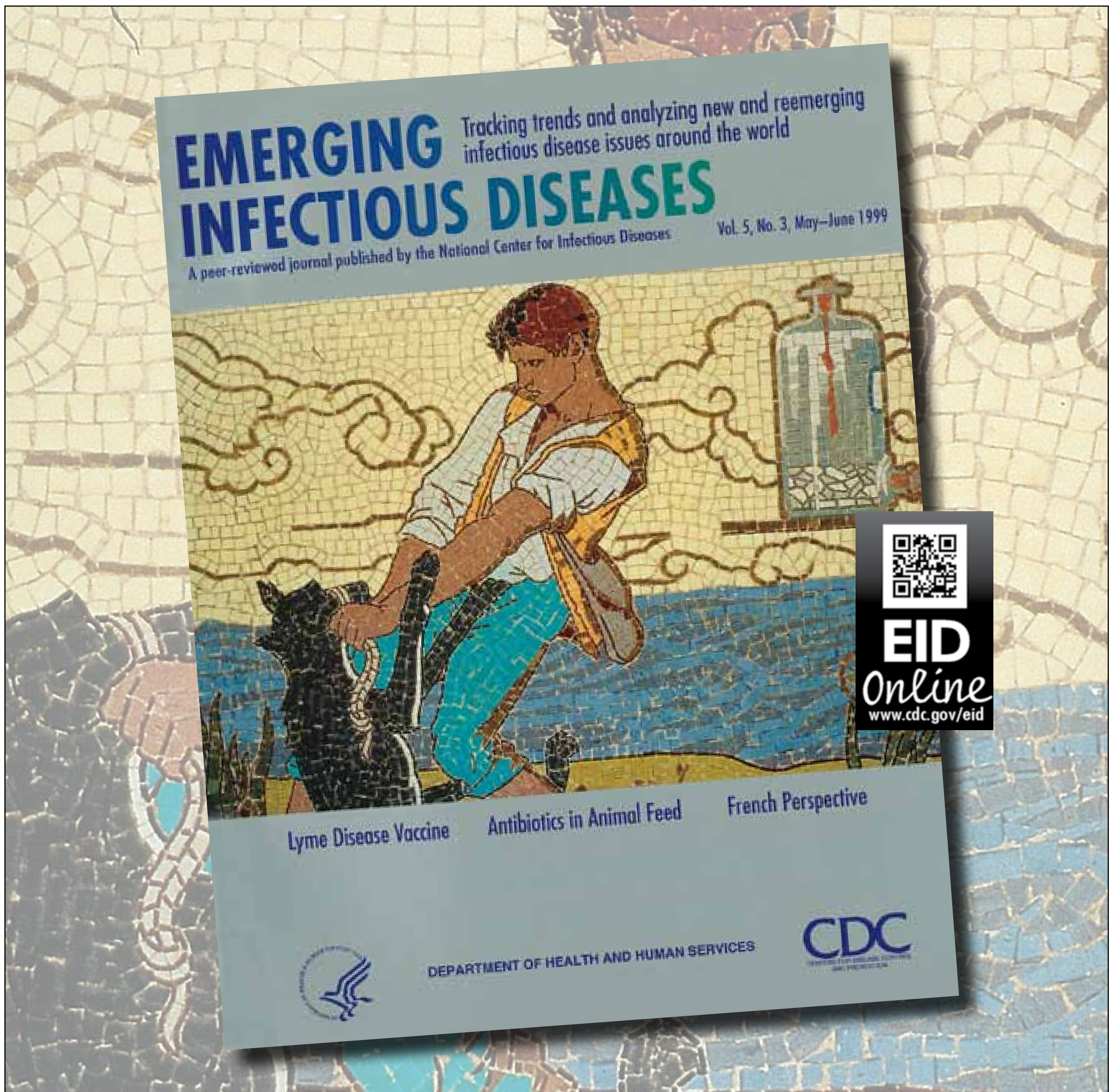

Underreporting of Viral Encephalitis and Viral Meningitis, Ireland, 2005–2008

Tara A. Kelly, Piaras O'Lorcain, Joanne Moran, Patricia Garvey, Paul McKeown, Jeff Connell, and Suzanne Cotter

Viral encephalitis (VE) and viral meningitis (VM) have been notifiable infectious diseases under surveillance in the Republic of Ireland since 1981. Laboratories have reported confirmed cases by detection of viral nucleic acid in cerebrospinal fluid since 2004. To determine the prevalence of these diseases in Ireland during 2005–2008, we analyzed 3 data sources: Hospital In-Patient Enquiry data (from hospitalized patients following discharge) accessed through Health Intelligence Ireland, laboratory confirmations from the National Virus Reference Laboratory, and events from the Computerised Infectious Disease Reporting surveillance system. We found that the national surveillance system underestimates the incidence of these diseases in Ireland with a 10-fold higher VE hospitalization rate and 3-fold higher VM hospitalization rate than the reporting rate. Herpesviruses were responsible for most specified VE and enteroviruses for most specified VM from all 3 sources. Recommendations from this study have been implemented to improve the surveillance of these diseases in Ireland.

Encephalitis and meningitis are serious inflammatory diseases of the brain that require hospitalization for many patients and are a substantial cause of illness. Although the etiologic agent is not identified for most cases (*1*), viral infection has been reported as a major cause (*2*).

Acute encephalitis is characterized by a triad of fever, headache, and altered mental status (*3*). Common features include disorientation/depressed level of consciousness; disturbances of behavior, speech, or executive function; and diffuse or focal neurologic signs such as cranial nerve dysfunction, hemiparesis, or seizures (*3*). Capillary and endothelial inflammation of cortical vessels is a striking pathologic finding, occurring primarily in the gray matter or the gray–white junction (*4*). These features distinguish encephalitis from the more commonly encountered meningitis. The most common agents that cause acute viral encephalitis (VE) are herpes simplex virus (HSV) and varicella-zoster virus (VZV) (*5*).

A distinction must be made between acute VE and autoimmune/postinfectious encephalitis, which can occur with a variable latent phase between acute illness and the onset of neurologic symptoms (*6*,*7*). This distinction is critical because the management and prognosis are often quite different (*4*). Evaluation of cerebrospinal fluid (CSF) following lumbar puncture is essential for accurately diagnosing disease, unless its collection is contraindicated because of high intracranial pressure (*4*). In this study, we did not attempt to ascertain the prevalence of autoimmune/postinfectious encephalitis in Ireland.

Aseptic meningitis refers to a disease with acute onset of symptoms and obvious signs of meningeal involvement, in which an etiologic agent is not apparent after bacterial culture of CSF (*8*). The disease is often associated with lymphocytic pleocytosis without other cause. These patients usually lack altered sensorium or abnormal global or focal neurologic signs (*3*,*4*). Viruses are most commonly associated with these clinical manifestations, most frequently, enteroviruses, herpesviruses, and arboviruses (*9*). Under the 2003 case definitions covering this study period, laboratory evidence involving CSF analysis or immune response, in addition to clinical diagnosis, was necessary for the reporting of VE and VM to public health departments (*10,11*).

In this study, we examined 3 different available data sources to estimate how well data reported to public health authorities and captured by the Computerised Infectious

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DOI: http://dx.doi.org/10.3201/eid1909.130201

Disease Reporting (CIDR) passive surveillance system, during 2005–2008, reflected the incidence of VE and VM in Ireland. CIDR is the real-time Internet-based surveillance system for 93.9% of all notifiable infectious diseases reportable by statute in Ireland. We compared cases reported to CIDR with laboratory detection of cases from the National Virus Reference Laboratory (NVRL) and cases identified from hospitalized patient discharge information in the Hospital In-Patient Enquiry (HIPE) scheme. In 2005, eight (24%) of the 34 public hospital laboratories, in addition to the national reference laboratories, were connected directly to CIDR. This connection increased to 16 (47%) in 2008. Hospital/community physicians who manage VE or VM patients using laboratories which were not directly connected to CIDR, were obligated to report notifiable diseases to CIDR through the local department of public health.

The data sources and the process of reporting notifiable diseases in Ireland are shown in Figure 1. HIPE is an active system that monitors hospital activity and is independent of either NVRL or CIDR. The diagnoses and procedures recorded on the patient's chart are coded by hospital administration staff according to the International Classification of Diseases and Related Health Problems, 10th Revision (ICD-10). A copy of this database, which includes only publicly funded hospitals, was accessed by using Health Intelligence Ireland (HII), an open source software program developed by the Irish Health Services Executive (www.healthatlasireland.ie/f1live).

A previous study suggested an underestimation of the incidence of acute encephalitis and VM in the northeastern part of Ireland (*12*,*13*). In that study, only hospital activity and CIDR data were compared, and no distinction was made between laboratory or clinical criteria for diagnosis of these diseases at that time.

Materials and Methods

CIDR Events

Events are composed of ≥1 clinical diagnoses and/or laboratory test results for a single patient (Figure 1). We extracted recorded events of VE and VM from CIDR. The organism responsible for each disease is captured as enhanced surveillance.

Hospitalizations

HIPE collects information regarding in-patient and day-case hospital activity. Each HIPE discharge record represents 1 episode of care. Patients may have been admitted to hospital(s) more than once with the same or different diagnoses. A HIPE discharge record is generated after a patient is discharged from, or dies, in the hospital (*14*). The record accessed through HII contains an anonymized medical record number. Duplicate discharges for the same anonymized medical record number were found by HII and removed.

Hospital discharges from January 1, 2005, through December 31, 2008, coded with the ICD-10-AM (Australian Modification), fourth edition diagnosis codes A83, A84, A85, A86, B00.4, B01.1, B02.0, B05.0, and B26.2 were defined as VE. Discharges associated with the codes A87, B00.3, B01.0, B02.1, B05.1, and B26.1 were defined as

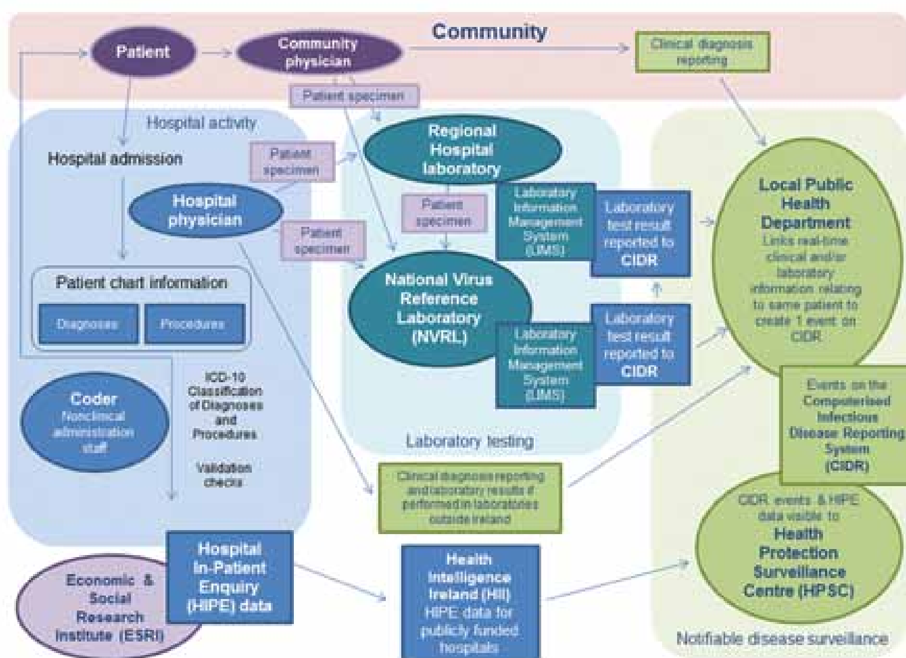

Figure 1. Sources of information of clinical and laboratory diagnoses of notifiable infectious diseases in Ireland and the routes of reporting such diagnoses to the national Computerised Infectious Disease Reporting system.

VM. We determined the proportion of these codes recorded in any of the diagnosis fields and in the primary diagnosis field only.

Hospitalizations in which a lumbar puncture (ICD-10-AM code 39000–00) was listed were extracted from HIPE for comparison with the number of CSF samples received at NVRL for virologic testing and with those in which a virus was detected from patients with symptoms of acute encephalitis or meningitis. Deaths were determined by searching for patients on HIPE who "died with post mortem" or "died no post mortem."

Calculations

Denominator data for population-based incidence rates (of CIDR events, laboratory confirmations, and patient discharges) correspond to published Irish Census 2006 Principle Demographic Results (n = 4,239,848; Central Statistics Office, 2007; www.cso.ie/en/statistics/population). To prevent the identification of individual hospital in-patients, when 5 or fewer discharges or patients for a particular diagnosis were found, we depicted the number as ≤5.

The statistical significance of the difference in proportions was tested by using the χ^2 test or Fisher exact test as appropriate (Stata 11.1; http://www.stata.com/stata11/point1.html). The 95% CIs were also calculated. Male-to-female ratios (MFRs) are presented as the number of male patients to 1 female patient.

Investigation of CSF Specimens at NVRL

Investigation of CNS infection is primarily based on detection of HSV-1 DNA, HSV-2 DNA, or VZV DNA. When appropriate, on the basis of clinical manifestations/underlying clinical issues and in collaboration with the clinical teams managing a patient's condition, testing can be performed for human herpesvirus 6 DNA, Epstein-Barr virus DNA, cytomegalovirus DNA, JC virus DNA, enterovirus RNA, enterovirus 71 RNA, measles virus RNA, mumps virus RNA, and lymphocytic choriomeningitis virus RNA. When encephalitis or meningitis is suspected because of arboviral infection, the infection is diagnosed by detecting IgM in serum, CSF, or both. On the basis of the patients' clinical and travel history, tests were performed for serologic evidence of the following: West Nile virus, Venezuelan equine encephalitis virus, Japanese encephalitis virus, yellow fever virus, dengue virus, eastern equine encephalitis virus, western equine encephalitis virus, St. Louis encephalitis virus, Powassan virus, La Crosse virus, and tick-borne encephalitis virus.

In addition to the investigation of CSF, molecular analysis and culture of fecal samples were also performed. The detection of enterovirus provides additional circumstantial evidence for the viral etiology of the CNS manifestations.

NVRL does not routinely receive convalescent-phase serum samples; therefore, investigations of acute- or convalescent-phase serum are uncommon. Intrathecal antibody testing is not performed at NVRL. On the rare occasion when such specimens are sent to an international reference laboratory, the results of such testing would be known to NVRL if the specimens were originally sent through NVRL to that laboratory.

Herpesvirus DNA is often not detectable in CSF from encephalitis/meningitis patients. It can nonetheless be confirmed through evidence of elevated specific intrathecal IgG. The absence of such testing in our analysis may have led to underdetection of meningitis attributable to herpes group viruses, particularly VZV, which other studies have found to be the most common viral cause for VM, including a 2001 study in Finland (*15*).

We acknowledge that molecular investigation may not always detect DNA or RNA soon after onset of symptoms or following antiviral treatment. However, collaboration with the clinical teams dealing with the patient can often highlight whether the lack of DNA or RNA could cause a diagnostic problem. Results were extracted from the laboratory information management system (LIMS) WinPath (CliniSys Solutions Ltd, Chertsey, UK) and configured for upload to CIDR.

Results

Reporting to CIDR

Between 2005 and 2008, a total of 40 VE events and 341 VM events were reported to CIDR (Table 1). Of these, 39 VE events and 261 VM events were classified as confirmed. The rates of VE and VM by data source and year are shown (Figure 2, panels A, B) with the highest rates of VM occurring in 2006.

VE (62.5%) and VM (58.7%) occurred more frequently in male patients (MFRs, 1.67 and 1.42, respectively). The greatest age-standardized incidence rates (ASIR) of VE (27.5%) and VM (29.9%) were for children <4 years of age (Figure 3, panels A, B). For VM, 24.6% of all events involved children <1 year of age.

The highest number of VE events were caused by HSV (40.0%), followed by VZV (27.5%) and mumps virus (12.5%) as shown in Table 1. Only 12.5% of herpesviral events had the type recorded, as opposed to 95% in 2011 (when all laboratories had live links to CIDR, T. Kelly, unpub. data). Most VM events were caused by enteroviruses (61.9%, which included coxsackie A and B viruses, enterovirus, echovirus and echovirus type 6), mumps (4.7%), and HSV (3.5%).

No deaths from either VE or VM were reported to CIDR. VE incidence did not display seasonality, whereas VM reporting was greatest in the month of August; overall rates were highest in August 2006 (Figure 4, panels A, B). Two

Table 1. Viral encephalitis and viral meningitis CIDR events, laboratory-confirmed cases, and patient hospitalizations by causative virus, Ireland, 2005 to 2008*

ICD code	Description of code	No. CIDR events	No. NVRL confirmed cases	HIPE data						
				No. discharges	No. patients	No. deaths	Case-fatality ratio, %	Total no. bed days	Mean no. bed days	Mean no. ICU days
A83	Mosquito-borne encephalitis	–	–	≤5	≤5	0	0.0	≤5	≤5	0.0
A84	Tick-borne encephalitis	–	–	≤5	≤5	≤5	50.0	91	45.5	26.0
A85	Other viral encephalitis	–	–	42	25	≤5	4.0	512	12.2	1.2
A86	Unspecified viral encephalitis	4	–	283	223	10	4.5	4,112	14.5	1.2
B00.4	Herpesvirus encephalitis	17†	42‡	195	95	11	11.6	2,857	14.7	0.9
B01.1	Varicella encephalitis	12§	35	28	28	≤5	7.1	706	25.2	2.3
B02.0	Zoster encephalitis			43	43	8	18.6	789	18.4	1.5
B05.0	Measles encephalitis	–	–	–	–	–	–	–	–	–
B26.2	Mumps encephalitis	5	–	≤5	≤5	0	0.0	18	18.0	3.0
	Enteroviral encephalitis	2	–	–	–	–	–	–	–	–
Total viral encephalitis		40	77	595	418	33	7.9%	9,086	15.3	1.2
A87.0	Enteroviral/ coxsackievirus/echovirus meningitis	210¶	215#	60	52	0	0.0	428	7.1	0.8
A87.1	Adenoviral meningitis	–	–	≤5	≤5	0	0.0	25	5.0	0.6
A87.2	Lymphocytic choriomeningitis/ lymphocytic meningoencephalitis	–	–	57	47	≤5	6.3	886	15.5	0.1
A87.8	Other viral meningitis	–	–	36	34	0	0.0	267	7.4	0.2
A87.9	Viral meningitis unspecified	91	–	1,048	990	≤5	0.1	5,701	5.4	0.1
B00.3	Herpesvirus meningitis	12**	32††	14	14	0	0.0	365	26.07	4
B01.0	Varicella meningitis	11‡‡	22	11	10	0	0.0	80	7.3	0.0
B02.1	Zoster meningitis			13	12	0	0.0	231	17.8	0.1
B05.1	Measles meningitis	–	–	≤5	≤5	0	0.0	6	6.0	0.0
B26.1	Mumps meningitis	16	–	≤5	≤5	0	0.0	22	4.4	0.0
	Parechovirus meningitis	1	–	–	–	–	–	–	–	–
Total viral meningitis		341	269	1,250	1,170	≤5	0.3%	8,011	6.4	0.2

*CIDR, Computerised Infectious Disease Reporting system; ICD, International Classification of Diseases, 10th Revision; NVRL, National Virus Reference Laboratory; HIPE, Hospital In-Patient Enquiry; ICU, intensive care unit.
†Includes 14 herpes simplex virus (HSV), untyped; 2 HSV-1 and 1 human herpesvirus type 6 events.
‡Includes 37 HSV-1, 1 HSV-2, and 4 human herpesvirus type 6 detections.
§Includes 11 varicella-zoster and 1 varicella species events.
¶Includes 205 enterovirus, 1 coxsackie A virus, 1 coxsackie B virus, 2 echovirus, and 1 echovirus type 6 events.
#Includes 211 enterovirus, 3 coxsackie B3 virus, and 1 coxsackie B5 virus detections.
**Includes 10 HSV untyped, 1 HSV-1, and 1 HSV-2 events.
††Includes 7 HSV 1, 5 HSV-2 and 20 human herpesvirus type 6 events.
‡‡Includes 10 VZV and 1 varicella species detections.

outbreaks of enteroviral meningitis were reported to CIDR; one in July 2006 (which began in late June) and one in July 2008. No outbreaks of VE were reported in these years.

HIPE Hospitalizations

During 2005–2008, a total of 418 patients were discharged on 595 occasions with a diagnosis of VE, giving a mean annual patient hospitalization rate of 2.49/100,000 population (95% CI 2.31–2.68) and a mean annual discharge rate of 3.51/100,000 population (95% CI 2.15–4.87); Figure 2, panels A, B). There were more hospital discharges for VM; 1,170 patients were discharged on 1,250 occasions, which corresponds to a mean annual patient hospitalization rate of 6.88/100,000 population (95% CI 4.76–9.00) and a mean annual discharge rate of 7.37/100,000 population (95% CI 5.17–9.17).

A total of 83.0% of discharges of patients with VE and 92.2% of those with VM were considered primary diagnosis discharges. The mean length of stay for a patient discharged with VE was 15.3 days (range 1–254 days; median 7 days), and mean length of stay in an intensive care unit was 1.2 days (range 0–55 days; median 0 days). The average cost per discharge for VE was €9,783.31. For those with VM, the mean length of stay was 6.4 days (range 1–128 days; median 5 days), the mean length of stay in an intensive care unit was 0.2 days (range 0–56 days; median 0 days), and the average cost per discharge was €4,612.77.

Male patients accounted for 53.4% of all VE patients and 53.0% of VM patients (MFRs of 1.24 and 1.13, respectively). The ASIR of patients hospitalized with VE was highest among elderly (80–84 years of age) and very young patients (0–4 years of age; Figure 3, panel A). The ASIR for

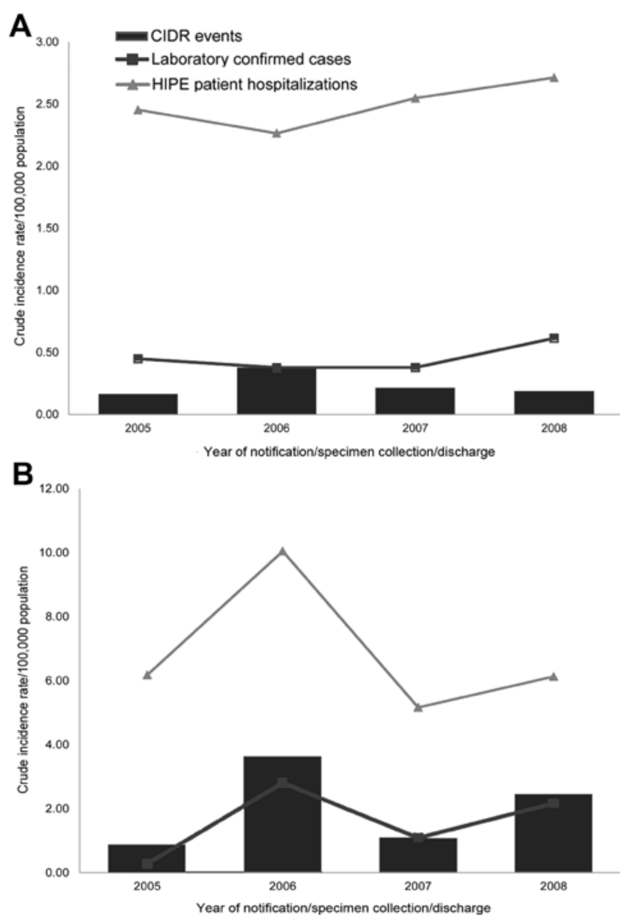

Figure 2. A) Crude incidence rates per 100,000 population of viral encephalitis events (Computerised Infectious Disease Reporting system [CIDR]), hospitalizations (Hospital In-Patient Enquiry [HIPE] patients), and laboratory-confirmed cases (National Virus Reference Laboratory [NVRL]), Ireland, 2005–2008. B) Crude incidence rates per 100,000 population of viral meningitis events (CIDR), hospitalizations (HIPE patients), and laboratory-confirmed cases (NVRL).

VM was highest among children aged 0–4 years, followed closely by adolescents ages 15–19 years (Figure 3, panel B).

For the majority (53.3%) of persons hospitalized with VE, viral cause was unknown (Table 1). When an agent was specified (n = 195), herpesvirus was the causative agent of 48.7%, zoster of 22.1%, other (including enteroviral, adenoviral, arthropod-borne, mosquito-borne, and tick-borne viruses) of 14.9%, and varicella of 14.4% of patient hospitalizations. For most (84.6%) patients hospitalized with VM, cause was also unspecified, and when a virus was specified (n = 180), enteroviruses (including coxsackie B virus and echovirus) were the most common etiologic agents at 28.9%, followed by lymphocytic choriomeningitis virus at 26.1% and other at 18.9%.

Thirty-three hospitalized patients with a diagnosis of VE died, giving a case-fatality rate (CFR) of 7.9% (Table 1; Figure 3, panel A); CFR was highest among those 80–84 years of age. Eleven of the 33 VE deaths were associated with herpesvirus encephalitis (CFR 2.6%) and 8 with zoster encephalitis (CFR 1.9%). Ten deaths were from unspecified cause, and the remaining 4 deaths were caused by varicella, tick-borne encephalitis, or other encephalitis-causing viruses. In contrast, VM resulted in fewer (≤5) deaths (Table 1), which were either caused by lymphocytic choriomeningitis virus or unspecified.

A peak of both VE and VM hospitalizations occurred in the summer months, most notably in July and August (Figure 4, panels A, B). For both diseases, the highest number of hospitalizations occurred in July 2006. Of the patients hospitalized for VE, 52.61% had a lumbar puncture, compared with 73.98% of patients hospitalized for VM (Table 2).

NVRL CSF Analysis

The NVRL received 6,502 CSF specimens collected during 2005–2008. Of those, 1.3% tested positive for a virus causing encephalitis, and 4.2% tested positive for a virus causing meningitis (Table 2). A listing of causative pathogens of laboratory-confirmed cases is shown in Table 1.

Most CSF specimens indicating VE were from male patients (63.6%; MFR 1.75), as were those indicating VM (56.8%; MFR 1.31). The highest ASIR of laboratory confirmations for viruses causing encephalitis was from patients ≥55 years of age (Figure 3, panel A) and of viruses causing meningitis were from those 0–4 years of age (Figure 3, panel B). No seasonality was shown in laboratory detections of viruses causing encephalitis (Figure 4, panel A), but a higher number of viruses causing meningitis were detected in the summer months; most were detected in July (Figure 4, panel B).

Statistical Differences between Data Sources

We analyzed the differences between hospital activity, laboratory confirmations, and CIDR events created by using several parameters. When these factors were compared by age of patient, we found a significant difference in the distribution of VE laboratory confirmations and events (Fisher exact test, $p<0.001$).

Of VE cases with a specified cause, which should have been reported to public health authorities according to the case definitions in use at the time, a significant difference was found in the distribution of VE cases by causative agent between laboratory confirmations and events (Fisher exact test, $p<0.001$). Less than half of laboratory confirmations and 18.5% of hospitalizations were reported.

Among VM cases for which an organism was specified, a significant difference was found in the distribution of cases by causative agent between hospitalizations and events ($\chi^2 = 139.83$, $p<0.001$) and laboratory confirmations

and events ($\chi^2 = 9.91$, $p = 0.01$). Only 9.2% of patients with illness of unspecified cause were reported. On the other hand, the number of enteroviral events or laboratory confirmations was 4-fold higher than the number of hospitalizations. No deaths had been reported to CIDR, in contrast to the 33 VE deaths and ≤5 VM deaths recorded in HIPE.

Discussion

During 2005–2008, only 9.6% of cases in hospitalized patients with VE and 29.2% of cases in hospitalized patients with VM were reported to CIDR. Attempting to ascertain the proportion of the difference due to underreporting, we looked at the difference in the percentage of patients hospitalized with a diagnosis for VE and VM and events of these diseases created in CIDR in 2011. By then, 100% of public hospital laboratories were connected to CIDR, which facilitated the reporting mechanism. We found that hospitalized patients reported to CIDR with a diagnosis of VE increased from 9.6% to 20.2% in 2011 and that patients with VM increased from 29.2% to 69.6% (T. Kelly, unpub. data). The laboratories conducting the testing probably contributed to the underreporting. Underreporting or misclassification of diagnoses by hospital physicians without confirmatory tests may also have occurred.

Surveillance data are routinely used to quantify the incidence of disease and to identify outbreaks or emerging

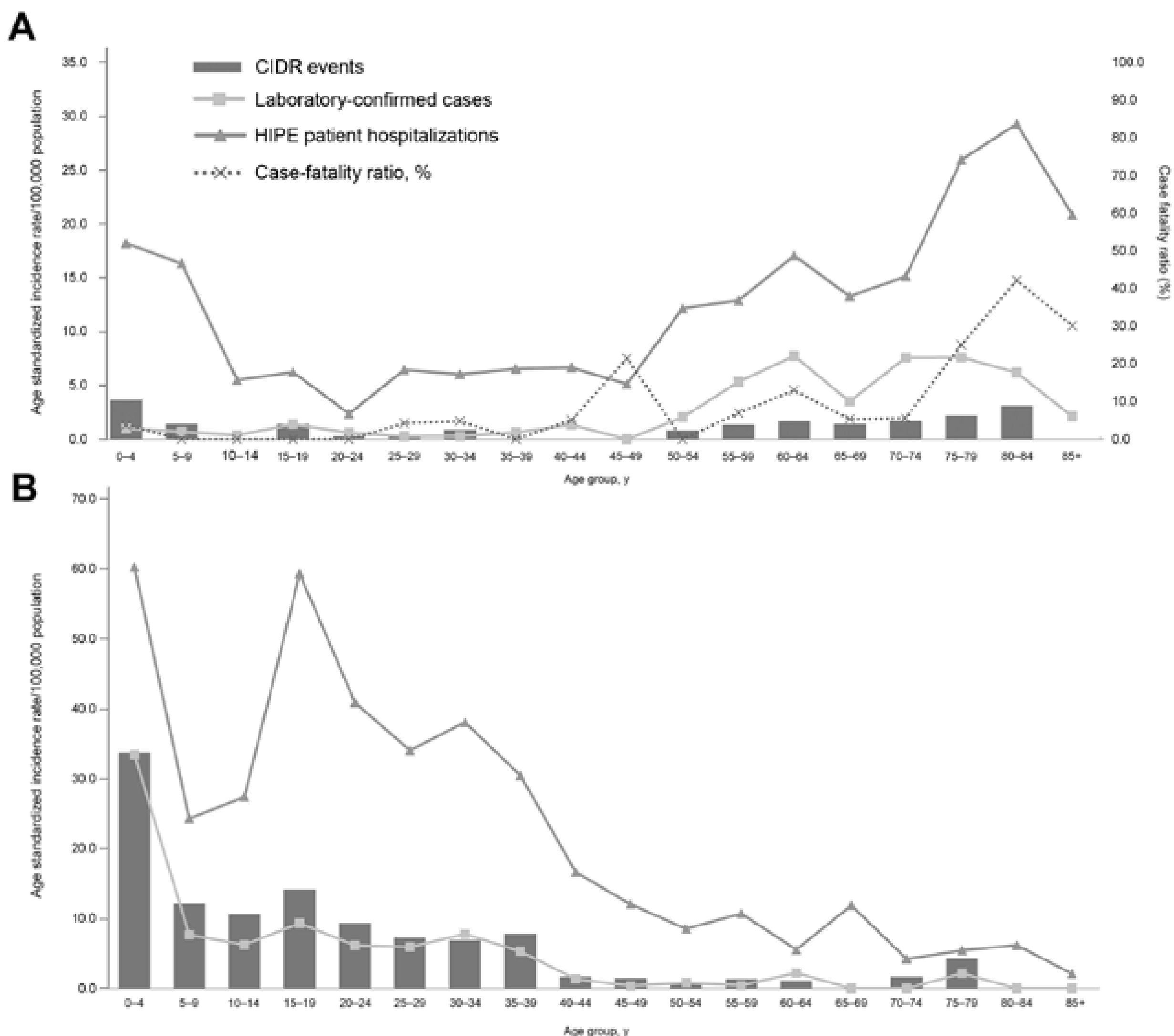

Figure 3. A) Viral encephalitis age-specific incidence rates of events (Computerised Infectious Disease Reporting system [CIDR]), hospitalizations (Hospital In-Patient Enquiry [HIPE] patients), and laboratory-confirmed cases (National Virus Reference Laboratory [NVRL]) by age group, Ireland, 2005–2008. B) Viral meningitis age-specific incidence rates of events (CIDR), hospitalizations (HIPE), and laboratory-confirmed cases (NVRL) by age group, Ireland, 2005–2008. The figure excludes 4 CIDR events and 1 laboratory-confirmed case with patient age unknown.

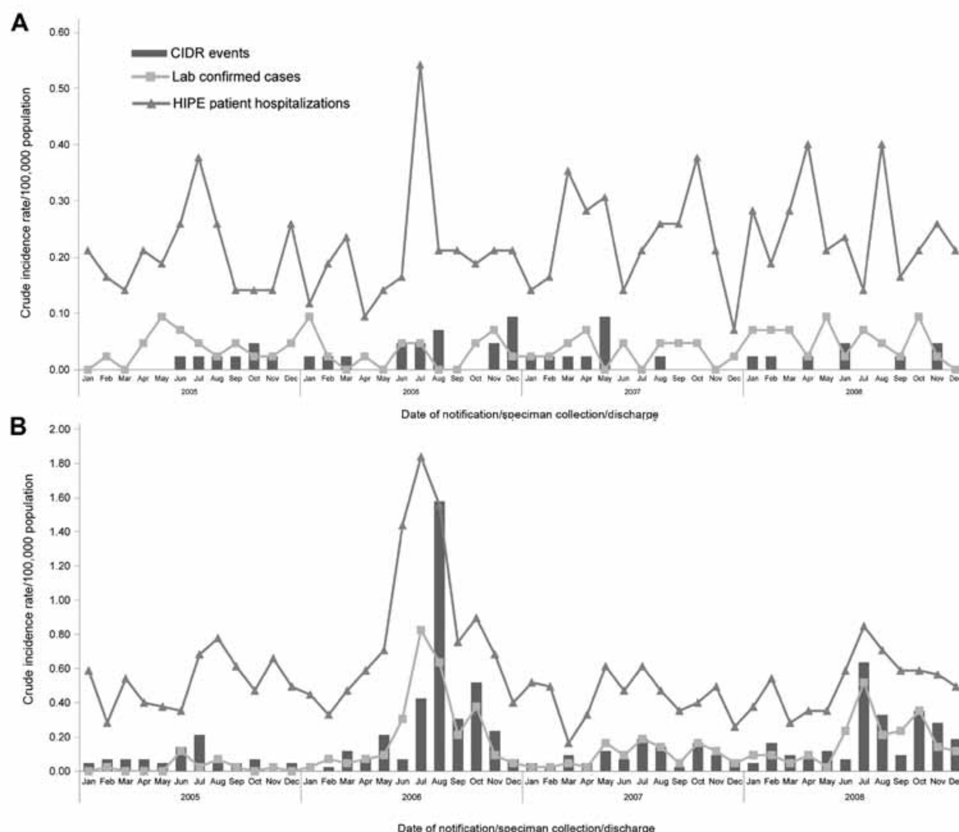

Figure 4. A) Viral encephalitis crude incidence rates of events (Computerised Infectious Disease Reporting system [CIDR]), laboratory-confirmed cases (National Virus Reference Laboratory [NVRL]), and patient hospitalizations (Hospital In-Patient Enquiry [HIPE]), by month and year, Ireland, 2005–2008. B) Viral meningitis crude incidence rates of events (CIDR), laboratory-confirmed cases (NVRL), and patient hospitalizations (HIPE), by month and year.

viral diseases, and this underidentification of VE and VM in Ireland is of concern. With the emergence of West Nile fever in Italy (*16*), dengue fever in France (*17*) and other European countries, and changes in the mosquito populations of different countries, CIDR must be able to detect possible emerging pathogens.

VE was shown to have greater effects on the Irish health service than VM in terms of deaths, length of hospital stay, repeat hospitalizations, ongoing hospital care, financial costs, and residual damage, although VM caused a greater number of hospital admissions. Similar to the situation in the United States, those seeking treatment for VE and VM were more likely to be male (*9*,*18*). However, a UK study found no difference in VE by sex and showed a lower hospitalization rate for patients with VE (*19*).

Table 2. Crude incidence rates per 100,000 population and proportions of lumbar puncture procedures among hospitalized patients with a diagnosis of VE or VM, Ireland, 2005–2008*

Rate or proportion	Year of discharge/laboratory confirmation				Average annual rate or proportion
	2005	2006	2007	2008	
Hospitalization rate for all patients with lumbar puncture†	99.20	116.30	120.05	129.53	116.27
VE patient hospitalization rate	2.45	2.26	2.55	2.71	2.49
Hospitalization rate among VE patients with lumbar puncture	1.18	1.18	1.44	1.44	1.31
% VE hospitalizations with lumbar puncture	48.16	52.21	56.47	53.14	52.61
VM patient hospitalization rate	6.18	10.05	5.17	6.13	6.88
Hospitalization rate among VM patients with lumbar puncture	4.34	7.38	3.89	4.74	5.09
% VM hospitalizations with lumbar puncture	70.23	73.43	75.24	77.32	73.98
CSF collection rate of samples sent for virologic testing	31.46	40.71	39.65	41.53	38.34
Laboratory CSF confirmation rate of VE	0.50	0.42	0.42	0.71	0.51
% Laboratory confirmed CSF for VE	1.59	1.03	1.06	1.71	1.33
Laboratory CSF confirmation rate of VM	0.28	2.83	1.11	2.24	1.62
% Laboratory confirmed CSF for VM	0.89	6.95	2.80	5.39	4.23
Laboratory CSF confirmation rate of VE or VM	0.78	3.25	1.53	2.95	2.13
% Laboratory confirmed CSF for VE or VM	2.48	7.98	3.69	7.10	5.56

*VE, viral encephalitis; VM, viral meningitis; CSF, cerebrospinal fluid.
†All hospitalized patients who underwent lumbar puncture for any reason, diagnostic or therapeutic.

VE hospitalizations and events were most prominent in the youngest and oldest age groups. VM followed the same pattern for all 3 data sources (without statistical difference), with peaks in the 0–4 and 15–19 year age groups, which had also been found in Denmark (*20*). The peak in adolescents may reflect a large mumps outbreak that occurred during this period which primarily affected adolescent boys (*21*). The mumps outbreak in this age group may be attributable to a combination of factors, including social and environmental exposures and waning immunity to mumps (*22*). A previous seroepidemiologic study had identified inadequate immunity to measles, mumps, and rubella in school-aged children in Ireland (*23–25*).

Similar to results of studies in other countries (*15*,*19*,*26*), herpes and varicella-zoster viruses were the most commonly specified causes of acute aseptic encephalitis. As for VE, most (84.6%) VM hospitalizations had an unspecified cause, which is similar to the 92% reported in the United States by the Centers for Disease Control and Prevention (*9*), which also reported that the highest rates of specified VM were due to enterovirus infection. The large number of enteroviral meningitis events, which are not reflected in hospital activity, suggests that many enteroviral meningitis cases are classified as unspecified.

We did not identify seasonal trends in VE incidence but did identify a higher incidence of VM during the summer months, as found in other studies (*3*,*9*). Across all 3 data sources, the highest rates of VM were found in July 2006 and July 2008, correlating to periods when community outbreaks of enteroviral meningitis were reported to CIDR. This seasonality for VM continued in subsequent years (*27*).

Because lumbar puncture has a key role to play in the accurate diagnosis of VE and VM, we evaluated the number of patients with VE and VM who had undergone this procedure and found that a higher percentage of patients with suspected VM had undergone lumber puncture than did patients with suspected VE. We also found that a higher percentage of specimens tested positive for a virus causing meningitis. The percentage positive for VE was remarkably low. This finding may reflect either a higher contraindication rate to lumbar puncture in VE patients, a problem confirming the diagnosis of encephalitis, or an increase in the rate of intrahospital transfer of severely ill VE patients to other hospitals with expertise in VE case management (in which case, repeat lumbar punctures would not be usual). It is also possible that alternative noninvasive diagnostic tools were used in patients for whom lumbar punctures were contraindicated, such as magnetic resonance imaging or electroencephalographic testing (*5*).

As a result of this study, new case definitions (*28*) have been implemented that incorporate a case classification for "possible" VE (enabling the reporting of patients meeting clinical criteria without laboratory confirmation). Only 52% of NVRL confirmations were reported to CIDR, but the NVRL Laboratory Information Management System extract used to report positive test results to CIDR was updated to capture omitted results. HIPE coding errors had also been identified in this study, and improved training and data entry validation measures were put in place. Clinical guidelines are being prepared by the Health Protection Surveillance Centre, which aim to improve both the investigation of VE and VM and reporting by physicians. Additional modifications to the list of notifiable diseases (*29*) include new case definitions for dengue fever, West Nile fever, and chikungunya disease, specifically, as well as new case definitions for hospitalized patients with chickenpox caused by VZV. The enhanced surveillance for influenza will now capture encephalitis.

Potential limitations of this study include the presentation of hospitalizations as numbers of patients hospitalized rather than numbers of discharges. Patients obtain a new medical record number when admitted to a different hospital, and patients who are transferred could be counted twice, but the transfer of patients is considered more likely to affect numbers of VE cases than VM, because of disease severity. We do not have a unique patient identifier in Ireland or a single health information system, as do some Scandinavian countries. HIPE data are collected with the sole purpose of being a source of hospital activity information and, under the Data Protection Act, cannot be used for any other purpose; as such, hospital information cannot be linked with surveillance data (*30*).

This study underscores a key disconnection between health care providers, diagnostic laboratories, and disease reporting. Further investigation of the perspective of attending physicians, laboratory directors, and public health officials may identify approaches for establishing more effective communication between each group on the essential issues of expeditious linking of pathogen identification with clinically apparent disease and reporting to public health. We recommend a follow-up study comparing rates with those when the new case definition (implemented March 2012), improved laboratory reporting, and clinical guidelines have been put in place, to evaluate whether there has been an effect by these changes. Analysis of the referral source of clinical diagnoses and laboratory results to CIDR, specimen type, and laboratory testing performed would facilitate better understanding of the proportion of difference between HIPE and CIDR because of underreporting. Such analysis would also provide feedback and education to the partners involved in health protection as an aid in highlighting the value of the surveillance of these diseases in Ireland and the detection of possible threats to public health.

Acknowledgments

We thank the staff of Health Intelligence Ireland and the HIPE and National Perinatal Reporting System Unit at the Economic and Social Research Institute, Ireland.

This work was supported by the Irish Health Service Executive.

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High Rates of *Mycobacterium tuberculosis* among Socially Marginalized Immigrants in Low-Incidence Area, 1991–2010, Italy

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Migration from low- and middle-income countries to high-income countries increasingly determines the severity of tuberculosis (TB) cases in the adopted country. Socially marginalized groups, about whom little is known, may account for a reservoir of TB among the immigrant populations. We investigated the rates of and risk factors for *Mycobacterium tuberculosis* transmission, infection, and disease in a cohort of 27,358 socially marginalized immigrants who were systematically screened (1991–2010) in an area of Italy with low TB incidence. Overall TB and latent TB infection prevalence and annual tuberculin skin testing conversion rates (i.e., incidence of new infection) were 2.7%, 34.6%, and 1.7%, respectively. Prevalence of both TB and latent TB infection and incidence of infection increased as a function of the estimated TB incidence in the immigrants' countries of origin. Annual infection incidence decreased with time elapsed since immigration. These findings have implications for control policy and immigrant screening in countries with a low prevalence of TB.

Migration from low/middle income countries with high tuberculosis (TB) incidence increasingly accounts for most TB cases in high-income countries with low TB incidence; the greatest risk for active TB is within the first few years of arrival (*1–4*). Screening for active pulmonary TB when documented immigrants enter a new country has found ≈3.5 cases per 1,000 documented immigrants (*5*,*6*). The prevalence of smear-negative cases of TB reported for US-bound immigrants and refugees was 9.6 cases per 1,000 persons (*7*).

In countries with low incidence of TB, vulnerable populations, such as persons living in prisons (*8*) and shelters (*9*) and hard-to-reach populations (*10–13*), are at high risk for TB (*3*). Marginalized immigrants have the combined risk of coming from countries with high incidence of TB and being vulnerable because of their relegated social position in countries of destination (*14*). Recent evidence suggests that the distribution of latent TB infection (LTBI) and TB among immigrants is uneven. LTBI prevalence among recent immigrants to the United Kingdom increased as a function of TB incidence in the country of origin (*15*). In high-income countries, refugees, asylum seekers, and immigrants, who were screened for TB when entering the new country, had 11.9, 2.7, and 2.8 cases of TB/1,000 persons, respectively (*5*).

Among easy-to-reach immigrants, LTBI prevalence is around 40%, measured by tuberculin skin test (TST) (*16*,*17*), and 15%–19%, measured by interferon-γ release assays (IGRAs) (*11*,*16*). The largest study assessing TST results enrolled <1,000 undocumented immigrants (*17*); the studies that used IGRAs recruited no more than 125 undocumented immigrants (*11*,*16*). Estimates of prevalence of active TB among undocumented immigrants (range 0.0065%–1.6%) are based on <10 cases in each study (*11*,*16*). No data have been published on incidence of infection, the key indicator of *Mycobacterium tuberculosis* transmission, among socially marginalized immigrants. Knowledge of these parameters among socially marginalized groups could inform control strategies for TB in countries in which TB incidence is low.

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DOI: http://dx.doi.org/10.3201/eid1909.120200

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We estimated the prevalence of and risk factors for active TB and LTBI, as well as the incidence of infection, among socially marginalized immigrants in an area of western Europe where incidence of TB is low. The study was conducted at the Regional Reference Centre for Tuberculosis Prevention in Turin, Italy. The estimated TB incidence during the study period, 1991–2010, was relatively stable (≈20 cases/100,000 persons), and in 2010, an increasing proportion of cases (»70%) occurred among foreign-born persons (*18–20*).

Methods

Study Population

The immigrant population was recruited through a screening program designed to complement the National Health Service routine program to detect LTBI and TB in the general population, i.e., passive case finding and contact tracing of patients with active TB. The screening program was set up to systematically identify and test local persons and immigrants regardless of their documented status, as a mandatory prerequisite to access public and private health and social care facilities in Turin, such as shelters, canteens, charity-run outpatient clinics. According to national recommendations (*21*), these persons were tested for LTBI and, if clinically suspected, for active pulmonary TB. Because the social group is less likely to be detected through passive case finding and contact tracing, we defined this group of persons as a socially marginalized population. This population is usually made up of the homeless, drug abusers, and former prisoners and is recognized by the European Centre for Disease Prevention and Control as a group of persons who are particularly difficult to find and test and treat for LTBI and active TB (*22*).

Details of the screening procedures were reported elsewhere (*17*). In brief, LTBI was diagnosed by means of a TST performed by using intradermal injection of PPD-5IU (Biocine Sclavo, Siena, Italy). If active pulmonary TB was suspected, chest radiography was performed at the first consultation. Patients were then asked to return for a second consultation at which the TST result would be read and chest radiography performed for those with a positive TST result (i.e., induration diameter at least 10 mm). Patients with suspect TB on the basis of a chest radiograph were further tested by smear examination and culture. All patients with suspected TB who were capable of producing sputum had at least 3 sputum specimens submitted for microscopic examination for acid-fast bacilli detection and culture. Since 1988, the culture methods used were BACTEC radiometric blood culture system (Johnston Laboratories, Inc., Towson, MD, USA) and after 1998, MGIT 960 (Becton Dickinson Diagnostic Instrument Systems, Sparks, MD, USA) and confirmed by growth on conventional Lowenstein-Jensen media. Finally, patients with a TB diagnosis were treated with standard short-course chemotherapy, whereas patients with no abnormalities on chest radiographs and positive TST results were offered preventive treatment (*23*). Patients who moved into or transferred between social care facilities were rescreened. Regional TB surveillance programs provided quality assurance and regular training of screening personnel (*24*). No patients with known contact with a person with active TB were included in the analysis. We have analyzed the data collected during January 1991–December 2010. The set information included in the analyzed dataset is reported in the online Technical Appendix (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/9/12-0200-Techapp1.pdf ,Table 1).

The geographic origin of each patient was categorized according to TB incidence data from the World Health Organization (WHO). In particular, each patient was assigned to one of the following categories: very low incidence (<25 annual cases/10^5 population), low incidence (25–49 annual cases/10^5 population), intermediate incidence (50–99 annual cases/10^5 population), high incidence (100–299 annual cases/10^5 population), and very high incidence (≥300 cases/10^5 population). The estimated annual incidence for each country at specific year of immigration was obtained from the WHO Global Atlas (*25*). If data relative to the specific year of immigration were not available, we considered the closest available year. For the annual incidence of active TB in the former Soviet Union, before 1995, we used the estimates reported by WHO (*26*).

Case Definitions

Cases of active pulmonary TB were defined according to WHO and International Union Against Tuberculosis and Lung Disease recommendations (*23*). Cases were defined as microbiologically confirmed or "definite cases" (i.e., sputum smear examination positive for acid-fast bacilli or culture positive for *M. tuberculosis* complex) and "other than definite" cases (i.e., with negative smear sputum and missing or without culture examination but with radiographic and/or clinical picture consistent with TB). TB cases diagnosed at first visit were considered as prevalent cases. Patients with cutaneous induration of at least 10 mm diameter at 48–72 hours after TST inoculation, normal chest radiographs, and absence of symptoms were considered to have prevalent cases of LTBI, regardless of bacillus Calmette–Guérin (BCG) vaccination history. We considered alternative methods of interpreting TST results (*27*), but given the highly heterogeneous nature of our population in terms of geographic origin (>100 countries), the predominance of prior BCG vaccination, and the need for a well-defined TST cutoff for clinical decision-making, we adopted the above cutoff as recommended

Table 1. Descriptive characteristics of socially marginalized immigrants investigated, Italy*

Variable, group	No. (%)
TB incidence rate in the country of origin of the immigrants, (x 10^5 person-years)	
<25	679 (2.5)
25–49	3,500 (12.8)
50–99	1,668 (6.1)
100–299	20,050 (73.3)
≥300	1,461 (5.3)
Age at test	
<20	2,500 (9.1)
20–29	10,705 (39.1)
30–39	8,659 (31.7)
≥40	4,938 (18.0)
Missing	556 (2.0)
Age at immigration	
<20	3,416 (12.5)
20–29	12,415 (45.4)
30–39	6,318 (23.1)
≥40	4,778 (17.5)
Missing	431 (1.6)
Sex	
F	11,116 (40.6)
M	16,242 (59.4)
Year of immigration	
<1990	1,978 (7.2)
1990–1994	5,121 (18.7)
1995–1999	8,206 (30)
2000–2004	8,911 (32.6)
2005–2010	2,092 (7.6)
Year of first testing	
1991–1994	2,040 (7.5)
1995–1999	10,783 (39.4)
2000–2004	11,400 (41.7)
2005–2010	3,055 (11.2)
First TST within	
2 y	60.4 (59.8–61)
5 y	81.9 (81.4–82.3)
10 y	95 (94.8–95.3)

*TB, tuberculosis; TST, tuberculin skin test. Given missing data the totals do not always sum up to 100%.

by national (*21*) and international guidelines (*28*). Finally, incident LTBI was defined as TST conversion in the absence of active TB; TST conversion was defined as an increase of at least 10 mm from the previous negative TST result in accordance with national (21) and international guidelines (*29*).

Statistical Analysis

We describe the crude distribution of prevalent and incident LTBI cases and prevalent active pulmonary TB cases by using the following variables: sex, estimated annual TB incidence rate in the country of origin of the immigrants, age at first test, age at immigration, and year of immigration. The 95% CIs of LTBI and TB prevalence were calculated by assuming a binomial distribution of the prevalent cases and a Poisson distribution of the LTBI incident cases.

To assess the role of selected determinants on the risk for LTBI and TB occurrence, we performed both univariate and multiple regression analyses. In particular, using logistic models we tested the mutually adjusted effect of sex, estimated annual TB incidence rate in the country of origin of the immigrants, and age at test and time elapsed since immigration as determinants of LTBI and TB prevalence. Incidence of *M. tuberculosis* infection was estimated for immigrants who had had a second TST performed and read at least 1 year after the initial TST; each of these immigrants whose TST result converted from negative to positive was deemed to have acquired infection during the time between the TSTs. Finally, we tested the interaction between sex and the other model covariates.

Results

From 1991 through 2010, a total of 27,358 socially marginalized immigrants who attended the screening program in Turin were considered at risk for active pulmonary TB. A total of 804 cases of TB (557 [69%] definite cases and 247 [31%] other than definite cases) were identified through the screening program, 744 (93%) of which were diagnosed at first visit. The prevalence of LTBI and incidence of TB among the remaining 26,554 immigrants who did not previously have TB were estimated with at least 1 and 2 valid screening examinations, respectively, (Figure 1).

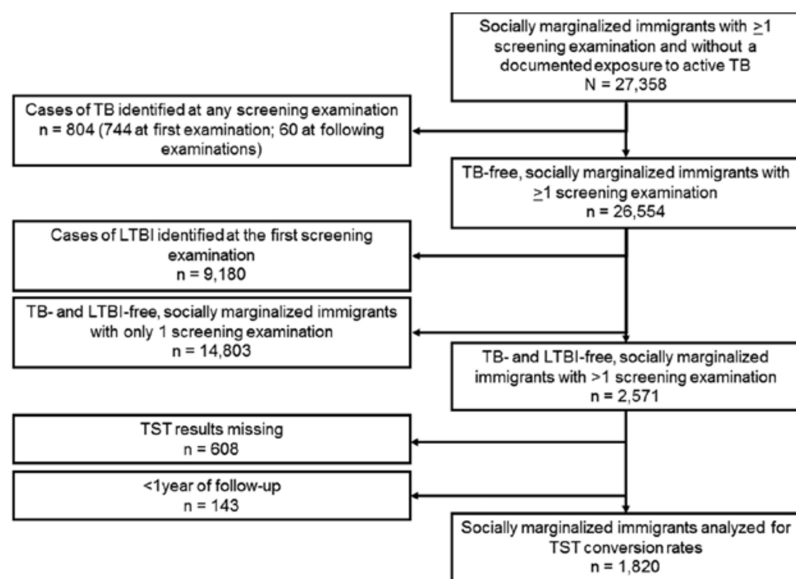

Figure 1. Immigrant selection process. TB, tuberculosis; LTBI, latent TB infection; TST, tuberculin skin testing.

Table 1 summarizes the main characteristics of the study population. Nearly 60% of the socially marginalized immigrants screened were men (n = 16,242), and 75% (n = 20,050 [73.3%]) were from countries with a high estimated annual incidence of TB (i.e., 100–299 cases/100,000 population); nearly 70% of the immigrants were 20–40 years of age at the time of immigration and screening. More than 80% entered Italy and were tested before 2005. The cumulative fraction of immigrants tested was 60%, 82%, and 95%, within 2, 5, and 10 years since immigration, respectively. Finally, the median interval between first and second TSTs for socially marginalized immigrants who had 2 TSTs and whose 1st TST result was negative was 5.6 years (interquartile range 2.9–8.6); for 143 immigrants, the interval was >1 year. Further details about the geographic origin of the immigrants can be found in the online appendix (online Technical Appendix, Table 2).

The distribution of TB and LTBI cases and TST conversions by sex, age at testing, and incidence rate of TB in the country of origin of the immigrants is reported in Table 2 along with crude estimates (and 95% CIs) of TB prevalence, LTBI prevalence, and infection incidence rates. The overall prevalence of active pulmonary TB was 2.7% (2.5%–2.9%), LTBI prevalence at first TST was 34.6% (34.0%–35.1%), and annual infection incidence was 1.7% (1.4%–2.1%). The prevalence of TB and LTBI among men was higher than that among women, 3.0% (2.8%–3.3%) vs. 2.3 (2.0%–2.6%) and 35.1% (34.4% –35.9%) vs. 3.8% (32.9%–34.7%), respectively (Table 2). However, the difference in infection incidence between men and women was not statistically significant, 1.6% (1.2%- 2.1%) and 1.9% (1.4–2.5%), p = 0.5, respectively (Table 2). As expected, the prevalence of TB and LTBI increased as a function of age at the time of testing, whereas the infection incidence rates did not appear to be affected by age at the time of testing. Finally, the overall distribution of the TB and LTBI cases suggests that TB and LTBI prevalence and infection incidence rates increased as a function of TB incidence rate in the country of origin of the immigrants (Figure 2). We have provided additional tables showing microbiologically confirmed cases of TB (online Technical Appendix, Tables 4 and 5).

The crude and adjusted effects of sex, estimated TB incidence rate in the country of origin of the immigrants, age at testing, and time elapsed since immigration on TB and LTBI prevalence and TST conversion are reported in the online Technical Appendix, Table 3, and Table 3 in this article. The odds ratio for TB and LTBI and the incidence rate ratio for TST conversion increased as a function of TB incidence rate in the country of origin of the immigrants (Figure 3, panel A). In particular, considering immigrants from countries with an estimated TB incidence of <25 cases/10^5population as a reference category, the odds ratio for TB reached a plateau, ranging from 4.6 to 5.3, as the estimated TB incidence rate in the country of origin of the immigrants was >50 TB cases/10^5 population (Table 3). The odds ratio for LTBI prevalence in immigrants from countries with an incidence in the country of origin >25 annual cases/10^5 person-years was higher than that observed in the reference category, although no specific patterns could be identified (Table 3). The estimated TB incidence rate in the country of origin of the immigrants displayed a linear effect on the incidence rate ratio for LTBI conversion with an incidence rate ratio of 1.2 (1.1–1.4) for an increase of 50 cases/10^5 person-years;

Table 2. Distribution and prevalence/rate of TB and LTBI cases and LTBI conversions*

	TB		LTBI			
Variable	No. cases/ population at risk	Prevalence, % (95% CI)	No. cases/ population at risk	Prevalence, % (95% CI)	No. cases/ p-y at risk	Conversion rates, % (95% CI)
Total	744/27,358	2.7 (2.5–2.9)	9,180/26,554	34.6 (34.0–35.1)	90/5,241	1.7 (1.4–2.1)
Sex						
F	254/11,116	2.3 (2.0–2.6)	3,663/10,849	33.8 (32.9–34.7)	46/2,462	1.9 (1.4–2.5)
M	490/16,242	3.0 (2.8–3.3)	5,517/15,705	35.1 (34.4–35.9)	44/2,777	1.6 (1.2–2.1)
Age, y, at test						
<20	30/2,498	1.2 (0.8–1.7)	405/2,465	16.4 (15.0–17.9)	4/245	1.6 (0.1–4.3)
20–29	190/10,705	1.8 (1.5–2.0)	3,163/10,500	30.1 (29.2–31.0)	26/1,436	1.8 (1.2–2.7)
30–39	253/8,659	2.9 (2.6–3.3)	3,480/8,382	41.5 (40.5- 42.6)	41/2,301	1.8 (1.3–2.4)
≥40	271/5,414	5.0 (4.4–5.6)	1,889/4,671	40.4 (39.0–41.9)	19/1,258	1.5 (1.0–2.4)
TB incidence (cases/10^5 p-y) in immigrant's country of origin						
<25	4/679	0.6 (0.2–1. 6)	111/674	16.5 (13.9–19.5)	1/74	1.3 (0.2–9.6)
25–49	56/3,500	1.6 (1.2–2.1)	1,008/3,440	29.3 (27.8–30.8)	6/814	0.7 (0.3–1.6)
50–99	48/1,668	2.9 (2.2–3.8)	407/1,615	25.2 (23.1–27.4)	3/311	1.0 (0.3–3.0)
100–299	602/20,050	3.0 (2.8–3.2)	7,196/19,400	37.1 (36.4–37.8)	74/3,764	2.0 (1.6–2.5)
≥300	34/1,461	2.3 (1.7–3.2)	458/1,425	32.1 (29.8–34.6)	6/278	2.1 (1.0–4.8)

*TB, tuberculosis; LTBI, latent TB infection; p-y, person years.

such an effect was not discernible when the categorical definition of estimated TB incidence was used (Figure 3, upper panel). As observed for crude estimates reported in Table 2, men were at a higher risk than women for TB and LTBI (odds ratio 1.5 [1.3–1.8] and 1.1 [1.0–1.2], respectively); this difference disappeared when the incidence rate ratio for infection incidence (0.9 [0.6–1.3]) was considered. Similarly, age at testing displayed a linear and more than linear effect on the risk for TB and LTBI, respectively, whereas it did not show any effect on the LTBI conversion rates. The risk for TB and LTBI and TST conversion rate did not change and increased and decreased as a function of time elapsed since immigration (Figure 3, panel B). No interaction between sex and the other model covariates was observed.

Discussion

Our study shows that socially marginalized immigrant populations as a whole may act as a reservoir for *M. tuberculosis*. In particular, the number of cases of TB and LTBI among socially marginalized immigrants, as measured through prevalence, was much higher than that reported among other immigrants or socially marginalized subgroups (*5*,*6*,*10*,*12*). Furthermore, the estimated TST conversion rates indicate that considerable *M. tuberculosis* transmission is occurring among socially marginalized immigrants in their adopted countries.

The estimated prevalence of active TB cases (2.7%), 69% of which were microbiologically confirmed (online Technical Appendix, Table 4), is considerably higher than the estimated incidence rates among in the general population in the respective different countries of origin. This finding may reflect demographic and socioeconomic differences between socially marginalized immigrants and the general population in the countries of origin, such as age distribution and the poor social and living conditions of socially marginalized immigrants. Undocumented immigrants may also be less likely to seek medical attention. The estimated TB prevalence is higher than that found when screening for active pulmonary TB among refugees at entry into the new country (1.2%) (*5*). The prevalence of microbiologically confirmed cases (1.8%) is also higher

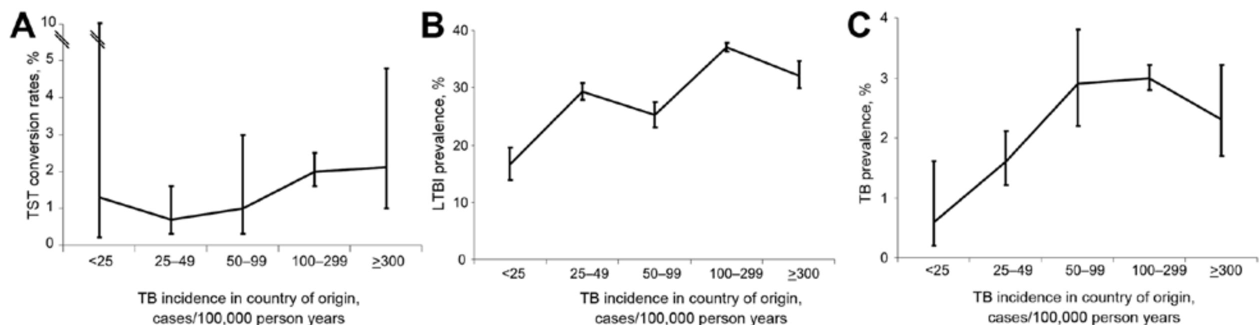

Figure 2. TB and LTBI among socially marginalized immigrants in an area of Italy where incidence of TB is low, by tuberculosis incidence rate in their country of origin, 1991–2010. A) TST conversion rates. B) LTBI prevalence. C) TB prevalence. Vertical bars indicate 95% CIs. TB, tuberculosis; LTBI, latent TB infection; TST, tuberculin skin testing.

Table 3. Multivariate model (logistic and Poisson) estimates of the odds and incidence rate ratios for TB, LTBI prevalence, and LTBI conversion*

Variable	Odds ratio (95% CI) for prevalence		Incidence rate ratio (95% CI) for TST conversion
	TB	LTBI	
TB incidence rate in the country of origin of the immigrants (per 10^5 p-y)			
<25	1 (reference category)	1 (reference cat)	1 (reference category)†
25–49	2.9 (0.9–9.3)	2.0 (1.6–2.6)	0.5 (0.1–4.1)†
50–99	5.3 (1.6–17.2)	1.6 (1.2–2.1)	0.7 (0.1–6.5)†
100–299	4.9 (1.6–15.2)	2.6 (2.1–3.3)	1.3 (0.2–9.3)†
≥300	4.6 (1.4–14.9)	2.2 (1.7–2.9)	1.4 (0.2–11.9)†
Sex			
F	1 (reference category)	1 (ref category)	1 (reference category)
M	1.5 (1.3–1.8)	1.1 (1.0–1.2)	0.9 (0.6–1.3)
Time elapsed since immigration, y			
<3	1 (reference category)	1 (ref cat)	1 (reference category)
3–6	1.1 (0.9–1.3)	1.1 (1.0–1.2)	0.8 (0.4–1.5)
7–9	1.1 (0.9–1.3)	1.2 (1.1–1.3)	0.5 (0.3–1.0)
≥10	0.9 (0.6–1.2)	1.2 (1.1–1.4)	0.4 (0.2–0.9)
Age, y, at test			
<20	1 (reference category)	1 (reference category)	1 (reference category)
20–29	1.4 (1.0–2.2)	1.9 (1.7–2.2)	1.0 (0.4–3.0)
30–40	2.4 (1.6–3.5)	3.1 (2.8–3.5)	1.2 (0.4–3.5)
≥40	4.3 (2.9–6.4)	3.1 (2.7–3.5)	1.0 (0.3–3.1)

*The effects of the variables reported in the table were mutually adjusted. TB, tuberculosis; LTBI, latent TB infection; p-y, person-years.
†Incidence rate ratio for tuberculin skin test conversion (95% CI): 1.21 (1.07–1.36) for an increase of 50 cases/105 p-y.

for this population than for the homeless populations in London (*10*) and Rotterdam (*12*) (0.8% and 0.9%, respectively). The TST conversion rates for our cohort far exceed those for the general population (0.5%), as measured by using the same TST methods used by clerical workers of health care services in Turin between 1997 and 2004 (*30*).

The size of our cohort yielded the largest dataset on TB infection and disease among immigrants in Europe; this dataset allowed for stratified analyses, which indicated that different subgroups of socially marginalized immigrants carry considerably different risks for LTBI and TB. Knowledge of such differences is highly pertinent for determining which subgroups of socially marginalized immigrants should be screened and when the screening should be conducted. The decline in TST conversion rates some 6 years after immigration provides robust empiric evidence for concentrating screening of LTBI on immigrants within the first few years of arrival, as already recommended in several countries. Furthermore, the strong relationship between TB incidence in the country of origin and the risk for LTBI, active TB, and TST conversion prioritizes certain subgroups of socially marginalized immigrants for screening. The correlation of LTBI and TB prevalence with TB incidence rate in the immigrant's country of origin probably reflects TB transmission that occurred in the countries of origin before immigration. By contrast, the increased risk for TST conversion, ≈20% for each increase of TB incidence rate in the country of origin of the immigrants of 50 cases/10^5 person-years, reflects ongoing transmission in Italy after immigration.

Differences in TST conversion rates between immigrants originating from different countries are consistent with assortative mixing between immigrants from the same geographic area. Assortative mixing would favor the transmission of TB between immigrants from the same geographic area. As a result, immigrants from areas where the prevalence of TB is higher would be at a higher risk for TST conversion. Social contacts, mixing patterns, and health-related behaviors in the countries of origin before immigration may also explain the higher prevalence of TB and LTBI among men than among women (adjusted odds ratios were 1.5 and 1.1, respectively), a finding consistent with other published data (*31*).

In contrast with what has been observed among immigrants in other industrialized countries such as the United Kingdom, Denmark, and Canada, where the risk for TB decreases as a function of time elapsed since immigration (*1*,*2*,*4*), in our population the risk for active TB did not decrease with time since immigration. The reason for this unexpected finding is unclear but may be explained by an arising difference in exposure to conditions promoting LTBI reactivation. At entry, any socially marginalized immigrant must attend the screening program to have access to health and social care facilities. Over time since immigration, an increasing proportion of socially marginalized immigrants experience improvements in their socioeconomic condition and become easy to reach, and the attendant environmental changes, including nutrition and housing, might be expected to decrease their risk for LTBI reactivation. Such immigrants would no longer be required to be screened. The remaining long-term socially marginalized immigrants, who would continue to be screened, are persistently exposed to social and

living conditions presumably favoring the reactivation of LTBI. Moreover, the occurrence of new infections, as measured through TST conversion, decreased with time elapsed since immigration regardless of the sex, age, and geographic origin of the immigrants. This decrease may represent a reduction of the transmission of *M. tuberculosis* among socially marginalized immigrants as a result of changes in contact patterns and difference of exposure resulting from the dilution of assortative mixing over time since immigration.

Our study has several limitations. The screening program was devised to reachthose persons accessing public and private health and social care facilities or the socially marginalized population less likely to be timely identified through routine passive case finding and contact tracing. For this reason, our target immigrant population is by definition socially marginalized and a credible denominator of immigrants cannot be determined; thus, we could not assess the coverage of screening program. Because the screening program does not collect detailed information on the reasons for immigration and for referral, we were unable to incorporate these factors in our analyses. Rates of completion of LTBI therapy after arrival in the destination for the study population are also unknown. LTBI prevalence and conversion rates were assessed by using TST rather than IGRAs, which became available in our setting in 2005 but for cost reasons have not hitherto been introduced for LTBI screening of immigrants. Our data would therefore tend to overestimate LTBI prevalence relative to data generated by IGRAs, which are more specific for LTBI (*11*,*16*,*32*); notwithstanding, the measures of association reported in Table 3 should not be affected, assuming that TST false-positive results are not differentially distributed across the variables investigated. Despite the relatively high specificity of TST conversion for recent infection, we cannot exclude the possibility that a fraction of the observed conversion is attributable to a boosted reaction as a result of repeated TST. To limit this possibility, we included in the analyses repeat TST performed not <12 months later (Figure 1). Estimated TST conversion rates may also have been affected by LTBI acquired by traveling to the countries of origin to visit friends and relatives (*33*), as suggested by the increased risk for TST conversion according to the TB incidence rate in the country of origin of the immigrants. On the other hand, the estimated reduction of conversion rates over time since immigration conflicts with this hypothesis. Overall, how much this hypothesis applies to the investigated immigrants, who are not representative of the general immigrant population, is uncertain. Finally, since reliable data on the BCG status of the socially marginalized immigrants were not available, we were unable to control for this in our analyses.

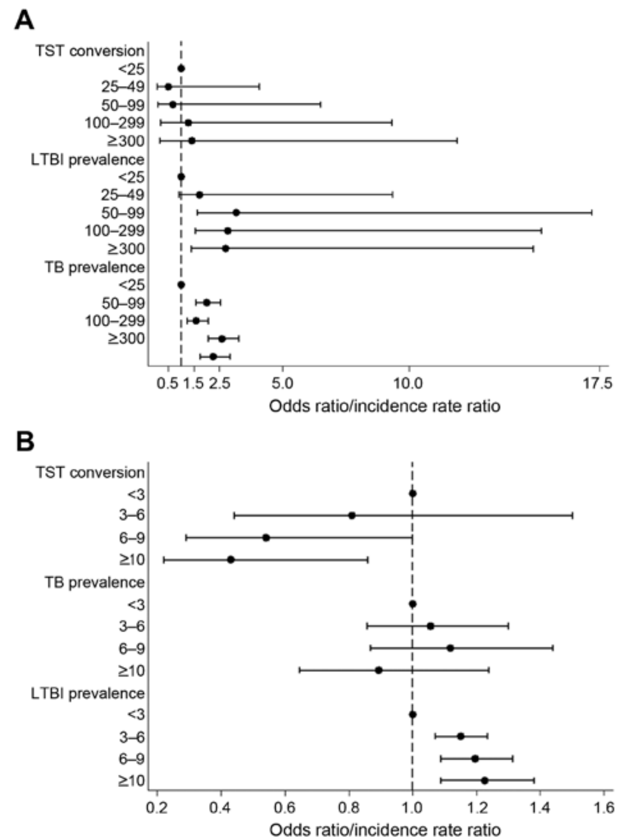

Figure 3. Adjusted odds ratio and incidence rate ratios for TB and LTBI occurrence among socially marginalized immigrants in a low-incidence area of Italy, by TB incidence rate in the country of origin (A) and time elapsed since immigration (B), 1991–2010. *Indicates reference category. Horizontal bars indicate 95% CIs. TB, tuberculosis; LTBI, latent TB infection; TST, tuberculin skin testing.

In conclusion, we have identified socially marginalized immigrants as a key reservoir of *M. tuberculosis* with substantial ongoing transmission in the first few years after arrival, which suggests that this population should be prioritized for screening for active TB and LTBI in countries where TB incidence is low. Our findings help to inform targeted interventions by identifying which immigrant subgroups should be prioritized for screening.

A.L. is a Wellcome Trust Senior Research Fellow in Clinical Science and a National Institute of Health research senior investigator. S.M. acknowledges the scientific support of the Master in Epidemiology of the Turin University. This study was funded by "Ricerca Finalizza–2009," Piedmont Region, Italy. M.P. is funded by a Medical Research Council Capacity Building Studentship.

A.L. is the inventor for several patents underpinning T cell–based diagnosis. The ESAT-6/CFP-10 IFN-gamma ELISpot assay was commercialized by an Oxford University

spin-out company (T-SPOT.*TB*, Oxford Immunotec Ltd, Abingdon, UK) in which the University of Oxford and A.L. have minority shares of equity and royalty entitlements. All other authors declare no conflicts of interest.

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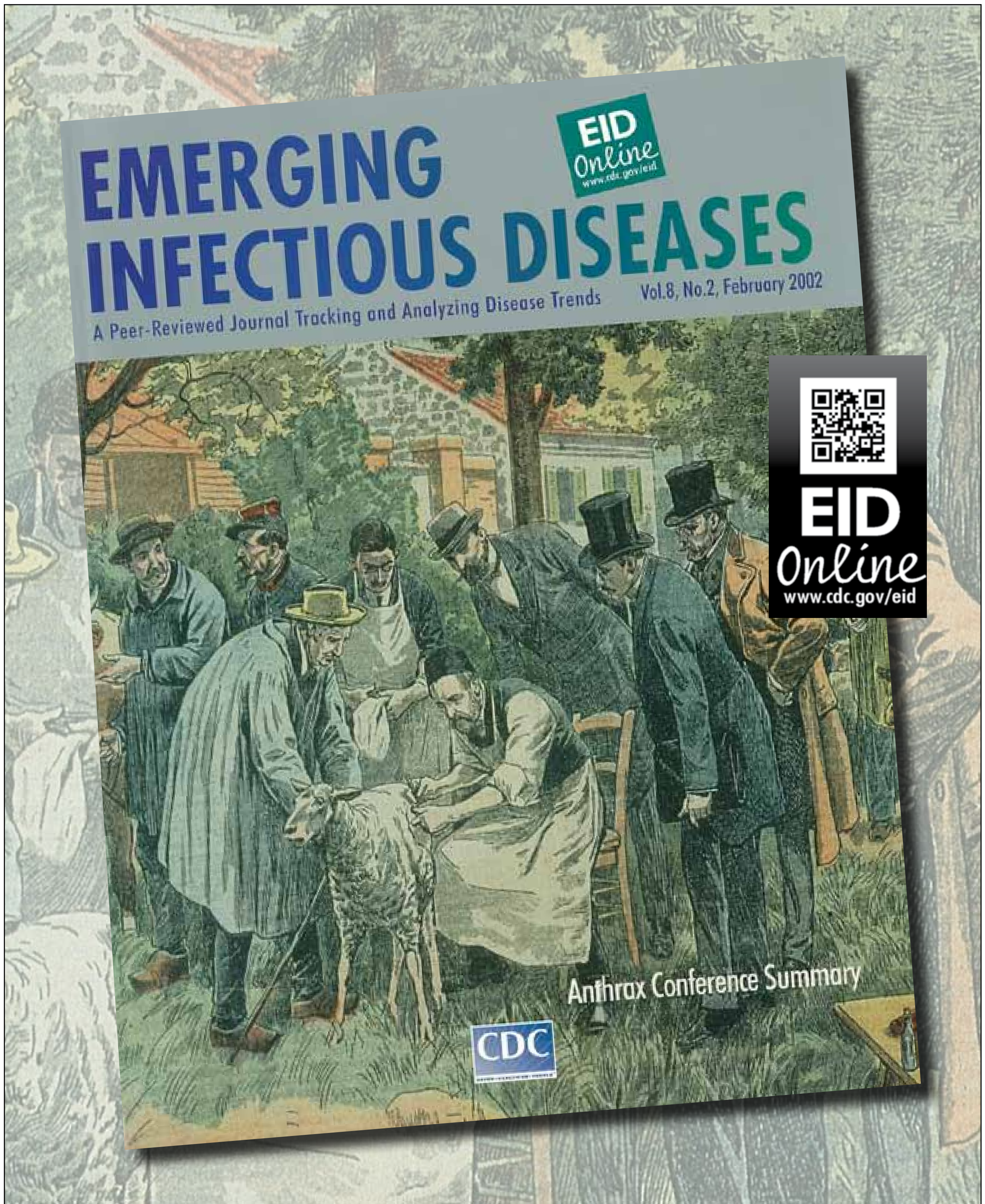

Plasmodium falciparum Mutant Haplotype Infection during Pregnancy Associated with Reduced Birthweight, Tanzania

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Intermittent preventive treatment during pregnancy with sulfadoxine–pyrimethamine (IPTp-SP) is a key strategy in the control of pregnancy-associated malaria. However, this strategy is compromised by widespread drug resistance from single-nucleotide polymorphisms in the *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthetase genes. During September 2008–October 2010, we monitored a cohort of 924 pregnant women in an area of Tanzania with declining malaria transmission. *P. falciparum* parasites were genotyped, and the effect of infecting haplotypes on birthweight was assessed. Of the genotyped parasites, 9.3%, 46.3%, and 44.4% had quadruple or less, quintuple, and sextuple mutated haplotypes, respectively. Mutant haplotypes were unrelated to SP doses. Compared with infections with the less-mutated haplotypes, infections with the sextuple haplotype mutation were associated with lower (359 g) birthweights. Continued use of the suboptimal IPTp-SP regimen should be reevaluated, and alternative strategies (e.g., intermittent screening and treatment or intermittent treatment with safe and effective alternative drugs) should be evaluated.

Pregnancy-associated malaria is a leading cause of maternal anemia and low birthweight (*1*). Measures to prevent pregnancy-associated malaria include insecticide-treated nets, treatment with effective antimalarial drugs, and administration of intermittent preventive treatment during pregnancy with sulfadoxine–pyrimethamine (IPTp-SP) (*2*). IPTp-SP is given at least twice during pregnancy, with doses 1 month apart (*3*,*4*). Studies have shown that IPTp-SP reduces the incidence of anemia, clinical malaria, low birthweight, and parasite prevalence at delivery (*3*,*5*–*9*).

SP acts by inhibiting the *Plasmodium falciparum* dihydropteroate synthetase and dihydrofolate reductase enzymes, respectively (*10*,*11*). However, resistance to the combined drug (SP) is widespread among the *P. falciparum* population in sub-Saharan Africa; this resistance is caused by accumulation of point mutations in the *P. falciparum* dihydropteroate synthetase *(Pfdhfr)* and dihydrofolate reductase *(Pfdhps)* genes (*12*,*13*). An increased number of point mutations in these genes is associated with augmented resistance to SP in vivo (*14*). There is sufficient evidence to support that the triple *Pfdhfr* mutation asparagine 51 to isoleucine (N51I), cysteine 59 to arginine (C59R), and serine 108 to asparagine (S108N) in combination with double *Pfdhps* mutant alanine 437 to glycine (A437G) and lysine 540 to glutamic acid (K540E)—forming quintuple mutant haplotypes—confer a high risk for treatment failure in malaria-infected children and nonpregnant adults who receive SP treatment (*14*). In addition, recent reports have shown that an increase in *Pfdhps* mutations at alanine 581 to glycine (A581G), further escalating the risk for even higher levels of resistance (*15*).

Because *P. falciparum* parasite resistance to SP is high, most likely because of the high prevalence of quintuple mutant haplotypes, use of the drug to treat uncomplicated malaria has been abandoned in many parts of eastern Africa. In Tanzania, SP was replaced in 2006 by artemether-lumefantrine for the management of uncomplicated malaria

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DOI: http://dx.doi.org/10.3201/eid1909.130133

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(*4*). The high prevalence (>50%) of the K540E mutation, which is found almost exclusively as the quintuple mutant haplotype, has also resulted in poor SP efficacy when used as intermittent preventive treatment in infants (*16*). However, other studies have indicated that IPTp-SP is still efficacious in some areas with high resistance (*17*). Nonetheless, with increased *P. falciparum* resistance, the usefulness of IPTp-SP might be compromised (*18–20*).

It has not been known whether there is an association between *P. falciparum* sextuple mutant haplotypes and poor pregnancy outcome. To determine if there is a relationship, we conducted a prospective cohort study in northeastern Tanzania in an area with declining malaria transmission (*21*). The study received ethical approval from the Tanzania Medical Research Coordinating Committee (reference no. NIMR/HQ/R.8a/Vol. IX/688). All procedures were conducted in accordance with the Declaration of Helsinki and Good Clinical and Laboratory Practices. All participants gave written informed consent.

Methods

Study Design and Samples

The study was conducted during September 2008–October 2010 in Korogwe District in the Tanga Region of northeastern Tanzania, where *P. falciparum* is the predominant malaria-causing species. A prospective cohort of 924 pregnant women was monitored from first attendance at the antenatal clinic through delivery. The study has been described in detail (*22–24*). In brief, pregnant women with a gestational age of ≤24 weeks, as estimated by using ultrasound, were enrolled if they had lived in Korogwe District for ≥6 months and were willing to give birth at Korogwe District Hospital. Study participants attended 3 additional prescheduled antenatal visits at weeks 26, 30, and 36 of pregnancy, and they were attended to by a study nurse/clinician. Obstetric history and maternal anthropometric measurements were recorded for all women (*23*). A venous blood sample was collected at each antenatal clinic visit, and venous and placental blood samples were collected at the time of delivery.

Diagnosis, Treatment, and Prevention of Malaria

All blood samples were tested for malaria parasites by using a rapid diagnostic test (RDT) (Parascreen, Zephyr Biomedicals, Goa, India; Paracheck Pf, Orchid Biomedical Systems, Mumbai, India; or ParaHIT-f, Span Diagnostics Ltd, Surat, India) and by microscopy. Blood smears for women with negative RDT results were examined retrospectively, whereas those for women with positive RDT results were examined immediately if deemed necessary by the study physician for a treatment decision (*25*). Parasite density was determined as the number of asexual stage parasites/200 leukocytes (500 leukocytes if <10 parasites) and converted to the number per microliter, as described (*22,25*); ≥100 fields were double-examined before a blood smear was declared negative.

Women with positive RDT results were treated with the antimalarial drug artemether-lumefantrine (Coartem Dispersible, Norvatis Corporation, Suffern, New York, USA) or with quinine. For infections occurring during the first trimester of pregnancy, quinine sulfate–coated tablets (ELYS Chemical Industries Ltd, Nairobi, Kenya) were used, and for severe cases, quinine dihydrochloride injection (Vital Healthcare PVT Ltd, Mumbai, India) was used.

Two doses of IPTp-SP (Sulphadar, Shelys Pharmaceutical Ltd, Dar es Salaam, Tanzania) were given ≥1 month apart as directly observed treatment; each dose contained 1,500 mg of sulfadoxine and 75 mg of pyrimethamine. Women with a gestational age of ≥20 weeks at enrollment were given the first IPTp-SP dose at the study-inclusion visit and the second dose during the third trimester. Women with a gestational age of <20 weeks at enrollment were given the first dose at 20 weeks of gestation. Women who had received IPTp-SP before study inclusion but earlier than recommended by the World Health Organization (i.e., after quickening in the second trimester) received a second dose after 20 weeks of gestation, and a third dose was given in the third trimester. Use of SP from private pharmacies/drug shops for malaria treatment before and after study inclusion was also documented. All study participants were provided with a voucher for procuring insecticide-treated nets.

Laboratory Methods and Birthweight Measurements

EDTA-preserved venous blood was used to estimate hemoglobin levels (KX-21N Automated Hematology Analyzer, Sysmex, Kobe, Japan). Live newborns whose birthweights were measured by using a spring scale (Fazzini, Vimodrone, Italy) with an accuracy of ≤50 g or a digital strain gauge scale (ADE, Hamburg, Germany) with an accuracy of ≤10 g within 24 h of delivery were included in the birthweight analysis. Newborns with severe malformations, twins, and those born to women with preeclampsia were excluded from analyses because these conditions can severely affect birthweight (*23*).

EDTA-preserved blood (50 μL) was spotted on Whatman number 3 filter paper (VWR– Bie & Berntsen, Herlev, Denmark), dried at room temperature, and stored in separate zip-lock bags. DNA was extracted by using the Chelex 100 method, as described (*26*). The DNA supernatant was transferred to a 96-well PCR plate and stored at -20°C until use. The parasite DNA was amplified by outer and nested *P. falciparum*–specific PCRs, as described (*27*); the products were analyzed by electrophoresis in 1.5% ethidium bromide–stained gel, as described (*22*).

To determine the multiplicity of infections, block 2 of the merozoite surface protein 2 domain was amplified by using fluorescent PCR (*28*). The results were analyzed by using GeneScan software, version 3.7 (Applied Biosystems, Naerum, Denmark).

Parasite DNA was amplified by outer and nested PCR with specific primers targeting the *Pfdhfr* and *Pfdhps* genes, as described (*29*). Single-nucleotide polymorphisms (SNPs) in the *Pfdhfr* and *Pfdhps* genes were identified by using a sequence-specific oligonucleotide probe ELISA technique, as described (*29*) with minor modifications. In brief, we used sequence-specific oligonucleotide probes targeting *Pfdhfr* codons c50/51 CI/CN, c59 (C/R), c108 (S/N/T), and c164 (I/L) and *Pfdhps* codons c436/437 (AA/AG/SA/SG/FG), c540 (K/E), c581 (A/G), and c613 (A/S). Individual SNPs were combined to deduce the different infecting mutant haplotypes.

Data Management and Statistical Analyses

We double-entered and validated data in Microsoft Access version 2007 (Redmond, WA, USA). Statistical analyses were conducted by using Stata version 10 (StataCorp, College Station, TX, USA) deploying parametric and nonparametric methods, as appropriate. The effect of infecting allelic haplotypes on birthweights was investigated by using multiple linear regression and dichotomized (as 6 and <6 SNPs) to infecting haplotypes; variables with a $p \leq 0.20$ in univariate analysis were included in the multivariate models. By using a stepwise backward elimination approach, we obtained final models including only variables with a $p \leq 0.10$. A 2-sided p-value of ≤ 0.05 was considered significant. Final models included only women without missing values.

Results

Demographic and Parasitologic Characteristics of the Study Cohort

Of 1,171 screened pregnant women, 995 met the study inclusion criteria; 924/995 women completed follow-up. For the entire study cohort, 5,555 venous and placental blood samples were collected during antenatal care and at delivery. Among the women completing follow-up, 76 had a total of 96 episodes of malaria. Some women had >1 infection, as determined by RDT and/or blood smears. The median asexual parasite density for study participants was 2,570 asexual stages/μL (range 40–390,749).

A total of 91 samples from women with RDT parasite-positive results were examined by *P. falciparum*–specific PCR, and 65 (71.4%) were positive. Of these 65 samples, 54 (83.1%) from 49 women were successfully typed in subsequent molecular analyses; 21 (38.9%) of these 54 samples were obtained at study enrollment. The median age of women with samples included in the genotyping analysis was 22 years (range 17-35), and at study inclusion, they had a median gestational age of 17.7 weeks (range 6.9 23.9). Overall, 91.8% of the women in the study cohort received 2 doses of IPTp-SP.

Multiplicity of Infections and *P. falciparum dhfr*/*dhps* Genotypes

Sixty percent of the genotyped parasites were polyclonal, with a mean of 2.5 clones/sample (range 1.0–8.0 clones/sample). Of the few women with repeat *P. falciparum* infections and sequence-specific oligonucleotide probe ELISA data (n = 5), 1 had a recrudescent and 1 had a new infection; genotype results of the 3 remaining infections were inconclusive.

Of the 54 parasite isolates, 49 (90.7%) were triple *Pfdhfr* mutants (CIRNI [C50, 51I, 59R, 108N, and I164]); 4 (7.4%) isolates were double *Pfdhfr* mutants CICNI/CNRNI; and the remaining 1 (1.9%) isolates was wild-type CNCSI (Figure 1, panel A). Two major *Pfdhps* haplotypes were identified in the 54 isolates: the double mutant AGEAA/SGEAA (A436/S436, A437G, and K540E) haplotype in 28 (51.9%) isolates and the triple mutant *Pfdhps* SGEGA (S436A, A437G, K540E, and A581G) haplotype in 25 (46.3%) isolates (Figure 1B). An AAKAA wild-type haplotype was identified in only 1 (1.9%) of the 54 isolates (Figure 1, panel B).

The different SNPs were combined to generate the following mutant *Pfdhfr*/*Pfdhps* haplotypes: quadruple haplotype (double *Pfdhfr* [CICNI/CNRNI] with double *Pfdhps* [AGEAA/SGEAA]); quintuple haplotypes (triple *Pfdhfr* with double *Pfdhps* [CNRNI–AGEAA/SGEAA] or double *Pfdhfr* with triple *Pfdhps* [CICNI–SGEGA]); and sextuple haplotype (triple *Pfdhfr* [CIRNI] and triple *Pfdhps* [SGEGA]). The only *Pfdhfr* CNCSI wild-type isolate was a double *Pfdhps* (SGEAA) mutant, and the only *Pfdhps* AAKAA wild-type haplotype was a triple *Pfdhfr* (CIRNI) mutant. The isolates with these 2 haplotypes were grouped as quadruple or less when generating the combined *Pfdhfr*/*Pfdhps* haplotypes. The quadruple or less, quintuple, and sextuple haplotypes were observed for 9.3%, 46.3%, and 44.4% of the malaria infections, respectively (Figure 1, panel C). For the woman with repeated infections, the highest level of mutation was used in the analyses on pregnancy outcome.

Trends of *Pfdhfr* and *Pfdhps* Allelic Haplotypes by Gestational Age

Quadruple and less mutated haplotypes were observed mainly during early pregnancy, but the quintuple and sextuple haplotypes were observed throughout pregnancy (Figure 2); there was no clear trend indicating a selection by IPTp-SP use for the most mutated haplotypes (Table 1). Among the women who did not receive IPTp-SP before

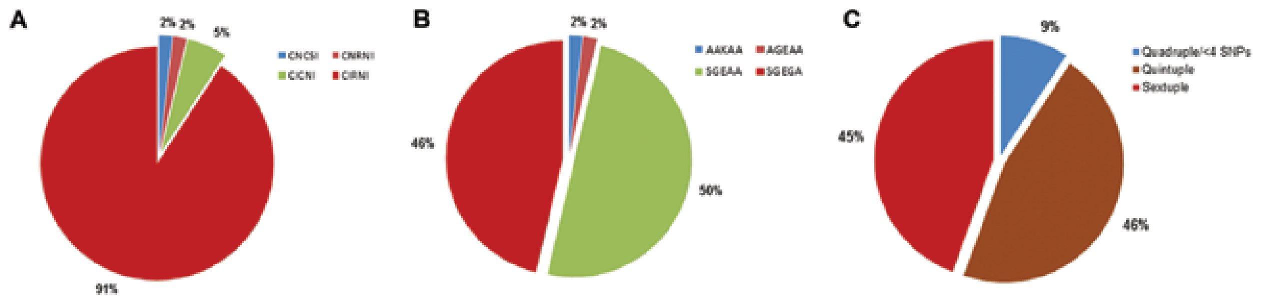

Figure 1. *Pfdhfr/Pfdhps* haplotyping results for *Plasmodium falciparum* parasite isolates from 54 parasite isolates in 49 pregnant women, Korogwe District, Tanga Region, Tanzania, September 2008–October 2010. A) Proportion of single nucleotide polymorphisms (SNPs) conferring sulfadoxine–pyrimethamine resistance on the *P. falciparum* dihydrofolate reductase gene (*Pfdhfr*) at codons C50, N51I, C59R, S108N, and L164I, resulting in allelic haplotypes CNCSI (wild-type), CICNI (double *Pfdhfr* mutant), CNRNI (double *Pfdhfr* mutant), and CIRNI (triple *Pfdhfr* mutant). B) Proportion of SNPs conferring sulfadoxine–pyrimethamine resistance on the *P. falciparum* dihydropteroate synthetase (*Pfdhps*) gene at codons S436A, A437G, K540E, A613S/T, and A581G with allelic haplotypes AKAA (wild-type), AGEAA (double *Pfdhps* mutant), SGEAA (double *Pfdhps* mutant), and SGEGA (triple *Pfdhps* mutant). C) Proportions of *Pfdhfr/Pfdhps* quadruple or less, quintuple, and sextuple mutant haplotypes from the cohort of pregnant women. The derivations of the allelic haplotypes were based on a combination of 2 or 3 *Pfdhfr* SNPs with 2 or 3 *Pfdhps* SNPs forming quadruple (4 SNPs), quintuple (5 SNPs), and sextuple (6 SNPs) haplotypes. Quadruple haplotype or less included 4 SNPs (quadruple) and triple *Pfdhfr* (CIRNI, n = 1) or double *dhps* (SGEAA, n = 1) with wild-type *dhfr* (CNCSI, n = 1) or wild-type *dhps* (AKAA, n = 1). Less than 4 haplotypes had 1 triple or double mutation in 1 gene combined with a wild-type mutation in the other gene A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/09/13-0133-F1.htm).

malaria infection, 3 with quintuple and 1 with sextuple haplotype infections reported using SP for treatment of suspected malaria before study inclusion. Even when both exposures to SP (i.e., as IPTp-SP and as SP treatment) were taken into account, no trend toward accumulation of resistant haplotypes as a result of SP use was observed (Table 1).

Effect of Sextuple Haplotypes on Pregnancy Outcome

There were no statistically significant differences in the characteristics of women infected with highly mutated parasites and those with less mutated parasites with respect to age, gravidity, and anthropometry (Table 2). Of the 26 women with quintuple or less haplotype infections and 23 with sextuple haplotype infections, 24 and 20, respectively, received 2 doses of IPTp-SP. Two women with quintuple or less haplotype infections and 2 with sextuple haplotype infections received no or 1 dose of IPTp-SP, and 1 woman with sextuple haplotype infection received 3 doses. The levels of parasitemia were also similar between the 2 groups, and there were no differences in the incidence of fever (axillary temperature >37.5°C) or level of hemoglobin (g/dL) at the time of infection or at delivery (Table 2).

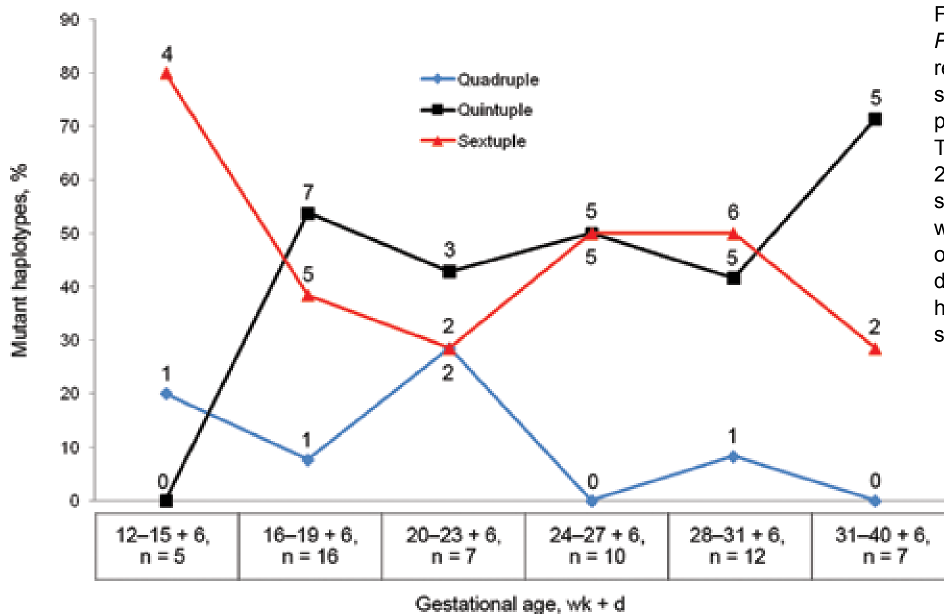

Figure 2. Proportion of mutant *Plasmodium falciparum* dihydrofolate reductase and dihydropteroate synthetase haplotypes among pregnant women, Korogwe District, Tanga Region, Tanzania, September 2008–October 2010. Proportions are shown by gestational age; partial weeks are indicated by the number of days. Numbers above and below data points are the number of mutant haplotypes; total numbers (n) are shown below the graph.

Table 1. Stratification of *Plasmodium falciparum* mutant haplotypes among pregnant women, by exposure of the women to SP before infection, Korogwe District, Tanga Region, Tanzania, September 2008–October 2010*

Participant exposure to SP	No. (%) women with infecting allelic haplotype		
	Quadruple or less, n = 5	Quintuple, n = 25	Sextuple, n = 24
Did not receive IPTp–SP (n = 28)	4 (14.3)	12 (42.9)	12 (42.9)
Received first dose of IPTp–SP (n = 18)	1 (5.6)	8 (44.4)	9 (50.0)
Received second dose of IPTp–SP (n = 8)	0	5 (62.5)	3 (37.5)
Had previous exposure to SP (n = 30)†	1 (3.3)	16 (53.0)	13 (43.0)

*SP, sulfadoxine–pyrimethamine; IPTp–SP, intermittent preventive treatment during pregnancy with SP.
†Received IPTp-SP or SP used as treatment for malaria before study inclusion.

Among the 49 newborns born to women for whom *P. falciparum* strains were genotyped, there was 1 case of preeclampsia, 2 twin deliveries, and 1 newborn with congenital malformation. Among the remaining 45 singleton newborns, 36 had their birthweight measured within 24 hours of delivery and, thus, were eligible for analysis. There was no difference in the distribution of haplotypes between mothers of the included and excluded newborns (data not shown).

The median birthweight for newborns of women with sextuple haplotype infections was significantly lower than that for newborns of women with less mutated haplotype infections (Table 2). When using *z* scores (adjusting for gestational age at delivery and sex of newborn) based on a Tanzanian reference chart from the same cohort (*23*), borderline significance toward lower *z* scores in women with sextuple mutant haplotype infections was observed (p = 0.06). There was no difference in the incidence of low birthweight newborns or of newborns that were small for gestational age at birth among women with sextuple haplotype and less mutated haplotype infections (Table 2). When the effect on birthweight was further analyzed by using a stepwise multiple linear regression model adjusting for covariates, newborns of women with sextuple mutant haplotype infections had a significantly reduced birthweight (mean reduction 359 g; 95% CI, -601 to −117) compared with newborns of women with quintuple or less haplotype infections (p = 0.005) (Table 3) Covariates for the linear regression model were mid upper arm circumference at study inclusion, parasite density, hemoglobin level, number of IPTp-SP doses, gestational age at inclusion and at delivery, number of infections, maternal age, weight/weight gain during pregnancy, HIV status, place of delivery, sex of the newborn, and gravidity.

Women in whom sextuple haplotype parasites were identified more often had repeated infections than those in whom less mutated haplotypes were identified (Table 2). In the univariate analysis, there was a borderline significance toward decreased birthweight after repeated infections (p = 0.08). However, this effect was not observed in the multivariate model (Table 3).

There was a trend toward decreased birthweight with increasing numbers of IPTp-SP doses received (Table 3). This trend was explained by the fact that newborns of the 3 women who received 0 or 1 IPTp-SP dose had much higher birthweights than newborns of the 33 women who received 2 doses (3,690 g vs. 2,920 g, respectively; p = 0.04). However, in the entire cohort (924 women) there was no association between the number of IPTp-SP doses and birthweight (data not shown). Furthermore, the number of IPTp-SP doses received before time of infection and the time between the most recent IPTp-SP dose and when infection occurred was not associated with birthweight. Adding these variables to the multivariate model did not alter the result (data not shown).

Discussion

IPTp-SP is still being used in areas where prevalence of *Pfdhfr/Pfdhps* mutations is high (*17*), although widespread resistance is likely to affect the protective effect of this strategy in preventing pregnancy-associated malaria. The continued use of this suboptimal drug for IPTp might promote further development of resistance because resistant strains are likely to have a fitness advantage.

This study showed that despite administration of a full IPTp-SP course to most of the study participants, the women were not fully protected, and malaria infections occurred in 8.2% (76/924) of the women. Point mutations in *Pfdhfr* and/or *Pfdhps* genes were observed in all genotyped parasites. Quadruple or less mutated haplotypes were mainly observed early during pregnancy, whereas quintuple and sextuple mutated haplotypes were encountered throughout pregnancy. Harrington et al. (*20*) indicated that IPTp-SP use was associated with increased prevalence of parasites with mutations at codon A581G. However, we did not observe an association between exposure to SP before infection and increased A581G mutant parasite prevalence (Table 1). This lack of association in this study could be due to the fact that the parasite populations were already largely saturated with the highly mutated strains.

Of concern, the high prevalence of the *Pfdhfr/Pfdhps* sextuple haplotype was associated with reduced birthweight. Previous studies have reported an association between mutation level and adverse pregnancy outcomes (*18–20*) rather than, as we show, an adverse effect on birthweight specifically associated with sextuple haplotype parasites. For the entire cohort of 924 women, a negative

Table 2. Demographic and biological characteristics for 49 pregnant women infected with *Plasmodium falciparum* sextuple or less mutant haplotypes, Korogwe District, Tanga Region, Tanzania, September 2008–October 2010*

	P. falciparum mutant allelic haplotype						
	Less than sextuple			Sextuple			
Characteristic	No.†	Median†	Range or %	No.†	Median†	Range or %	p value‡
Maternal age, y	26.0	22.5	17.0–35.0	23.0	21.0	17.0–32.0	0.38
Gravidity	26			23			0.81
Primi-/secundigravidae	20		77%	17		74%	
Multigravidae (3–8 pregnancies)	6		23%	6.0		26%	
Gestational age, wk							
At study inclusion	26.0	17.6	6.8–23.8	23.0	16.3	8.3–22.3	0.54
At delivery	26.0	39.7	32–43.1	22.0	40.0	35.1–42.6	0.82
Owned bed net	26	19	73%	23	11	48%	0.07
MUAC							
At study inclusion, cm	26	26	20–32	23	24	22.0–37.0	0.07
At delivery, cm	23.0	25.5	21.4–31.6	20.0	25.0	21.2–30.0	0.22
Child's birthweight, g§	19	3,148 ± 434¶		17	2,822 ± 436¶		**0.03**
Children with birthweight <2,500 g	19	1	5%	17	2	12%	0.82
z-score at delivery	18.0	–0.11 ± 1.27¶		17.0	–0.88 ± 1.07¶		0.06
SGA at delivery	19	2	11%	17	6	35%	0.11
Weight of placenta, g	17	645	381–780	15.0	492	307–800	0.16
Hemoglobin level, g/dL							
At delivery#	16.0	11.2 ± 1.8¶		13.0	11.1 ± 1.8¶		0.80
At time of infection	25.0	10.0 ± 1.4¶		22.0	10.3 ± 1.8¶		0.56
Fever at time of infection	25	1	4%	23	2	8.7%	0.60**
Parasitemia, IE/µL††	17	2,565	42–10,1208	18	1,895	40–390,749	0.64
>1 infection‡‡	26	1	4%	23	7	30%	**0.02**
>2 doses of IPTp	26	24	92%	23	21	91%	0.90

*MUAC, mid upper arm circumference; SGA, small gestational age; IE, infected erythrocytes; IPTp, intermittent preventive treatment during pregnancy.
†Data are no. or median no. unless otherwise indicated in column one or by ¶.
‡Unless otherwise indicated, all medians were compared by using the Mann Whitney Rank sum test, means were compared by using the Student *t*-test, and proportions were compared by using the χ^2 test. **Bold** font indicates statistical significance ($p<0.05$).
§Among the 49 study participants, 1 had preeclampsia, 2 delivered twins, and 1 delivered a newborn with severe malformation. The birthweight of 39 newborns (including 1 pair of twins and the child born with severe malformation) was measured within 24 h of birth. Only the 36 singleton newborns without malformation were included in the analyses.
¶Data are mean ± SD.
#Hemoglobin levels were measured for many women after delivery; however, only levels measured before delivery were included in the analyses. Low hemoglobin after delivery might be due to ante- and postpartum bleeding rather than antenatal events (e.g., malaria infection).
**Fisher exact test.
††Parasitemia is only stated for 17 nonsextuple and 18 sextuple infections because some infections were rapid diagnostic test–positive but blood smear–negative. Sequence-specific oligonucleotide probe ELISA on filter paper was, however, still possible despite the very low level of parasitemia.
‡‡No. infections is based on all infections detected in the woman by using a rapid diagnostic test and/or blood smear, regardless of whether sequence-specific oligonucleotide probe ELISA was conducted.

association between malaria and fetal growth/birthweight has been reported (*25*).

We converted birthweights to *z* scores by using a reference chart of birthweights for infants born to healthy women in the 924-person study cohort (*23*). By using the *z* scores, we standardized the difference between individual birthweight and the mean population birthweight adjusted for sex of newborn and gestational age at delivery. The disadvantage of this conversion is that any inherent uncertainty in the reference chart is imputed into the birthweight measure. This could explain why the difference in *z* scores between the sextuple and less mutated haplotypes was only borderline significant (Table 2). The multivariate model on the effect of infection with the sextuple haplotype on birthweight was already adjusted for gestational age at delivery, and the results were not altered when sex of the newborn was included (data not shown).

Harrington et al. (*18*) observed that the use of IPTp-SP increased parasite growth. We did not find a substantial difference in parasite density between the different haplotypes, nor did we find that the time between IPTp-SP use and infection altered the effect of the haplotype on birthweight. Therefore, IPTp-SP does not seem to increase parasite density, and differences in parasite density cannot explain the observed effect of the sextuple haplotype on birthweight. The presence of highly mutated *P. falciparum* genotypes that cannot be cleared by IPTp-SP exposes women to persistent and chronic malaria infections. In our study, sextuple haplotype parasite infections were associated with reduced weight of the placenta, although this difference did not reach statistical significance. Low placental weight indicates poor development of the placenta, which has been associated with pregnancy-associated malaria (*30, 31*). In vitro assays with extravillous trophoblasts showed that serum and plasma from *P. falciparum*–infected pregnant women inhibited extravillous trophoblast invasion and migration, offering a possible explanation for the pathophysiologic events that may cause impaired placentation, reduced placental weight, and low infant birthweight (*32*). Therefore, longer lasting infections, altering placental

Table 3. Determinants of birthweight for children born to 49 pregnant women with *Plasmodium falciparum* genotype data, Korogwe District, Tanga Region, Tanzania, September 2008–October 2010*

	Univariate analysis			Multivariate analysis†		
Variable	Coefficient	95% CI	p value	Coefficient	95% CI	p value
MUAC at study inclusion, cm	49	1 to 97	0.047	15	–28 to 59	0.47
Gestational age, wk						
At study inclusion	–20	–63 to 22	0.340			
At delivery	90	–23 to 204	0.120	90	1 to 179	**0.048**
Sex of newborn, F	–106	–432 to 221	0.520			
Maternal age, y	37	10 to 64	0.009	2	–30 to 35	0.890
Gravidity (>2 pregnancies)	477	166 to 788	0.004	434	152 to 716	**0.004**
Maternal weight at study inclusion, kg	9	–12 to 30	0.370			
Maternal weight gain, kg						
From study inclusion to ANV3	38	–47 to 124	0.372			
From ANV3–ANV4	–22	–72 to 28	0.370			
Infecting haplotype, sextuple	–326	–621 to –31	0.031	–359	–601 to –117	**0.005**
Parasite density, per 1,000 IE/μL	0.09	–3 to 3	0.95			
No. infections‡	–308	–649 to 34	0.08	–143	–426 to 140	0.31
No. IPTp doses before delivery	–303	–614 to 8	0.06	–231	–498 to 35	0.087
Maternal HIV status positive	230	–308, to 769	0.390			
Place of delivery						
Hospital	–86	–381 to 209	0.56			
Other than hospital	–12	–696 to 671	0.971			

*Effect on birthweight in the multivariate analyses is stated for all variables with p<0.2 in the univariate analyses. In the final model, only variables with p<0.10 were included in the model. MUAC, mid upper arm circumference; ANV3, antenatal clinic visit at gestational week 30; ANV3–4, ANV at gestational week 30–36; IE, infected erythrocytes.
†Blank spaces indicate no data/information. **Boldface** indicates statistical significance (p<0.05).
‡No. infections is based on all infections detected in the woman by using a rapid diagnostic test and/or blood smear, regardless of whether sequence specific oligonucleotide probes–ELISA was conducted.

development among the women with sextuple haplotype infections, could explain the reduced birthweights observed in our study.

Harrington et al. (*18*) reported that SP in itself could have detrimental effects on the health of newborns. Our findings do not indicate that SP in itself causes lower birthweight. We did find a borderline significant association between the number of IPTp-SP doses and reduced birthweight among the 36 newborns; however, this association was not seen when we evaluated the effect of IPTp-SP doses on birthweight in the entire cohort of 924 women (data not shown).

Despite regular screening, using RDTs, for malaria among pregnant women and treatment with effective antimalarial drugs, it is worrisome that birthweights for newborns of women infected with sextuple mutated parasites were lower than those for newborns of women infected with less mutated parasites. This finding underscores the need to evaluate the effect of these mutations among populations using IPTp-SP at a wider scale.

Because of the changing epidemiology of malaria, which has transformed the transmission pattern from high to low in large parts of sub-Saharan Africa (*21,33*), including the study area, it is likely that many pregnant women, irrespective of gravidity, will have little immunity against pregnancy-associated malaria. The decreasing prevalence of malaria and the fact that isolates with sextuple mutations had a significant effect on birthweight underscores the question of whether IPTp-SP should be continued or replaced by intermittent screening and treatment and/or with an alternative drug for IPTp. SP might be teratogenic in the first trimester of pregnancy (*34*), and most other currently available antimalarial drugs are either teratogenic or their efficacy and safety profiles among pregnant women are still poorly known (*35*); thus, there is an urgent need to conduct further efficacy and safety studies to determine alternative drugs for IPTp. Until then, we suggest that screening by RDTs during pregnancy may allow early case detection and prompt treatment with effective antimalarial drugs (*22*).

This study demonstrates that sextuple *Pfdhfr*/*Pfdhps* mutated haplotypes are prevalent in the study area and that these highly SP-resistant parasites are associated with a significant reduction in birthweight of newborns of malaria-infected women. Presumably, the presence of these highly mutated *P. falciparum* genotypes is largely unaffected by IPTp-SP and expose women to persistent and chronic malaria infection, and this effect, rather than SP by itself, has detrimental effects on the health of newborns. We observed highly mutated haplotypes even before IPTp-SP was used and throughout pregnancy, indicating saturation of the population with resistant parasites. Therefore, continued use of the suboptimal IPTp-SP regimen should urgently be reevaluated, and its replacement with screening and treatment or with an alternative safe and effective antimalarial drug for IPTp should be considered.

Acknowledgments

We thank all study participants for their willingness to participate; Tilaus Gustav, Thomson Mwampamba, Rashid

Madebe, Deusdedith Makingi, Deusdedith Ishengoma, and Ulla Abildtrup for excellent technical support; Charles Tunuka, Sophia Kabome, Lydia Massawe, Francis Assenga, Latifa Shaweji, Prisca Mavindi, Rose Mutua, Halima Mpambile, and Aziz Seiph for clinical support; Christopher Mhagama, Hassan Kilavo, Silas Msangi, Stella Mkandawile, and Eva Rimoy for timely and organized data management; and Allen Mrango, Francis Mkongo, and Frank Mnango for excellent logistics support; Hannah Elena Suhrs, Martyna Gassowski, Pernille Kofoed, Line Holm, Peter Cordes, Alisha Walker, Neeltje Rutten, Nicolien Beld, and the staff of National Institute for Medical Research–Korogwe Research Laboratory for their wonderful support and cooperation; Phillip Deloron for assisting in merozoite surface protein 2 genotyping and overall coordination; Christopher Masaka, Obedi Ole Kaondo, Lydia Lugomora, and Bibiana Reuben for administrative support; and the National Institute for Medical Research–Tanga Centre and Headquarters' Administration and Joint Malaria Programme Office and Korogwe District Hospital for their wonderful administrative support. Vito Baraka is also thanked for his useful comments.

This work was supported by the European Union through the Seventh Framework Programme STOPPAM (contract no. 200889) and the Danish International Development Agency (grant no. DFC file no.87-08-KU).

D.T.R.M., C.S., A.S., A.J.F.L., M.L., T.T., J.L., and M.A. designed the study. D.T.R.M., C.S., S.B., M.O., P.M., C.P., D.J., and J.L. conducted the study and participated in the laboratory analyses. D.T.R.M. drafted the manuscript. C.S., B.M., MA, and T.T. assisted in data management and analyses.

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New Estimates of Incidence of Encephalitis in England

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Encephalitis causes high rates of illness and death, yet its epidemiology remains poorly understood. To improve incidence estimates in England and inform priority setting and treatment and prevention strategies, we used hospitalization data to estimate incidence of infectious and noninfectious encephalitis during 2005–2009. Hospitalization data were linked to a dataset of extensively investigated cases of encephalitis from a prospective study, and capture–recapture models were applied. Incidence was estimated from unlinked hospitalization data as 4.32 cases/100,000 population/year. Capture–recapture models gave a best estimate of encephalitis incidence of 5.23 cases/100,000/year, although the models' indicated incidence could be as high as 8.66 cases/100,000/year. This analysis indicates that the incidence of encephalitis in England is considerably higher than previously estimated. Therefore, encephalitis should be a greater priority for clinicians, researchers, and public health officials.

Encephalitis is associated with severe illness, appreciable mortality rates, and high health care costs (*1*), but its epidemiology remains poorly understood (*2*). The sole previous incidence estimate for encephalitis in England of 1.5 cases per 100,000 population per year was for viral encephalitis only and was based on hospitalization data from 1989–1998 (*3*). Incidence should be understood; as an increasing number of viruses have been found to cause encephalitis in humans, more cases might be found among the high proportion of cases of unknown etiology (*2*,*4*–*6*). Climate change and increasing international travel raise the possibility of wider geographic spread of microbes, which may have important public health implications. Clarifying incidence is also important clinically with the increasing recognition of novel immune-mediated forms of encephalitis, especially because treatment is available if started early (*7*–*9*).

Although encephalitis is a statutorily notifiable disease in England, it is grossly underreported, making notification data unsuitable for incidence estimation (*3*). Almost all encephalitis case-patients require hospitalization; thus, routinely collected hospitalization data provide a possible source of data from which to estimate incidence. However, diagnosis of encephalitis is complicated by the lack of a standard case definition or pathognomonic symptoms and signs. Many patients with suspected encephalitis ultimately are found to have conditions with neurologic signs that mimic encephalitis. We recently reported on the Public Health England (PHE) study, the largest population-based prospective cohort of encephalitis patients to date in England (*10*). Data from this study, which included exhaustive multistage diagnostic investigations of cases, provided a unique opportunity to complement routinely collected hospitalization data to enable detailed analyses of encephalitis incidence in England. We linked the 2 data sources and performed capture–recapture analyses to estimate the number of encephalitis cases in England attributable to infectious and noninfectious causes.

Methods

Data Sources

PHE Study

Patients with encephalitis were recruited over a 2-year period from 24 hospitals in 3 regions of England. Suspected cases were actively identified and investigated by using extensive systematic laboratory testing, a specific case definition, and classification of cases by a multidisciplinary expert panel. Details are described elsewhere (*10*).

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DOI: http://dx.doi.org/10.3201/eid1909.130064

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Hospital Episode Statistics

These routinely collected data include all admissions to National Health Service hospitals in England, recorded by financial year (April–March). Each record represents an episode, a continuous period of hospital care under 1 consultant; patients may have multiple episodes within 1 hospitalization. For example, 2 separate records and hence episodes are generated in Hospital Episode Statistics (HES) if a patient is initially admitted to Accident and Emergency and subsequently transferred to a neurology ward. Each episode has up to 20 diagnoses, recorded by using codes from the International Classification of Diseases, 10th Revision (http://apps.who.int/classifications/icd10/browse/2010/en). The primary diagnosis usually reflects the main reason for hospital admission. However, incident encephalitis cases can also be recorded in secondary diagnostic fields if the primary diagnosis was a symptom or sign, if encephalitis was initially misdiagnosed, or if encephalitis developed during hospital admission.

All episodes with an admission date from April 1, 2005 through February 28, 2009 containing an encephalitis code (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/9/13-0064-Techapp1.pdf) in any diagnostic field were obtained from HES. Data were obtained in May 2009; data from 2008–2009 were provisional and did not include cases for March 2009.

Data with an encephalitis code in any diagnostic field (the main analysis), as well as data with an encephalitis code in the primary diagnostic field only, were examined (see Table 1 for definition of numerators). Mid-2007 resident population estimates for England from the Office for National Statistics were used as denominators. Bootstrap resampling of hospital trusts and 1,000 repetitions were used to obtain 95% CIs.

Rates of HES encephalitis admissions were calculated by year, sex, age, and region by using data with an encephalitis code in any diagnostic field. We performed multivariable negative binomial regression analyses to investigate how the rate of incident encephalitis admissions varied with each variable.

Table 1. Definition of numerator for encephalitis incidence estimates, England

First-ever episode*	Subsequent episodes
Encephalitis code in any diagnostic field (1–20)	Subsequent encephalitis code/s represented a different etiology to that recorded for the first-ever episode; for identical codes, both were in the primary diagnostic field and the end date of the first episode and start date of the subsequent spell were >6 mo apart
Encephalitis code in primary diagnostic field only	Subsequent encephalitis codes were also in the primary diagnostic field and represented a different etiology to that recorded for the first-ever episode; for identical codes, both were in the primary diagnostic field and the end date of the first spell and start date of the subsequent spell were >6 mo apart

*Refers to the first-ever episode within this dataset.

Capture–Recapture Analyses

For these analyses, PHE and HES data were restricted to patients admitted from November 1, 2006, through October 31, 2007, when all 24 hospitals in the PHE study were recruiting simultaneously, and to the 19 HES hospital trusts that included the 24 PHE study hospitals (hospital data are coded to trust level in HES). Cases were linked by date of birth, date of admission, sex, and postcode. Fuzzy (approximate) matching was also performed to allow for possible coding errors in the matching variables.

For 2 PHE study hospitals, the corresponding HES hospital trust included another hospital that had not participated in the PHE study and could have admitted encephalitis patients (e.g., had an accident and emergency department). Data from these 2 PHE hospitals and HES trusts were excluded in sensitivity analyses to assess their effect on capture–recapture estimates.

We determined the number of cases that matched between the 2 datasets and the numbers included only in the PHE study and only in the HES data. Fisher exact and χ^2 tests were used to compare characteristics (including age, sex, ethnic group, and etiology) of PHE study cases that matched and did not match to HES cases and characteristics of matched and unmatched HES admissions. Other comparisons between matched and unmatched HES cases included method of admission (e.g., elective or emergency), consultant specialty, and discharge destination. The 2-sample Wilcoxon rank sum test was used to compare length of hospital stay between matched and unmatched HES admissions.

Two-sample capture–recapture analyses were performed. The Lincoln-Peterson formula was used to estimate the number of encephalitis cases that occurred from November 1, 2006, through October 31, 2007, for the 24 included sites, which comprised the number listed in either source plus the estimated number of unlisted cases (*11*). The completeness of ascertainment (%) for each data source was estimated by using this total capture–recapture estimate. CIs were calculated from the standard errors of coefficients obtained from fitting a log linear model. To assess potential violation of the capture–recapture assumption that every patient has the same probability of being ascertained by each source, data were stratified by 2 characteristics that could affect the likelihood of being ascertained: age (<18 years and ≥18 years) and region (South West, London, and North West). Capture–recapture analyses were rerun for each stratum, and the results were combined and compared with those from unstratified analyses.

Another key assumption of capture–recapture analyses is the absence of false-positive cases, which could have arisen in the HES data because of misdiagnosis of nonencephalitis cases or coding errors. Sensitivity analyses were thus conducted by using a range (30%–80%) of positive predictive values (PPVs) for an HES encephalitis code to reduce the number of HES-only cases included in capture–recapture models. Within this range of PPVs, we focused on a best estimate of 54%, based on the proportion of patients with suspected cases of encephalitis who were initially screened in the PHE study and ultimately included in the study (203/379), assuming that all screened PHE cases would have been coded as encephalitis in HES (*10*).

HES admissions from November 1, 2006, through October 31, 2007, for the trusts that corresponded to PHE study hospitals represented 19% of all encephalitis admissions in England. We assumed the accuracy of coding in these trusts is generalizable to all England admissions; therefore, we multiplied the capture–recapture estimate of the number of cases in these trusts by 5.3 to obtain ascertainment-adjusted national incidence estimates.

Results

HES Incidence

Annual incidence estimates are displayed in the Figure. On the basis of HES data with an encephalitis code in any diagnostic field, the overall mean incidence was 4.32 cases/100,000 population/year (95% CI 3.74–4.96/100,000/year). Two percent of patients (n = 216) had >1 encephalitis admission during the study period; incidence did not change (4.20 cases/100,000/year) when subsequent admissions of these patients were excluded from the analysis. By using data restricted to the primary diagnostic field, the overall mean incidence was 2.75 cases/100,000/year (95% CI 2.39 cases–3.10/100,000/year).

Results of multivariable analyses showed that, compared with 2005–2006, incidence in all subsequent years was slightly higher but with little evidence of a trend ($p = 0.19$). The incidence rate was highest among patients <1 and ≥65 years of age. The rate for female patients (adjusted for year, age, and region) was 8% less than the adjusted rate for male patients (online Technical Appendix Table 2)

Capture–recapture Analyses

For the defined 1-year period, the overall number of incident encephalitis admissions recorded in the relevant HES trusts was almost 4 times the number of cases in the PHE study (439 admissions vs. 113 cases) by using HES data with an encephalitis code in any diagnostic field and was 2.5 times the number (287 admissions vs. 113 cases) when HES data were restricted to the primary diagnostic field.

Fifty-nine (52%) of 113 PHE study cases matched to HES cases, and 1 case was linked by using fuzzy matching. Fifty-four cases were registered only by the PHE study and 380 were registered only by HES. The characteristics of the 59 matched HES cases and the 380 unmatched HES cases are presented in Table 2. A high proportion (91%) of encephalitis cases of unknown etiology indicated in the HES data were unmatched. Of the 125 case-patients admitted electively and 38 case-patients treated by neurosurgery in the HES data, 95% and 100%, respectively, did not match the PHE data; unmatched HES patients with admissions also had a shorter length of hospital stay (11 vs. 31 days; $p<0.001$; Table 2). The results were similar when HES data restricted to the primary diagnostic field were used (data not shown).

Heterogeneity in etiology was evident between unmatched PHE cases and HES-matched PHE cases ($p = 0.01$; Table 2). A high proportion (80%) of bacterial encephalitis cases in the PHE data were unmatched, whereas

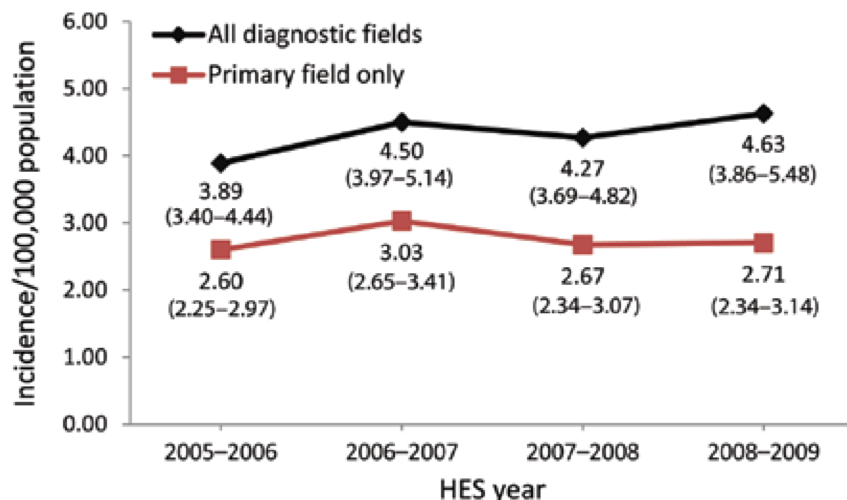

Figure. Rate of incident encephalitis admissions by year per 100,000 population, England, April 2005–February 2009. Values indicated are rate (95% CI). Overall rate for all diagnostic fields: 4.32 (3.74–4.96); for primary field only: 2.75 (2.39–3.10).

a lower proportion of PHE cases designated as immune-mediated encephalitis (22%) or herpes simplex virus (HSV) encephalitis (33%) were unmatched. Further comparison of the etiologic classification of the 59 cases that matched in the 2 datasets showed some discrepancies (online Technical Appendix Table 3); 1 HES varicella zoster virus encephalitis case was diagnosed as HSV encephalitis in the PHE study, and 2 "other mixed" HES cases were diagnosed as immune-mediated in the PHE data. The 21 HES encephalitis admissions of unspecified etiology that matched to PHE

Table 2. Characteristics of HES-only admissions compared with those that matched to PHE study cases and characteristics of cases included in the PHE study only compared with those of PHE study cases that matched to HES, England, November 1, 2006–October 31, 2007*

	HES			PHE study		
Characteristics	HES only, n = 380	Matched,† n = 59	p value	PHE only, n = 54	Matched,‡ n = 59	p value§
Age group†						
<1	14 (77.8)	4 (22.2)	0.11§	5 (55.6)	4 (44.4)	0.22
1–4	23 (71.9)	9 (28.1)		5 (38.5)	8 (61.5)	
5–19	51 (86.4)	8 (13.6)		6 (40)	9 (60)	
20–44	113 (85.6)	19 (14.4)		15 (44.1)	19 (55.9)	
45–64	115 (88.5)	15 (11.5)		9 (39.1)	14 (60.9)	
≥65	52 (92.9)	4 (7.1)		14 (73.7)	5 (26.3)	
Sex						
M	192 (86.9)	29 (13.1)	0.89§	34 (54)	29 (46)	0.18
F	188 (86.2)	30 (13.8)		20 (40)	30 (60)	
Ethnicity**						
White	248 (86.7)	38 (13.3)	0.2§	40 (48.8)	42 (51.2)	0.66
Mixed	2 (100)	0		2 (50)	2 (50)	
Asian or Asian British	23 (82.1)	5 (17.9)		1 (16.7)	5 (83.3)	
Black or black British	27 (87.1)	4 (12.9)		8 (50)	8 (50)	
Chinese or other	12 (66.7)	6 (33.3)		2 (50)	2 (50)	
Etiology						
Herpes simplex virus	13 (46.4)	15 (53.6)	<0.001§	8 (33.3)	16 (66.7)	0.01
Varicella zoster virus	25 (89.3)	3 (10.7)		2 (50)	2 (50)	
Other specified viral	37 (88.1)	5 (11.9)		3 (50)	3 (50)	
Bacterial	10 (71.4)	4 (28.6)		12 (80)	3 (20)	
Parasitic	7 (100)	0		2 (100)	0	
Amoebic	0	0		0	0	
Fungal	0	0		0	0	
Immune-mediated	37 (82.2)	8 (17.8)		5 (21.7)	18 (78.3)	
Unknown	218 (91.2)	21 (8.8)		21 (56.8)	16 (43.2)	
Other mixed	33 (91.7)	3 (8.3)		1 (50)	1 (50)	
Admission type						
Elective	119 (95.2)	6 (4.8)	0.001§			
Emergency	218 (83.5)	43 (16.5)				
Transfer††	43 (81.1)	10 (18.9)				
Discharge destination‡‡						
Home	272 (86.3)	43 (13.7)	0.85§			
Other hospital/nursing home	37 (84.1)	7 (15.9)				
Died	40 (85.1)	7 (14.9)				
Treatment specialty						
Neurosurgery	38 (100)	0	<0.001¶			
General medicine	65 (81.2)	15 (18.8)				
Neurology	121 (90.3)	13 (9.7)				
Pediatrics	27 (73)	10 (27)				
Pediatric neurology	22 (75.9)	7 (24.1)				
Infectious diseases	11 (68.8)	5 (31.2)				
Intensive care medicine	10 (71.4)	4 (28.6)				
Other	86 (94.5)	5 (5.5)				
Median length of stay, d (range)	11 (0–737); n = 351	31 (0–414);§§ n = 57	<0.001#			

*Values are no. (%) except as indicated. HES, Hospital Episode Statistics; PHE, Public Health England.
†Using data from HES.
‡Using data from the PHE study.
§Fisher exact test.
¶χ^2 test.
#Mann Whitney test.
**For HES only. n = 312; HES matched, n = 53; PHE only n = 53.
††Transfer from other hospital, excluding emergency.
‡‡For HES matched, n = 57.
§§For HES only, n = 368.

study cases included 8 cases also classified as unknown etiology in the PHE study, 8 as immune-mediated, 2 as HSV, and 3 as due to other infectious causes.

The initial capture–recapture model estimated that 348 cases of encephalitis were not registered in either data source, giving an estimated number of encephalitis cases for the relevant period in that area of 841 (Table 3) and an ascertainment-adjusted incidence rate of 8.66 cases/100,000 population/year. The completeness of the 2 sources was estimated at 52% for HES and 13% for the PHE study. The estimated number of cases for children and adults combined following the stratified analyses (n = 830) was similar to the overall figure obtained in the crude analysis (n = 841); similar results were obtained when the data were stratified by region and recombined. When HES data were restricted to an encephalitis-specific code recorded in the primary diagnostic field, the capture–recapture model estimated 265 unlisted and 612 total encephalitis cases, giving an incidence estimate of 6.3 cases/100,000/year and estimated completeness of HES and the PHE study of 47% and 18%, respectively (Table 3). Similar incidence estimates were obtained after excluding from analyses the 2 sites for which additional hospitals with potential encephalitis patients were included in the HES data only (data not shown).

Results of sensitivity analyses to account for potential overdiagnoses of HES-only encephalitis cases are reported in Table 4. By assuming a best estimate PPV of 54% for HES-only admissions, the capture–recapture model estimated 508 total cases, giving an incidence of 5.23 cases/100,000/year and an estimated completeness of 22% for the PHE study; this figure represents our "best estimate" of the true incidence of encephalitis in England. By assuming a higher PPV for HES-only admissions, the number of estimated total cases increased and the completeness of the PHE study decreased. When HES data were restricted to the primary diagnostic field only and a PPV of 54% was assumed, the capture–recapture model estimated 384 total cases, equivalent to an incidence of 3.96 cases/100,000/year (Table 4).

Discussion

Our analyses provide estimates of the incidence of encephalitis in England attributable to infectious and noninfectious causes. We present a unique application of capture–recapture models to estimate the number of cases of encephalitis by using an original dataset of well-defined and extensively investigated cases of encephalitis.

Multiple scenarios were investigated to assess the sensitivity of the estimates to various assumptions. Depending on the scenario, estimated incidence ranged from 2.73 cases/100,000/year to 8.66 cases/10^5/year; all estimates were higher than the 1.5 cases/100,000/year previously reported (*3*). This unique study has brought together 2 distinct datasets to help address the inevitable limitations within such data sources, particularly those encountered with complex syndromes such as encephalitis. We consider our capture–recapture estimate of 5.23/100,000/year (assuming 54% PPV for HES data) to be the best estimate of encephalitis incidence in England; this is 3.5 times higher than that previously described by Davison et al (*3*). Our incidence analyses update the Davison et al. estimates; diagnostic advances, emerging etiologic agents, and introduction of new interventions and control strategies (e.g., vaccines) are all likely to have affected incidence estimates over

Table 3. Two-source capture–recapture estimates of encephalitis cases in England, November 1, 2006–October 31, 2007*

Coding and strata	HES only	PHE study only	Both sources	Estimated total no. cases (95% CI)	% Completeness (95% CI)	
					HES	PHE study
Encephalitis-specific code in any of the 20 diagnostic fields						
Crude analysis	380	54	59	841 (692–991)	52 (43–61)	13 (10–17)
Stratified by age group						
Children <18 y	78	15	20	172 (121–223)	57 (41–73)	20 (12–28)
Adults ≥18 y	290	39	39	658 (512–805)	50 (39–61)	12 (8–15)
Total for strata	368	54	59	830 (675–986)		
Stratified by region						
London	149	23	16	403 (254–552)	41 (26–56)	10 (5–14)
North West	168	29	38	364 (286–442)	57 (45–69)	18 (13–24)
South West	63	2	5	96 (49–143)	71 (38–100)	7 (1–14)
Total for strata	380	54	59	863 (689–1,038)		
Encephalitis-specific code in primary field only						
Crude analysis	234	60	53	612 (494–731)	47 (38–56)	18 (14–23)
Stratified by age group						
Children <18 y	49	16	19	126 (87–165)	54 (38–71)	28 (17–39)
Adults ≥18 y	173	44	34	475 (358–593)	44 (33–55)	16 (11–21)
Total for strata	222	60	53	601 (478–725)		
Stratified by region						
London	86	23	14	265 (159–371)	38 (22–53)	14 (7–21)
North West	114	29	34	275 (211–339)	54 (42–66)	23 (16–30)
South West	34	2	5	55 (27–83)	71 (38–100)	13 (2–23)
Total for strata	234	54	53	595 (469–722)		

*HES, Hospital Episode Statistics; PHE, Public Health England.

Table 4. Sensitivity analysis adjusting for variation in positive predictive value for HES-only admissions*

Positive predictive value, %	HES only	PHE study only	Both sources	Estimated total no. cases (95% CI)	% Completeness (95% CI) HES†	% Completeness (95% CI) PHE study	Estimated incidence
Encephalitis-specific code in any of the 20 diagnostic fields							
30	114	54	59	332 (272–391)	52 (43–61)	34 (27–41)	3.42
40	152	54	59	405 (332–477)	52 (43–61)	28 (22–34)	4.17
50	190	54	59	477 (392–562)	52 (43–61)	24 (18–29)	4.91
54	206	54	59	508 (418–598)	52 (43–61)	22 (17–27)	5.23
60	228	54	59	550 (452–648)	52 (43–61)	21 (16–25)	5.67
70	266	54	59	623 (512–734)	52 (43–61)	18 (14–22)	6.42
80	304	54	59	696 (572–819)	52 (43–61)	16 (12–20)	7.17
Encephalitis-specific code in primary field only							
30	71	60	53	265 (214–315)	47 (38–56)	43 (34–51)	2.73
40	94	60	53	314 (254–374)	47 (38–56)	36 (28–44)	3.23
50	117	60	53	363 (293–433)	47 (38–56)	31 (24–38)	3.74
54	127	60	53	384 (310–458)	47 (38–56)	29 (23–36)	3.96
60	141	60	53	414 (334–494)	47 (38–56)	27 (21–34)	4.26
70	164	60	53	463 (374–553)	47 (38–56)	24 (19–30)	4.77
80	188	60	53	514 (415–614)	47 (38–56)	22 (17–27)	5.29

*Incidence = cases/100,000 population. HES, Hospital Episode Statistics; PHE, Public Health England
†Due to the Lincoln-Petersen formula, if the number of HES-only admissions varies, the total HES admissions (HES only plus matched) is proportional to the estimated total number of cases; hence, the completeness of HES (ratio) does not change with varying positive predictive value.

time. Furthermore, our data included both infectious (not just viral) and noninfectious causes of encephalitis, in line with the increased recognition of new immune-mediated encephalitis etiologies.

A higher incidence (adjusted for year, age, and region) of encephalitis was observed among male patients, which is consistent with previous studies (*12–16*). We also observed higher incidence of encephalitis among patients <1 and ≥65 years of age. Hyporesponsiveness of the immune system in early life and later immunosenescence render these groups more susceptible to infection, to reactivation of latent infection, or development of encephalitis once infected (*17*).

Multiple admissions were included in our analyses only if they represented different etiologies or occurred >6 months apart, either of which is not a common occurrence in encephalitis as supported by our data. When multiple occurrences were excluded, incidence was unchanged. Infectious episodes of encephalitis are unlikely to recur in the absence of immunosuppression; relapses in immune-mediated cases are more frequent and were documented in the PHE study.

We extended our encephalitis incidence analyses beyond the use of HES and linked HES data to PHE study data and applied capture–recapture models. The number of incident encephalitis admissions recorded in HES was considerably higher than the number of cases included in the PHE study, even after restricting HES diagnoses to the primary diagnostic field. Conversely, nearly half of the PHE cases were not captured in HES. The poor agreement between these 2 data sources could have several possible reasons.

The likely explanation for nonmatched PHE cases is that testing in the PHE study went far beyond routine clinical practice, which highlighted the extent to which encephalitis can be underdiagnosed. The higher proportion of bacterial cases in the PHE study, classified as meningoencephalitis, suggests that these cases may be coded as meningitis rather than encephalitis in HES. Also, patients with unusual signs and symptoms, such as those with N-methyl-D-aspartate receptor-antibody encephalitis, which typically causes psychiatric symptoms, might not have been classified as encephalitis case-patients in HES (*18*). Unfortunately, we could not identify the HES codes used for the unmatched PHE cases because the HES data for this study included only patients with an encephalitis code.

Unmatched HES cases likely include true encephalitis cases not reported to the PHE study team and nonencephalitis cases misdiagnosed as encephalitis. Underascertainment in the PHE study is likely, as employing >1 research nurse per region to actively identify cases was not financially feasible: some centers relied on case notification by hospital staff alone. The likelihood that HES admissions coded as encephalitis included misdiagnoses of syndromes with signs that mimic encephalitis is highlighted by the PHE study, in which only 54% of suspected encephalitis patients initially screened during the 2-year period were ultimately included following a rigorous diagnostic process (*10*). The higher proportion of cases of unknown etiology in unmatched compared with matched HES admissions and their shorter length of hospital stay supports the possibility of misdiagnosis and suggests a more likely diagnosis of a mimicker syndrome such as septic encephalopathy.

Other reasons for mismatches need consideration. The catchment areas covered by the 2 data sources differed slightly, but the results of the capture–recapture model did not change when we excluded the 2 trusts that had hospitals with potential extra encephalitis cases in the HES data. The higher number of admissions in HES could be due in part to the inclusion of patients with postencephalitic sequelae, who would not have been notified to the PHE study. This

finding is supported by the higher proportion of cases admitted electively and treated under neurosurgery in unmatched HES admissions; alternatively, some of these cases could be miscoded nonencephalitis mimics, i.e., cases of nosocomial meningitis following surgery.

Three assumptions required for valid capture–recapture estimates also need consideration. First, for a given source every patient should have an equal chance of being ascertained by that source, although different sources may have different probabilities of identifying an individual case. Our analyses stratified by age or region of residence did not indicate any bias in the point estimate linked to these variables. Other variables may have influenced the probability of a patient appearing on a list, such as ethnicity, sociodemographic factors, or heterogeneity in coding between hospitals within regions. Because of the small sample sizes, we could not stratify by these variables. We did find evidence of heterogeneity in etiology, which suggested that in 1 or both datasets, patients with bacterial encephalitis had a different probability of being identified than did patients with encephalitis of other etiologies, which may have led to an overestimate of the number of encephalitis cases. Again, performing a stratified analysis is not easy, both because of small numbers and because we know misclassification and missing data about etiology were present and no pathognomonic features exist to allow different causes to be distinguished in these cases.

Second, the registers used in capture–recapture should be independent; having an encephalitis-specific diagnosis in HES should not affect being included in the PHE study or vice versa. A patient with suspected encephalitis seen by a hospital clinician was likely to be coded as such in HES and also be notified to the PHE study. Thus, these sources are likely to be positively dependent, and the capture–recapture will have underestimated the true number of cases. A less likely scenario is that the datasets were negatively dependent, for example, if encephalitis cases with bacterial etiology were simultaneously more likely to be excluded from the HES data and more likely to be included in the PHE data. This situation would have led to an overestimate in the number of cases. We did not have access to a third data source to evaluate the independence of data sources (*19*). Nevertheless, 2-source capture–recapture can indicate an upper or (as is likely here) the lower bound of estimates when the direction of dependency is known or highly suspected (*20*).

Third, no false positive cases should occur due to misdiagnoses or miscoding. As discussed above, coded HES encephalitis cases are likely to have included mimicker syndromes, which would inflate the capture–recapture estimate. We addressed this possibility by applying a range of PPVs to the HES data; even after assuming a PPV as low as 30% for HES-only admissions, the number of cases was still higher than previous estimates. A review of medical records would be necessary to determine the degree of misdiagnosis of true encephalitis cases in HES data. We could not do this because HES do not keep patients' names, and use of HES data are subject to strict protocols to prevent identification of individual patients.

With a mean length of hospital stay of 34 days, an incidence of 5.23 cases/100,000/year ("best estimate") equates to 90,852 bed-days of hospital occupancy. On the basis of a bed-day cost of £261 (US$394 million), the cost to the National Health Service would be >£23 million (US$35 million) per year (*21*). An incidence of 8.66 cases/100,000/year, our maximum estimate, would cost almost £40 million (US$60 million) per year. The actual cost is likely to be higher as patients often require intensive care, costly investigations, and in-patient rehabilitation. Additional costs include long-term care and loss of productivity among many working-age survivors.

In summary, the different scenarios used in this study provide strong evidence that the incidence of encephalitis is higher than that previously estimated in England. This higher incidence has clinical, research, and public health implications. A diagnosis of encephalitis should be considered for patients with compatible symptoms, especially given the increased recognition of immune-mediated encephalitides for which treatment is available and effective if instigated early. Early recognition is important to help reduce the substantial economic and societal costs of encephalitis suggested by our study. Stand-alone HES data are used extensively for public health research; our analyses highlight the extent to which HES-only data might over- or underascertain cases of complex syndromes and the advantages of linking these data to other sources to improve incidence estimates. Encephalitis incidence in this study was higher than that of other neurologic conditions, such as meningococcal meningitis and motor neuron disease, both of which have a higher profile and public focus (*22–24*). This study highlights the importance of accurate diagnosis and coding for complex syndromes with multiple etiologies to obtain accurate estimates of incidence and to further explore the epidemiology and outcomes of this devastating neurologic illness.

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Acknowledgments

We thank Andrew Grant and Elizabeth Turner for analytical advice on the capture–recapture analyses, Ross Harris for advice regarding STATA programming, and the Encephalitis Society (www.encephalitis.info) for their ongoing support to persons who have survived encephalitis.

Use of patient postcode of residence and date of birth to enable HES linkage to PHE study data was approved by the Database Monitoring Sub-Group. This is an independent report funded by the Policy Research Programme in the Department of Health, UK, with salary support from PHE for J.G. and N.C.

The views expressed in the publication are those of the authors and not necessarily those of the Department of Health. The authors have no conflict of interest.The opinions, results and conclusions reported in this paper are those of the authors. No endorsement by the Ontario Agency for Health Protection and Promotion is intended or should be inferred.

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Enzootic and Epizootic Rabies Associated with Vampire Bats, Peru

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During the past decade, incidence of human infection with rabies virus (RABV) spread by the common vampire bat (*Desmodus rotundus*) increased considerably in South America, especially in remote areas of the Amazon rainforest, where these bats commonly feed on humans. To better understand the epizootiology of rabies associated with vampire bats, we used complete sequences of the nucleoprotein gene to infer phylogenetic relationships among 157 RABV isolates collected from humans, domestic animals, and wildlife, including bats, in Peru during 2002–2007. This analysis revealed distinct geographic structuring that indicates that RABVs spread gradually and involve different vampire bat subpopulations with different transmission cycles. Three putative new RABV lineages were found in 3 non–vampire bat species that may represent new virus reservoirs. Detection of novel RABV variants and accurate identification of reservoir hosts are critically important for the prevention and control of potential virus transmission, especially to humans.

Rabies virus (RABV; family *Rhabdoviridae*, genus *Lyssavirus*) is a bullet-shaped, single-stranded, negative-sense RNA virus with a 12-kb genome that encodes 5 structural proteins: nucleoprotein (N), phosphoprotein, matrix protein, glycoprotein, and polymerase (*1*). Over the course of its evolutionary history, RABV has established independent transmission cycles in diverse species of mesocarnivores and bats. Rabies disease remains a serious public health concern in several countries of Asia, Africa, and the Americas, where it is estimated that >50,000 fatal infections occur annually (*2*).

In Latin America, rabies diseases are classified into 2 major epidemiologic forms, urban rabies and sylvatic rabies. For the former, dogs are the main viral reservoir host; for the latter, several species of wild carnivores and bats maintain independent rabies enzootics. Because of the widespread control of urban rabies through vaccination of domestic dogs, the common vampire bat (*Desmodus rotundus*) has emerged as the principal RABV reservoir host along the species' natural range from Mexico to South America (*3*,*4*). The transmission and maintenance of RABV in natural populations of *D. rotundus* bats remains poorly understood, particularly within ongoing epizootics and enzootics occurring in different regions of the Americas (*5*,*6*). Active programs for the control of vampire bat–associated rabies in Latin America rely primarily on reduction of vampire bat populations by culling (*7*,*8*). Nonetheless, cross-species transmission to humans and domestic animals persists, even in areas where culling occurs regularly.

In Peru and other countries within the Amazon rainforest region, RABV transmitted by vampire bats has acquired greater epidemiologic importance because of the more frequent detection of human rabies outbreaks. This increase may reflect enhanced laboratory-based surveillance; increased awareness among public health stakeholders; or ecologic changes that promote greater contact between bats and humans, such as depletion of vampire bats' natural prey community through hunting or habitat fragmentation. During 2002–2007, a total of 293 (77%) of the rabies cases diagnosed by the Instituto Nacional de Salud in Peru were associated with vampire bat RABV variants; the remaining 87 (23%) were attributed to RABV variants associated with dogs. In communities where vampire bats commonly feed on humans, the frequency of outbreaks depends on the transmission dynamics within the local vampire bat populations (*9*,*10*). Unfortunately, recent outbreaks in native communities of the Amazon region have been poorly characterized because of cultural constraints and local beliefs that have precluded investigators from obtaining diagnostic specimens (*11*).

Molecular epidemiology has been extensively used to determine RABV reservoir hosts in a given region or country, define the geographic distribution of the disease associated with those hosts, infer the temporal and spatial spread of the disease, identify spillover infections to nonreservoir

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DOI: http://dx.doi.org/10.3201/eid1909.130083

species, describe novel RABV variants, and detect putative host shifts (*12*). The spatiotemporal epidemiology and genetic diversity of vampire bat–associated rabies in Peru have not been explored; a laboratory-based investigation conducted in 1999 addressed the comprehensive characterization of RABV in only 2 humans (*11*). Given the increasing importance of vampire bat–associated rabies in the Peruvian Amazon, comprehensive surveys of virus diversity and elucidation of geographic boundaries are needed to clarify the frequency and duration of rabies outbreaks. The goals of our study were to 1) determine the genetic diversity and geographic distribution of RABV infection associated with vampire bats; 2) clarify disease dissemination trends among affected areas; 3) detect the origins of spillover infections to other mammals; and 4) identify novel RABV lineages.

Materials and Methods

Virus Samples

During 2002–2007, decentralized units of the Ministry of Health of Peru collected 157 brain samples from multiple species and geographic regions of Peru (online Technical Appendix Table 1, wwwnc.cdc.gov/EID/article/19/9/13-0083-Techapp1.pdf). Samples were selected on the basis of identification of vampire bat or any other bat-associated rabies virus variant by using a panel of 8 monoclonal antibodies, as described (*12*). The specimens included samples from 98 cows, 26 bats, 12 humans, 9 horses, 5 goats, 2 dogs, 2 donkeys, 1 kinkajou, 1 pig, and 1 sheep. Most samples (n = 118) originated from the departments of Apurimac, Ayacucho, Cusco, Madre de Dios, and Puno, located in the southern region of the country, which is made up of inter-Andean valleys and Amazon rainforest. Twenty-six samples were from the departments of San Martin, Amazonas, Cajamarca, and Lambayeque, located in the northern region, which comprises the Andean mountains and Amazonian forests. The remaining 13 samples were from the departments of Pasco, Huanuco, and Ucayali in the central Amazon. All samples were submitted to the reference laboratory of the Instituto Nacional de Salud for RABV confirmation by fluorescent antibody testing (*13*).

PCR and Sequencing

Total RNA was extracted from each sample after a single passage in mouse brains by using TRIzol (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's specifications. Amplification of the complete N gene was achieved by reverse transcription PCR through 2 overlapping reactions by use of 3 published primers (Lys 001, 550F, and 304) and a modified version of primer 1066degB (*14–16*). The primer sets were used in the following combinations: Lys001, 5′-ACGCTTAACGAMAAA-3′; 1066degB, 5′-TCYCTGAAGAATCTTCTYTC-3′; 550F, 5′-ATGTGYGCTAAYTGGAGYAC-3′; and 304, 5′-TTGACGAAGATCTTGCTCAT-3′ (*14–16*). PCR products were visualized on 1.5% agarose gels, and expected size amplicons were purified by using ExoSAP-IT (USB Products Affymetrix, Inc., Cleveland, OH, USA). Cycle sequencing reactions were conducted by using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions. Products were analyzed on an ABI 3730 DNA analyzer (Applied Biosystems, Grand Island, NY, USA). Chromatograms were edited by using BioEdit (*17*), and sequences were assembled by using the fixed RABV SADB19 (GenBank accession no. M31046) as a template (*18*). Multiple alignments were attained by using ClustalW (*19*)

Phylogenetic Analysis

For phylogenetic reconstructions, we retrieved complete and partial RABV sequences from GenBank that represented historical and ongoing rabies epizootics in the Americas (online Technical Appendix Table 2). Other *Lyssavirus* species, such as European bat lyssavirus (EBLV) 1 (U22845) and EBLV-2 (U22846), were included as outgroups (*20*). Phylogenetic reconstructions using complete N gene sequences of the 157 isolates from Peru and 83 from GenBank were generated by using the neighbor-joining (NJ) method in MEGA 4.0 (*21*), assuming the maximum composite likelihood nucleotide substitution model. The statistical significance of branch partitions was assessed with 1,000 bootstrap replicates. We also estimated a time-scaled phylogenetic tree for the dataset comprising RABVs associated with *D. rotundus* (154 isolates from Peru and 58 from GenBank) by using BEAST version 1.7 (*22*), which uses a Bayesian coalescent framework to estimate evolutionary parameters from many possible genealogies through Markov chain Monte Carlo sampling. Our analysis used the Bayesian skyline model of population growth as a flexible demographic prior and the relaxed lognormal molecular clock to allow for rate variation among branches of the tree. Substitution models for coding positions 1+2 (CP12) and CP3 were unlinked, and substitution models in each coding position were selected by Akaike Information Criterion in jModeltest (*23*). The general time reversible model, including invariant sites and Γ distributed site heterogeneity, was applied to CP12, and time reversible model + Γ was applied to CP3. Four replicate Markov chain Monte Carlo analyses were run for 60 million generations each and combined for final estimates and construction of the maximum-clade credibility tree. Convergence across runs, appropriate burn-in periods, and effective sample sizes >200 were assessed by using Tracer (http://beast.bio.ed.ac.uk/Tracer).

Results

Phylogeny of RABV Isolates

Complete N gene sequences (1,350 nt, excluding the stop codon) were obtained from 157 specimens from humans, domesticated animals, and wildlife from 12 of the 24 departments of Peru (online Technical Appendix Table 1). Pairwise similarity ranged from 85.9% to 100%, with an average pairwise identity of 97.3%. The NJ phylogenetic analysis demonstrated 2 major RABV clusters, 1 associated with *D. rotundus* bats and 1 associated with insectivorous bats. The *D. rotundus* cluster was subsequently subdivided into 4 lineages, I–IV, each with a distinctive geographic distribution within Peru; the RABVs associated with insectivorous bats segregated into 3 independent RABV lineages not previously reported in Peru (Figure 1).

Sequences within lineage I showed a widespread spatiotemporal distribution. Isolates were obtained from the departments of Amazonas, San Martin, Cajamarca, Huanuco, Ucayali, Pasco, Ayacucho, Cusco, and Madre de Dios. Inclusion of the reference sequences from GenBank revealed that lineage I had an extended spatiotemporal distribution over northern regions of South America, encompassing Ecuador and Colombia, during 1997–2007 (Figure 1) (*24*). Conversely, isolates in lineage II were detected predominately during a human rabies outbreak in the Madre de Dios and Puno departments in 2007. This lineage also grouped with an isolate from a sample found in the Cusco department in 2003 (GenBank accession no. JX648444) and with several RABV sequences reported in Brazil and Uruguay during 2004–2008 (*24*). These findings indicate that lineage II has been circulating within a larger geographic scale, perhaps reflecting virus dispersion across the Amazon region and southern South America (Figures 1). Two isolates (GenBank accession nos. JX648544 and JX648543) grouped into lineage III as an independent cluster unrelated to any previously described RABV (Figure 1). These samples were collected in 2006 from the Pozuzo district, which is located in the eastern side of the Pasco department in the central Peruvian Amazon.

Lineage IV was the most frequently identified lineage among the isolates collected in Peru, encompassing 98 of the 157 isolates captured during 2002–2007. These results indicate this lineage's high prevalence in cattle in the Andes. Its geographic distribution comprised the valleys of Ayacucho and Apurimac, located at 1,200–3,500 m above sea level and extended into Cusco and north into San Martin, Lambayeque, and northern Colombia. Although this

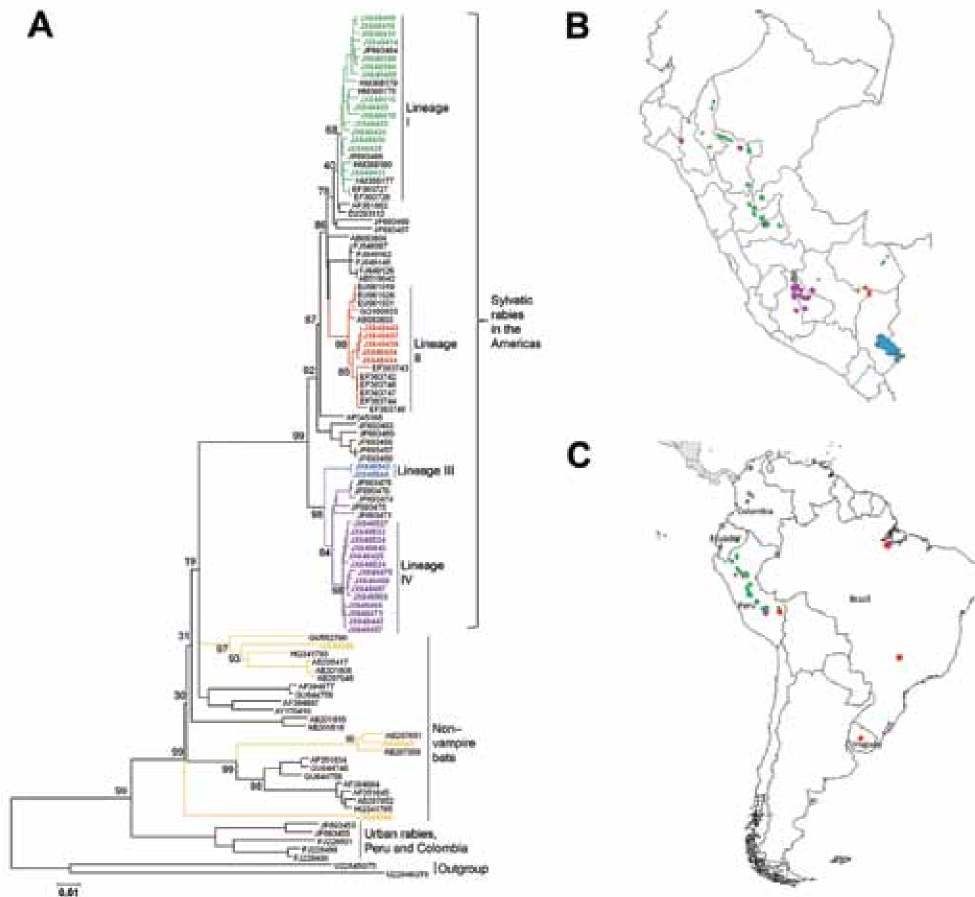

Figure 1. Phylogenetic and geographic comparisons of rabies virus isolates collected in Peru during 2002–2007 with representative rabies viruses circulating in South America. A) Phylogenetic tree showing relationships among virus isolates; B) locations from which viruses were isolated in Peru and South America. Colors indicate isolates from this study: green, lineage I; red, lineage II; blue, lineage III; purple, lineage IV. Gold indicates the 3 isolates collected in Peru from non–vampire bats. Additional lineage I isolates were found in Ecuador and Colombia; lineage II, Brazil and Uruguay; and lineage IV, Colombia. GenBank accession numbers are indicated. Scale bar indicates nucleotide substitutions per site.

lineage was predominately collected from livestock, it was also obtained from vampire bats (n = 24).

Evolution of Vampire Bat–Associated RABV in Peru

By applying a Bayesian coalescent analysis to 212 serially sampled partial N sequences (1,275-bp), we inferred the time scale of RABV evolution in lineages associated with vampire bats. Consistent with previous estimates, the median rate of nucleotide substitution of vampire bat–associated RABV was 9.76×10^{-4} substitutions per site per year (95% highest posterior density [HPD] 6.81×10^{-4} to 1.3×10^{-3}). These results would place the most recent common ancestor (MRCA) of contemporary vampire bat–associated RABVs as occurring in Peru in 1933 (95% HPD 1889–1962) (*25*). The maximum clade credibility tree (Figure 2) demonstrated similar topology to the NJ tree (Figure 1) when broader datasets were used, with vampire bat–associated RABVs differentiated into 4 phylogenetic lineages (Figure 2). As in the NJ trees, a deep division at the MRCA of vampire bat–associated RABVs separated lineages I and II from lineages III and IV (posterior probability [PP] 1.0). Major lineages appear to have been circulating for similar periods in Peru, each originating 33–44 years ago (when including the stem branch leading to current viral diversity), with extensive overlap of the 95% HPDs of the time since the MRCA for each lineage. Each RABV lineage in Peru except lineage III shared common ancestors with viruses circulating in other Latin American countries, indicating multiple viral dispersion events into or out of Peru; however, overlap of the 95% HPDs on the age of samples from Peru compared with those from other countries limited direct inference on the directionality of movement between countries. Within lineage I, samples from Ecuador and Colombia were interspersed with contemporary samples from Peru, which suggests a relatively recent spatial spread among countries. In addition, historical introductions of a similar RABV were indicated by strong posterior support (PP 0.99) for an MRCA between lineage I and samples from Colombia, Trinidad, and French Guyana in about 1973. Isolates related to lineage II were detected in Brazil and Uruguay; however, strong spatiotemporal clustering apparently separated distinct epizootics in Brazil in 2004 and Uruguay and Brazil in 2007–2008 from the human outbreak in southern Peru in 2007. A sample from a cow collected in 2003 in Peru was ancestral to samples from the 2007 human outbreak in Peru (PP 1), rather than grouping with the more contemporaneous viruses circulating in Brazil in 2004, indicating that this virus may have circulated in Peru for ≥4 years before the 2007 outbreak.

As in the NJ tree, lineage III was most closely related to lineage IV (PP 0.99) but was highly divergent, sharing an MRCA in 1963 (95% HPD 1940–1979). The large genetic distance from other lineages indicates that these sporadic cases were not recently introduced from other RABV lineages circulating elsewhere in Peru but rather were part of a previously unknown vampire bat–associated rabies enzootic. No samples from other countries clustered with the lineage IV samples from Peru, suggesting that this

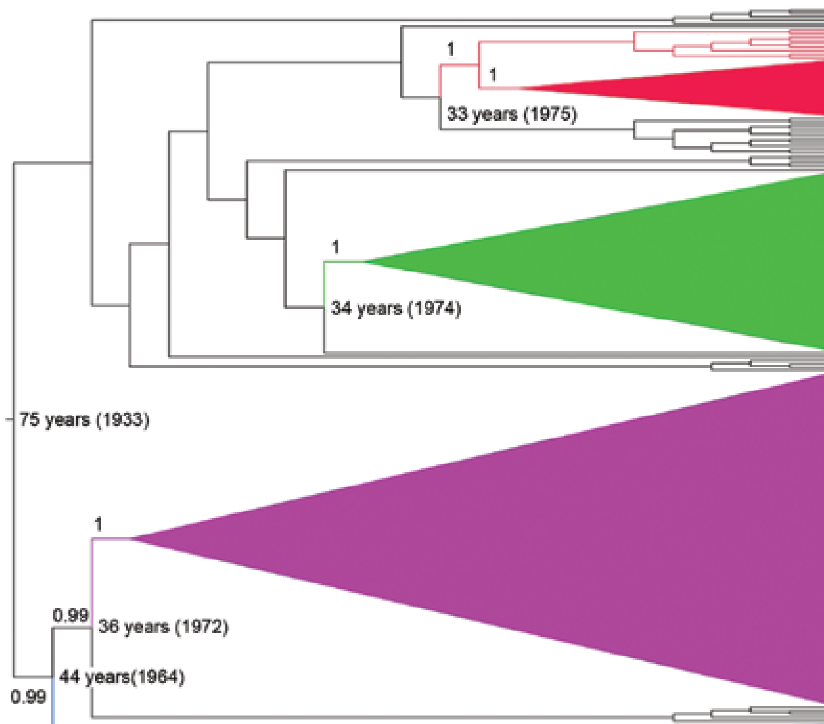

Figure 2. Phylogenetic tree generated by using Bayesian analysis for a 1,274-nt portion of the gene-coding sequences of rabies virus isolates collected in Peru during 2002–2007. Red, lineage II (found in Peru, Brazil, and Uruguay); green, lineage I (found in Peru, Ecuador, and Colombia); purple, lineage IV (found in Peru and Colombia); blue, lineage III (found in Peru). Posterior probabilities and lineage ages are shown for all major nodes.

virus has been maintained independently as a widespread rabies focus that covers the inter-Andean valleys (Ayacucho, Apurimac) and the rainforest of northern Peru (San Martin). The closest relatives of lineage IV were viruses collected in Colombia during 1994–2008, which diverged from the samples from Peru around 1972 (95% HPD 1957–1984), consistent with the enzootic maintenance of rabies over long periods within Peru.

Novel RABV Associated with Other Wildlife in Peru

We identified 3 novel RABV variants in wildlife other than vampire bats in the southeastern region of Peru. The first variant (GenBank accession no. JX648546) was isolated from a kinkajou (*Potus flavus*) in Madre de Dios in the Amazon rainforest in southern Peru. This variant was not closely related to any previously described RABV but grouped within the larger diversity of bat-associated RABVs in the Americas (Figure 1). A second RABV variant (GenBank accession no. JX648545) was isolated from a small big-eared brown bat (*Histiotus montanus*) in Puno in southern Peru. This variant was related to RABVs found in bats of the genera *Histiotus, Nyctinomops*, and *Tadarida* from Chile and Brazil but appears to be an independent lineage; its branch length and pairwise average divergence of 5% separate it from its closest relatives. A third variant (GenBank accession no. JX648547) was found during 2008 in Paucartambo, Cusco, which is located in an inter-Andean valley at 2,900 m altitude. This sample clustered with sequences from unidentified bats from Brazil (GenBank accession nos. AB297651 and AB297656). Unfortunately, the bat from which this sample was obtained was not available for taxonomic identification.

Discussion

Rabies epidemiology has experienced dramatic changes in Latin America during the past 4 decades because of the implementation of highly effective strategies for prevention and control of infection in dogs and the procurement of adequate postexposure prophylaxis for humans. In 2003, human rabies cases transmitted by bats outnumbered cases transmitted by dogs in Latin America (*4*), and that trend has continued. The increasing detection of RABV infection in humans in the Peruvian Amazon and the persistence of vampire bat–transmitted RABV infection in livestock highlight the need to clarify the diversity of RABV lineages circulating in Peru and the spatiotemporal dynamics of RABVs associated with vampire bats. We completed phylogenetic analysis of bat-associated RABVs collected in Peru, using samples collected from rabies-endemic areas in the Andes, during sporadic human outbreaks in the Amazon, and from previously unsurveyed wildlife host species. Our study revealed that at least 4 phylogenetic lineages of RABV are circulating in vampire bat populations in Peru; these lineages appeared to display distinctive spatiotemporal dynamics across their geographic ranges. Three of the lineages had wide geographic distributions in Peru and recent and historical relationships linked to rabies outbreaks occurring in other parts of South America (*24*,*26*–*32*). Dissemination of vampire bat–associated RABV appears to be gradual rather than involving long-distance dispersal events, as might be expected by the absence of long-distance migration and small home range of the reservoir species (*33*,*34*). Spatiotemporal analysis of lineage I, II, and IV RABVs showed that the ample distribution ranges were covered over periods no shorter than 3–4 decades. The specific movement of vampire bat–associated RABVs is difficult to assess, but the phylogenetic and evolutionary analyses we conducted indicate that lineages I and IV spread from north to south, whereas lineage II spread from south to north. Lineage III had restricted distribution in central Peru, which suggests it was part of a long-term vampire rabies enzootic that disappeared from Peru around 2006. Hence, in contrast to lineages I and IV, the local dynamics for lineage 3 were epizootic rather than enzootic. Understanding factors linked to the limited geographic distribution and apparent extinction of lineage III are important for preparing improved prevention and control practices.

Vampire bats are not a migratory species and usually inhabit places below 1,800-m altitude. Nonetheless, they may occasionally move relatively long distances and inhabit higher altitudes in response to limited food or roost availability. Movement encouraged by food supplementation may be illustrated by the distribution dynamics observed for lineage IV, which currently is mainly found along the inter-Andean valleys, an important cattle raising area in Per, which has an average altitude >2,000 m (*35*). Our data suggest that the incursion of lineage IV into the inter-Andean valleys is relatively recent (30–40 years ago) and probably occurred from northern lower lands, consistent with the likely ancestors of this lineage coming from Colombia and Ecuador (Figure 2). Because of the detrimental economic effects of vampire bat–associated rabies in the livestock industry in this region, in 2010, the government of Peru initiated intense control and prevention measures that included culling vampire bats. However, the frequency of rabies cases in livestock has been unaffected (*6*).

Our study showed that different RABV lineages may overlap temporally and geographically, which indicates that, within a rabies enzootic region, convergence or co-circulation of ≥1 RABV lineage may occur, perhaps in association with the maintenance of independent rabies foci by distinct vampire bat metapopulations. This observation could affect effective planning of prevention and control strategies because 1 focal point might be vulnerable to rabies reintroduction from adjacent foci, a process that could explain the persistence of the disease. Studies of population structure, gene flow, and dispersal of vampire bats

within Peru and throughout the South America are necessary for corroborating observations on the dissemination dynamics of rabies associated with this species.

Although it was not the intent of this study to identify the role of rabies transmission and maintenance among species other than vampire bats, we circumstantially discovered 3 potentially novel RABV lineages in non–vampire bat hosts. This finding stresses the potential emergence of novel RABV reservoirs in the country and the need for enhanced surveillance for lyssaviruses in potential wild animal reservoirs. In Peru, the surveillance system for the detection and monitoring of human rabies cases associated with bats and other wild animals is passive; that is, cases are recorded only as they are reported. Operationally, the system is less than ideal because, even though most clinical cases of rabies in humans may be recorded, few are laboratory confirmed; consequently, the RABV variants associated with them are not typed. Rabies associated with insectivorous bats is commonly encountered in countries such as the United States, where 1 or 2 cases of rabies occur in humans each year (*36*). A bat rabies surveillance system such as the one in place in the United States, which tests >20,000 bats and confirms ≈1,400 infections each year, relies heavily on submissions of sick or dead bats to rabies diagnostic facilities from the general public (*36*). This public participation in the process has been augmented by active educational programs that emphasize the potential risk for rabies transmission from bats to humans, pets, and livestock. Human rabies associated with insectivorous bats has been reported in other countries in Latin America, such as Chile and Mexico (*37*,*38*), but the role of these bats in rabies transmission to humans is largely unknown in Peru. Therefore, better understanding of these transmission cycles and better programs for the taxonomic identification of bats with rabies should be implemented.

We also identified RABV in a kinkajou; this strain that was not closely related to any known RABV. We could not determine whether this animal represented a single spillover infection from a previously unknown bat reservoir or an emerging host shift with ongoing transmission within kinkajous. Kinkajous are in the same taxonomic family (*Procyonidae*) as raccoons (*Procyon lotor*) (*39*), which are a well-established rabies reservoir in North America. This relationship suggests that kinkajou could serve as an emerging RABV reservoir if the traits that enable the establishment of RABV reservoirs are conserved along the phylogeny of procyonids. Serologic surveys and enhanced surveillance would be useful for further exploring this possibility.

In conclusion, our study demonstrates the presence of diverse RABV lineages associated with vampire bats and several other species in Peru. Although our research was limited by the restrictions of passive surveillance data, RABV lineages in vampire bats appear to show distinct spatiotemporal patterns, with 2 lineages that were abundant and widely distributed throughout the study period and 2 others that occurred more sporadically, consistent with enzootic and epizootic dynamics. Further discrimination of transmission cycles and their drivers will be crucial for prediction of the frequency of outbreaks in humans and domestic animals and, ultimately, for the design of informed strategies for rabies control in this region.

Acknowledgments

We thank Charles Rupprecht and Sergio Recuenco for their comments and suggestions on the manuscript, Cecilia Otero and Rebecca Alvarado and the American Fellows program for sponsoring R.E.C-C.'s visit to the Centers for Disease Control and Prevention, and the rabies team of the Instituto Nacional de Salud (Ricardo Lopez, Albina Diaz, Margarita Fernandez and Alejandro Arenas). We also thank all colleagues working in the national network of public health laboratories and personnel of the zoonosis program of Direcciones Regionales de Salud of the Ministry of Health in Peru for their valuable work in rabies surveillance.

This study was sponsored by the Centers for Disease Control and Prevention, Atlanta GA, USA; Instituto Nacional de Salud in Lima, Peru; and the American Fellows program, Partners of the Americas of the USA government.

Mr Condori is a guest researcher at the Centers for Disease Control and Prevention and has 10 years of experience in rabies diagnosis and molecular typing. He has a special interest in molecular epidemiology and ecology of rabies.

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Use of Staged Molecular Analysis to Determine Causes of Unexplained Central Nervous System Infections

Chien-Chin Hsu, Rafal Tokarz, Thomas Briese, Hung-Chin Tsai, Phenix-Lan Quan, and W. Ian Lipkin

No agent is implicated in most central nervous system (CNS) infections. To investigate cerebrospinal fluid samples from patients with CNS infections of unknown cause in 1 hospital in Taiwan, we used a staged molecular approach, incorporating techniques including multiplex MassTag PCR, 16S rRNA PCR, DNA microarray, and high-throughput pyrosequencing. We determined the infectious agent for 31 (24%) of 131 previously negative samples. Candidate pathogens were identified for 25 (27%) of 94 unexplained meningitis cases and 6 (16%) of 37 unexplained encephalitis cases. Epstein-Barr virus (18 infections) accounted for most of the identified agents in unexplained meningitis cases, followed by *Escherichia coli* (5), enterovirus (2), human herpesvirus 2 (1), and *Mycobacterium tuberculosis*. Herpesviruses were identified in samples from patients with unexplained encephalitis cases, including varicella-zoster virus (3 infections), human herpesvirus 1 (2), and cytomegalovirus (1). Our study confirms the power of multiplex MassTag PCR as a rapid diagnostic tool for identifying pathogens causing unexplained CNS infections.

Central nervous system (CNS) infections pose a diagnostic challenge because clinical manifestations are not typically pathognomonic for specific pathogens, and a wide range of agents can be causative. An infectious cause of encephalitis is determined for <40%–70% of cases worldwide (*1–5*). Culture is of limited use, particularly for viral infections. In recent studies, only 1.9% of cerebrospinal fluid (CSF) viral cultures were positive (*3*), and <0.1% of CSF cultures recovered viruses other than enteroviruses or herpesviruses (*6*).

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DOI: http://dx.doi.org/10.3201/eid1909.130474

PCR enables sensitive detection of microbial nucleic acids in clinical samples, which may be useful for identifying pathogens that are nonviable, uncultivable, or fastidious. MassTag PCR is a multiplex platform that enables inexpensive, sensitive, and simultaneous detection of multiple pathogens (*7–10*). Originally implemented for differential diagnosis of respiratory tract infections (*7*), MassTag PCR has been expanded to several syndrome-based panels for differential diagnosis of hemorrhagic fever and for detection of tick-borne pathogens (*9*,*11*).

Amplification and sequencing of the 16S ribosomal RNA (rRNA) gene is a well-established technique for identifying bacterial pathogens (*12*). Broad-range 16S rRNA PCR with subsequent sequencing is superior to bacterial culture for diagnosing bacterial meningitis, with a sensitivity of 86% and a specificity of 97% (*13*). It is particularly useful for slow-growing bacteria (e.g., *Mycobacterium tuberculosis*) and for diagnosis of cases that remain culture-negative as a result of antimicrobial drug treatment before lumbar puncture.

Microarray technology also has been applied to the detection and identification of infectious diseases (*10*,*14*) and has the potential to test for virtually all known viruses, bacteria, fungi, and parasites (*15*).The advent of high-throughput sequencing affords unique opportunities for pathogen surveillance and discovery with regard to CNS infections. We have successfully used high-throughput sequencing to identify causative agents of disease for patients with transplant-associated encephalopathy (*16*) and encephalitis associated with agammaglobulinemia (*17*).

Our staged molecular approach used complementary tools for pathogen detection and discovery that include syndrome-based multiplex PCRs, DNA microarray, and

high-throughput sequencing (*18*). We report the results of our investigation of CNS infections of unknown cause in 1 hospital in Taiwan, which we conducted by using MassTag PCR, 16S rRNA PCR, DNA microarray, and high-throughput sequencing.

Methods

Patients

Meningitis was diagnosed by clinical features of fever (≥38°C), headache, nuchal rigidity, abnormal CSF profile (CSF protein >45 mg/dL [reference 15–45 mg/dL] and leukocyte >5/mL [reference 0–5/mL]), and/or meningeal involvement noted by brain magnetic resonance imaging (MRI). Encephalitis was diagnosed when there was evidence of brain parenchyma involvement (e.g., paralysis or focal or generalized seizure) and when the finding of brain MRI or electroencephalogram (EEG) was compatible with encephalitis. CSF samples from meningitis or encephalitis patients with noninfectious diagnosis, including traumatic, metabolic, malignant, vascular, surgical, hypoxic, and toxic causes, were excluded.

CSF Samples

A total of 212 CSF samples from 212 patients (165 meningitis and 47 encephalitis cases) were obtained during 2006–2008 in Kaohsiung Veterans General Hospital, a tertiary care hospital in southern Taiwan. Tests conducted in the hospital included bacterial and viral culture, cryptococcal antigen test, VDRL (Venereal Disease Research Laboratory) test and human herpesvirus (HHV) 1 PCR. Pathogens were identified for 71 meningitis patients and included*Cryptococcus neoformans* (36 cases), *M. tuberculosis* (18), *Klebsiella pneumonia* (7), enterovirus (4), *Streptococous pneumoniae* (2), *Listeria monocytogenes* (2), and *S. viridians* (2). Pathogens were identified for 6 encephalitis patients and includedJapanese encephalitis virus (3 cases) and herpesviruses (3). The remaining 131 patients with unexplained meningitis or encephalitis with CSF pleocytosis and without a definitive diagnosis were included in this study. CSF samples were stored at –70°C until analysis.

Sample Preparation

Total nucleic acids were extracted from 250-µL CSF samples by the NucliSENS Easy Mag Extraction Method (bioMérieux, Marcy l'Etoile, France) and eluted in 35 mL nuclease-free water. We performed reverse transcription (RT) PCR for human b-actin gene to ensure the quality of the extracted DNA and RNA. For RT, total nucleic acids were enriched for RNA with a DNase I treatment (DNA-free; Ambion, Austin, TX, USA). RT was performed by using random hexamers with the Invitrogen Superscript II Kit (Invitrogen, Carlsbad, CA, USA).

MassTag PCR

MassTag PCR is a platform that enables inexpensive, sensitive, and simultaneous detection of multiple pathogens (*7–10*). In this study, we used a MassTag PCR platform that targets 29 known CNS pathogens, including 21 viruses, 5 bacteria, 2 fungi, and 1 parasite. Detailed gene targets and primer sequences are listed in Tables 1 and 2. We first tested unmodified (i.e., untagged) primer sets in singleplex reactions for specificity with the cognate and negative control targets. All primers were then used in multiplex PCR to test for interference. Primers yielding a single and specific PCR product band on agarose gel electrophoresis were taken forward for synthesis and conjugation with tags of varying mass. These primers were included in the panel. Target DNA standards for panel development were cloned into pCR2.1 TOPO (Invitrogen) by PCR amplification of pathogen DNA or cDNA templates (RNA viral targets). A sensitivity assay was performed with 10-fold dilutions of linearized plasmid against a background of 5 ng/mL of human placental DNA.

For the DNA agent test panel, total nucleic acids (4 mL) were added to MassTag PCR reaction mixes containing the DNA pathogens primer mix and were amplified by a standard cycling protocol, as follows: denaturation at 94°C for 20 s, annealing with a temperature reduction in 1°C increments from 65°C to 51°C during the first 14 cycles and then continuing for 35 cycles at 50°C for 20 s, then extension 72°C for 30 s in an MJ PTC200 thermal cycler (MJ Research, Waltham, MA, USA). For the RNA agent test panel, 4 mL of RT products were added to MassTag PCR reactions containing the RNA pathogens primer mix and were amplified by the same protocol.

PCR products were purified by using QIAquick 96 PCR purification cartridges (QIAGEN, Hilden, Germany) to remove unincorporated primers before the tags were released from PCR products by UV irradiation in a flow cell. Tags were analyzed in a single quadrupole mass spectrometer by using positive-mode atmospheric-pressure chemical ionization (Agilent Technologies, Wilmington, DE, USA) (*7*). The identity of a pathogen in the CSF was determined by the presence of its cognate tags. The negative controls consisted of irrelevant DNA. A positive sample was defined as a sample in which an agent was detected with both tags giving signal above a threshold defined as the 95th percentile cut point of the negative control distribution, and positivity was assessed by using the interquartile ranges of this distribution.

16S rRNA Gene PCR

For samples that were negative by MassTag PCR, we performed broad-range bacterial 16S rRNA PCRs to detect bacterial sequences not addressed in the MassTag panel. To minimize potential for spurious amplification of 16S rRNA

Table 1. Primers for MassTag central nervous system infections panel, RNA pathogens

Pathogen	Target gene	Primer sequence, 5′→3′*	Mass code
Eastern equine encephalitis virus	E1	Fwd: ACACTAAATTCACCCTAGTTCGAT Rev: GTGTATAAAATTACTTAGGAGCAGCATTATG	383/650
Nipah/Hendra virus	Phos	Fwd: GGGGGAATGYCTAAGRATGATG Rev: TCCGGTACATTCTCCTCCATG	519/566
Japanese encephalitis virus	NS5	Fwd: TCAACCTAGGGAGCGGAACA Rev: GGCTGAGCCAGTAGCCTTCA	582/698
Parechovirus	5UTR	Fwd: ACACTAGTTGTAAGGCCCACGAA Rev: GGTBTGGCCCACTAGACGTTTT	690/606
Powassan virus	NS5	Fwd: CATCCGACCATGCACCTAGA Rev: CCAAAGTGAGGATGTGTACCAAAG	622/375
La Crosse virus	S	Fwd: CTCAACCTTGCTGCAGTTAGGA Rev: CCACCTGCCACTCTCCAAA	686/590
Lymphocytic choriomeningitis virus	pol	Fwd: CCACTYTTGTCTGCACTGTCTAT Rev: CTTTTTGATGCGCAATGGAT	614/654
St. Louis encephalitis virus	NS5	Fwd: CATTTGTTCAGCTGTCCCAGTC Rev: CTCACCCTTCCCATGAATTGA	658/423
Enteroviruses	5UTR	Fwd: TCCTCCGGCCCCTGAATGCGGCTAATCC Rev: GAAACACGGWCACCAAAGTASTCG	495/702
West Nile virus	DF3	Fwd: CCACCGGAAGTTGAGTAGACG Rev: GCTTTGTTCACCCAGTCCTCCT	499/539
Western equine encephalitis virus	E1	Fwd: ACATCGAGCCCACAAGCA Rev: GCATAGAGCTGCAGACCAACAC	598/678
Venezuelan equine encephalitis virus	E1	Fwd: CTACGCGCCACTCCCTATCA Rev: TGGCAGGTGACGTACTCCAA	602/646
Rabies virus	N	Fwd: GGGTTYATAAAVCAGATWAATCTCAC Rev: GAAGTGRATGAAATARGAGTGAGG	475/558
Influenza A virus	M	Fwd: CATGGAATGGCTAAAGACAAGACC Rev: AAGTGCACCAGCAGAATAACTGAG	618/690

*Fwd, forward primer; Rev, reverse primer.

sequences in reagents, we adopted the DNase I (Ambion DNA-free; Austin, TX, USA) pretreatment as described in Heininger et al. (*19*) to the PCR master mix, including the incubation of AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA, USA), $MgCl_2$, PCR 10× buffer and dNTPs with 0.1 IU DNase I for 30 min at 37°C, then for 10 min at 95°C. Primers and templates were added subsequently. 16S rRNA was amplified (899-bp length) with 2 universal primers, 8UA (5′-AGAGTTTGATCCTGGCTCAG-3′) and 907B (5′-CCGTCAATTCMTTTAGTTT-3′) (*20*). Cycling conditions were 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, 45°C for 30 s, and 72°C for 60 s, with a final elongation at 72°C for 5 min.

DNA Microarray

Sample Preparation

RNA was enriched from total nucleic acids by DNase I treatment (DNA-free; Ambion, Austin, TX, USA) to eliminate human chromosomal DNA. First-strand RT was initiated by using Superscript II (Invitrogen) with a random octamer linked to a specific primer sequence (5′-GTT TCC CAG TAG GTC TCN NNN NNN N-3′) (*14*). After digestion with RNase H (Invitrogen), the cDNA was amplified by using a 1:9 mixture of the random octamer–linked primer and the specific primer sequence (5′-CGC CGT TTC CCA GTA GGT CTC-3′). A low annealing temperature (25°C) was used for the initial PCR amplification cycle; a stringent annealing temperature (55°C) was used thereafter to favor priming through the specific sequence (*15*). The products of this first PCR (18 cycles) were subsequently labeled by a primer that included the specific primer sequence linked to a capture sequence (49 cycles) that, after array hybridization, permitted the labeling through binding of 3 DNA dendrimers containing >300 fluorescent reporter molecules (Genisphere Inc., Hatfield, PA, USA).

Microarray Hybridization and Processing

Hybridization was performed by adding 30 mL of sodium dodecyl sulfate-based hybridization buffer (Genisphere Inc.) to the products of the second-labeling PCR, heating for 10 min at 80°C, and transferring the solution to the Greenchip Vir microarray (*15*) for hybridization for 16 hours at 65°C. The arrays were washed with 6X SSC (0.15 M NaCL plus 0.015 M sodium citrate), 0.005% Triton X-100, and 0.1´ SSC-0.005% Triton X-100 for 10 min at room temperature. Cy3 3DNA dendrimers (Genisphere Inc.) were then added for a secondary hybridization at 65°C for 1 hour before a final wash (*15*).

Microarray Scanning and Analysis

The microarrays were scanned by the NimbleGen MS 200 Microarray scanner (Roche NimbleGen, Madison, WI, USA), and analyzed with GreeneLAMP software (version 2.0) (*15*). A ranked list of candidate organisms

Table 2. Primers for MassTag CNS infections panel, DNA pathogens

Pathogen	Target gene	Primer sequence, 5′→3′*	Mass code
Virus			
Adenoviruses	Hexon	Fwd: CCCMTTYAACCACCACCG Rev: ACATCCTTBCKGAAGTTCCA	503/630
Cytomegalovirus	Pol	Fwd: CATGCGCGAGTGTCAAGAC Rev: ACTTTGAGYGCCATCTGTTCCT	610/626
Epstein-Barr virus	EBER	Fwd: AAACCTCAGGACCTACGCTGC Rev: AGACACCGTCCTCACCAC	570/463
Varicella-zoster virus	Gp 31	Fwd: CCGATTCTGGATTTTCGTTGTT Rev: AAAGTCGATTTCCCCCCAAA	471/515
Human herpesvirus 6	U7	Fwd: AAAATTTCTCACGCCGGTATTC Rev: CCTGCAGACCGTTCGTCAA	357/718
Human herpesvirus 1	Gp C1	Fwd: GATGCCGGTTTCGGAATTC Rev: CCCATGGAGTAACGCCATATCT	706/666
Human herpesvirus 2	UL3	Fwd: GGTCCCCTCTGCGTTTACTA Rev: TCGACTCTATGGGCGTCGTA	527/642
Bacterium			
Haemophilus influenzae	hgbC	Fwd: CGCTGGAAAGAGAACAAGCAA Rev: TTTCAGCTTGACGTAATCCATC	726/734
Streptococcus pneumoniae	plysin	Fwd: GACTCCTAAGGCTTGGGACAGAAAT Rev: TTCATAAACCGTACGCCACCATTC	694/714
Neisseria meningitides	ctrA	Fwd: TTCTGATGCGCGTGGTGTGT Rev: CGCATCAGCCATATTCACACGA	439/730
Leptospira interrogans	flaB	Fwd: GATCATGAAGCAGAGRGCGGATATG Rev: CCATATCGGCGTCYCGAATTC	634/383
Mycobacterium tuberculosis	pncA	Fwd: ACGTCAGGCCCACGACATTGA Rev: CCTGGGCAAGCTGAACCTCGAA	395/475
Parasite: *Toxoplasma gondii*	B1	Fwd: GAAGAGATCCAGCAGATCTCGT Rev: TGAGAGGAGGCAGCACAAG	548/562
Fungus			
Candida albicans	CaAG	Fwd: ACCAGTAGGAGTACAACGAACAGGAA Rev: ATTTCATTGAATATTGGTGTGGTTCA	602/670
Cryptococcus neoformans	cap59	Fwd: GCGAGGCAGCACAAGTACTT Rev: TTGTCTGGTCGTTGGAMCCGTT	650/638

*Fwd, forward primer; Rev, reverse primer.

was identified by linking probe sequences with positive signal on the array to the matching sequences in a viral sequence database; each of which corresponded to a taxonomic identifier (NCBI Taxon ID, TaxID). The individual TaxIDs were finally mapped to nodes in a phylogenetic tree that was constructed in accordance with data from the International Committee on Taxonomy of Viruses.

Pyrosequencing

The 100 samples for which no agent was identified after MassTag PCR and array analysis were pooled and analyzed by high-throughput pyrosequencing. The same sample preparation protocol as for array analysis up to the second-labeling PCR was used to amplify material for high-throughput sequencing analysis. Products >70 bp long were purified by using a MinElute kit (QIAGEN, Valencia, CA, USA). Purified products were pooled and sequenced on the GSL FLX platform (454 Life Sciences, Branford, CT, USA). Raw sequence reads were trimmed to remove sequences primer and highly repetitive sequences. In a second step, reads were clustered and assembled into contiguous fragments for comparison with the GenBank database by the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi) by using nucleotide and deduced amino acid sequence (*21*).

Confirmation of Pathogens

We confirmed the identify of pathogens using several methods. All pathogens indicated by MassTag PCR, microarray, and pyrosequencing were confirmed by targeted singleplex PCR amplification, sequencing of the products, and BLAST analysis of the obtained sequences.

Serology Analysis

For Epstein-Barr virus (EBV) DNA–positive CSF samples, we measured EBV antibodies by using the commercial kit Euroimmun (Medizinische Labordiagnostika, Lübeck, Germany), which tests for IgG and IgM against EBV viral capsid antigen (VCA), EBV early antigen, and nuclear antigen. Results were evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient samples over the extinction value of calibrator (ratio <0.8: negative; ratio ≥1.1: positive).

Results

A total of 131 CSF samples were collected during 2006–2008 from adults with unexplained meningitis or encephalitis. The patients in this study were 18–86 years of age (median 39 years); the male-to-female ratio was 2.4:1. Fourteen percent of patients had diabetes, and 8%

were HIV positive. In addition to fever and headache, mental status was abnormal for 22% of patients; 2% had paralysis/seizure.

Two patients with unexplained encephalitis died. Samples were analyzed by applying a staged molecular approach, including multiplex MassTag PCR, 16S rRNA-gene PCR, DNA microarray, and high-throughput sequencing (Figure). We identified 31 (24%) pathogens in 131 CSF samples from patients with meningitis and encephalitis of unknown cause. Candidate pathogens were identified for 25 (27%) of 94 patients with unexplained meningitis and 6 (16%) of 37 with unexplained encephalitis (Table 3). EBV (16 cases) accounted for most of the identified pathogens in unexplained meningitis cases, followed by *Escherichia coli* (5 cases), enterovirus (2), HHV-2 (1), and *M. tuberculosis* (1). Herpesviruses were identified for unexplained encephalitis cases and includedvaricella-zoster virus (3 cases), HHV-1 (2), and cytomegalovirus (1). We also tested 3 CSF samples from patients with noninfectious CNS disease as negative controls; no agents were identified in these samples.

Five bacterial pathogens were identified by 16S rRNA PCR (*E. coli*, 5 patients), and 1 HHV-1 was identified by microarray analysis. We pursued unbiased high-throughput pyrosequencing of RNA from CSF by pooling the remaining negative samples (screened previously by MassTag PCR and microarrays); however, no specific pathogen was identified by pyrosequencing. All positive samples detected by MassTag PCR, 16S rRNA PCR, and microarray were verified by targeted singleplex PCR amplification and sequencing.

Of 16 EBV-positive patients, 9 (56%) were HIV infected. To differentiate EBV latent infection from lytic infection, we further measured EBV-specific antibodies in EBV DNA–positive CSF samples; in 8 cases, paired CSF and serum samples were available for analysis (Table 4). IgG against EBV VCA was found in 11 (69%) of 16 EBV DNA–positive CSF samples; however, none of the CSF samples were positive for VCA IgM, and only 1 CSF sample was positive for both EBV early antigen and nuclear antigen, indicating current or recent EBV infection. All 8 serum samples, which were paired with 8 of the CSF samples, were positive for VCA IgG and EBV nuclear antigen but negative for VCA IgM and EBV early antigen, suggesting that latent EBV infections existed in those patients.

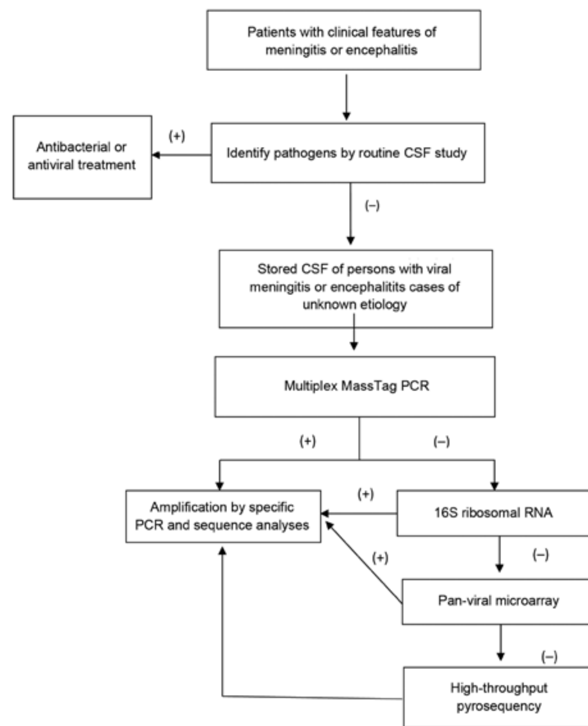

Figure. Molecular approaches used to investigate central nervous system infections of unknown cause. Routine study of CSF comprised chemistry, direct microbial examinations, antigen tests, and bacterial and viral cultures. CSF, cerebrospinal fluid; +, positive; –, negative.

Discussion

We have described results of a comprehensive, staged molecular analysis of unexplained encephalitis

Table 3. Pathogens identified by a staged molecular approach

Pathogen	No. (%) cases, Total	Meningitis	Encephalitis	No. HIV positive	Molecular method
Virus					
Enterovirus*	2 (6)	2 (8)	0	0	MassTag PCR
Human herpesvirus 1	2 (6)	0	2 (33)	0	MassTag PCR/microarray†
Human herpesvirus 2	1 (3)	1 (4)	0	0	MassTag PCR
Varicella-zoster virus	3 (10)	0	3 (50)	0	MassTag PCR
Epstein-Barr virus	16 (52)	16 (64)	0	9	MassTag PCR
Cytomegalovirus	1 (3)	0	1 (17)	0	MassTag PCR
Bacteria					
Mycobacterium tuberculosis	1 (3)	1 (4)	0	0	MassTag PCR
Escherichia coli	5 (16)	5 (20)	0	1	16S rRNA PCR
Total	31 (100)	25 (100)	6 (100)	10	

*Sequences of enteroviruses showed 1 infection each of echovirus 11 and echovirus 30.
†For 1 case, human herpesvirus 1 was identified by MassTag PCR; in the other, by DNA microarray.

Table 4. EBV antibody in CSF and serum of patients identified with EBV infections by MassTag PCR*

	CSF				Serum			
Case	VCA IgM	VCA IgG	EBV EA	EBV NA	VCA IgM	VCA IgG	EBV EA	EBV NA
1	–	–	–	–	–	+	–	+
2	–	–	–	–	–	+	–	+
3	–	–	–	–	ND	ND	ND	ND
4	–	+	–	–	ND	ND	ND	ND
5	–	+	+	+	ND	ND	ND	ND
6	–	+	–	–	–	+	–	+
7	–	+	–	–	–	+	–	+
8	–	+	–	–	ND	ND	ND	ND
9	–	+	–	–	ND	ND	ND	ND
10	–	+	–	–	–	+	–	+
11	–	–	–	–	–	+	–	+
12	–	+	–	–	–	+	–	+
13	–	+	–	–	ND	ND	ND	ND
14	–	+	–	–	ND	ND	ND	ND
15	–	+	–	–	ND	ND	ND	ND
16	–	–	–	–	–	+	–	+

*EBV antibody was measured by using commercial kit Euroimmun (Medizinische Labordiagnostika, Lübeck, Germany). Results were evaluated semiquantitatively by calculating a ratio of the extinction value of the control or patient samples over the extinction value of calibrator. Ratio <0.8: negative; ratio >1.1: positive. EBV, Epstein-Barr virus; CSF, cerebrospinal fluid; VCA, viral capsid antigen; EBV NA, EBV nuclear antigen indicating past EBV infections; EBV EA, EBV early antigen, indicating current or recent EBV infection; –, negative; +, positive; ND, not done.

and meningitis conducted by using CFS samples obtained over a 3-year period in a major referral hospital in Taiwan. Our investigation confirmed the presence of microbial sequences in 31 (24%) of 131 CSF samples; 25 were identified by MassTag PCR.

We detected EBV in 16 samples. EBV is a ubiquitous HHV that infects 90% of adults worldwide (*22*). In Taiwan, too, most persons are infected with EBV in early childhood (*23*). VCA was detected in all EBV-positive CSF samples. This finding is consistent with EBV-related CNS disease. In a study of 5 patients with mononucleosis, all had EBV DNA and EBV-specific antibodies in the CSF during the acute phase of disease in association with neurologic manifestations but not during convalescence (*24*). In contrast, in the same study, EBV DNA and EBV antibodies were not detected in the CSF of 17 EBV-seropositive patients with other CNS infections, such as mumps, meningitis, and rubella encephalitis (*24*).

It could be argued that any inflammatory process has the potential to carry EBV into the CSF as a passenger in infiltrating lymphocytes; however, PCR results for EBV in CSF are rarely positive for patients with other CNS infections (*25*). One study showed that only 11 of 2,233 specimens from 2,162 patients were EBV positive by PCR (*26*). In another study, EBV was not detected by PCR of CSF from patients with bacterial meningitis, despite the presence of more lymphocytes/monocytes in the CSF of control patients with bacterial meningitis than in the CSF of the EBV-positive patients (*27*). In accord with these results, we did not detect EBV in the CSF of patients with CNS infections of bacterial origin, such as *M. tuberculosis* and *E. coli*.

All of the 16 EBV-positive patients in our study had meningitis; none had encephalitis. EBV is an uncommon cause of meningitis (0.9%) (*28*) and of encephalitis (2.3%) (*29*). However, given the data reported here, EBV-associated CNS infections in immunocompromised patients may be underestimated. In our study, 56% of EBV-positive patients were HIV infected. EBV has been reported to be associated with primary CNS lymphomas in HIV-infected persons (*30*,*31*), and EBV DNA can be detected in the CSF of 80%–100% of HIV-infected patients with primary CNS lymphomas (*32–34*); however, EBV also may be detected in the CSF of 12% of HIV-infected patients with neurologic symptoms but without lymphoma (*35*). None of the EBV-positive patients in our study had evidence of CNS lymphoma by contrast computed tomography or MRI of the brain. Because EBV is a ubiquitous virus, the incidence of CNS infections caused by EBV may be underestimated. Our results suggest that EBV should be considered for all patients with CNS infections, especially in immunocompromised patients.

We used 16S rRNA gene amplification to screen for any bacteria in CSF before analysis by microarray and pyrosequencing. *E. coli* was detected in 5 samples that had been culture negative in previous analysis, most likely because of prior treatment with antibacterial drugs, which can confound the results of bacterial culture. Indeed, CSF can rapidly become sterile for bacteria after antibacterial treatment, with meningococci and pneumococci becoming noncultivable within 2 and 4 hours, respectively (*36*). *E. coli* meningitis is a common cause of neonatal meningitis (*37*), but it is a rare cause of acute bacterial meningitis in adults (*38*). In Taiwan, *E. coli* meningitis has been reported in adults with chronic underlying conditions (*39*). Among 5 patients identified with *E. coli* in our study, 3 had underlying clinical conditions, including pulmonary tuberculosis (2 patients) and HIV infection (1).

In our study, most of the candidate pathogens were identified by the multiplex MassTag PCR and 16S rRNA PCR. Although this finding may raise questions about the utility of more expensive and sophisticated techniques, such as high-throughput pyrosequencing for detecting CNS infections, the unique potential of high-throughput pyrosequencing has been demonstrated by its implication of an arenavirus in transplant-associated encephalitis (*16*) and a novel astrovirus in XLA-linked agammaglobulinemia-associated encephalitis (*17*). This approach may facilitate pathogen discovery for patients with CNS infections of unexplained cause as it becomes more popular in molecular diagnostics.

Our study has some limitations. First, because samples were collected from only 1 referral hospital, our results may not reflect the case distribution of CNS infections in Taiwan but instead might represent some of the more difficult-to-diagnose cases. Second, CSF samples were stored for 3 years after collection, and nucleic acid degradation, particularly of RNA, might have occurred before laboratory analysis, which may explain the low number of RNA viruses detected. Third, we cannot rule out misclassification of encephalitis with noninfectious causes, such as the newly described immune-mediated anti-N-methyl-D-aspartate receptor encephalitis (*40*), for which we did not test.

Rapid and accurate identification of the causative agent of a CNS infection can affect clinical management of individual patients. On the scale of populations, agent identification is crucial for determining the incidence of CNS infections caused by specific agents, enabling prioritization of targets for public health intervention and to prevent outbreaks of disease.

Our study confirms the power of multiplex MassTag PCR as a rapid diagnostic tool for identifying pathogens for patients with CNS infections and shows that viral and bacterial pathogens were detected in CSF from patients with CNS infections of unidentified cause. Additionally, the staged molecular approach incorporating complementary tools may enable detection of pathogens for patients with CNS infections of previously unrecognized causes, which would otherwise be missed. This approach may aid in explaining the observed worldwide high proportion of CNS infections of unknown cause.

Work in the Center for Infection and Immunity was supported by National Institutes of Health award AI057158-08 (Northeast Biodefense Center (to W.I.L.).

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Quinto Tiberio Angelerio and New Measures for Controlling Plague in 16th-Century Alghero, Sardinia

Raffaella Bianucci, Ole Jørgen Benedictow, Gino Fornaciari, and Valentina Giuffra

Plague, a zoonotic disease caused by the bacterium *Yersinia pestis*, has been responsible for at least 3 pandemics. During 1582–1583, a plague outbreak devastated the seaport of Alghero in Sardinia. By analyzing contemporary medical texts and local documentation, we uncovered the pivotal role played by the *Protomedicus* of Alghero, Quinto Tiberio Angelerio (1532–1617), in controlling the epidemic. Angelerio imposed rules and antiepidemic measures new to the 16th-century sanitary system of Sardinia. Those measures undoubtedly spared the surrounding districts from the spread of the contagion. Angelerio seems to have been an extremely successful public health officer in the history of plague epidemics in Sardinia.

The Black Death, a huge wave of epidemics of bubonic plague, spread across Europe during 1347–1353 CE. As detailed in nearly 200 local mortality studies relating to southern and western Europe, at least half of the population died of plague (*1*). The Black Death (1346–1353 CE) was the first outbreak of the second plague pandemic that occurred repeatedly until 1750 CE. Most likely it originated in the old plague reservoir (i.e., wild rodents) stretching from the northwestern shores of the Caspian Sea into southern Russia. Russian and Byzantine chroniclers mention the outbreak of terrible diseases there in spring 1346.

Kaffa, the far-outlying Italian trading station in Crimea, also was a source of infection. During spring 1347, Italian galleys fleeing Kaffa brought infection to Constantinople, where the plague began raging in the summer. From Constantinople, ships carried plague to ports along the Mediterranean littoral whence the infection fanned out from several epicenters, acquiring new momentum from these new centers as it spread (*1*).

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DOI: http://dx.doi.org/10.3201/eid1909.120311

Stimulated by earlier observations that epidemic diseases were transported by ships, the notion of quarantines began being developed in the early 14th century. Accordingly, a genuine quarantine was set up in 1377 in the Venetian trading station at Ragusa (present-day Dubrovnick, Croatia). Thirty-day isolation was imposed for ships from areas that were infected or suspected of being infected and 40-day isolation for land travelers from these areas (*2*,*3*).

During the 15th and 16th centuries, quarantine and sanitary cordons were imposed. Contacts and trades with infected regions were banned, and towns' gates and states' frontiers were closed, which prevented free movement of humans and merchandises to avoid the risk of spreading the contagion (*4*).

The first *lazaretto* (plague hospital/infirmary) was set up in 1423 in Venice. This institution soon became a model for isolating infected patients and preventing the spread of the epidemics (*3*). The *lazaretto* reflected the development of epidemiology and increasing administrative skills in Renaissance society.

During 1350–1520, ≈100 plague tracts were published (*5*). They shared a view of epidemic diseases: the final cause was God's anger over his human subjects' sins, and epidemic disease was His punishment. The means of punishment, the pathogenic causal agent, was miasma, a notion of Greek Hippocratic–Galenic medicine (*2*,*6*). Miasma was corruption or pollution of the air by noxious vapors containing poisonous elements caused by rotting putrid matter. Medieval medical theory added geophysical and astrologic elements; miasma also could be let out from the ground by volcanic activity or particular constellations of planets. Miasma was spread by wind and therefore could spread speedily; it could enter humans by inhalation or through the pores of the skin.

The theory was that miasma was the only cause of epidemic disease. The variety of epidemic diseases and their

clinical and epidemiologic manifestations were explained by miasma's ability to evolve into agents with different pathogenic properties, so a mild disease could develop into plague. According to miasmatic theory, plague patients were contaminated by the most dangerous type of miasma; air of the room also was contaminated by it (*6*).

The plague tracts warned all persons, including physicians, not to enter the rooms of plague patients or perform clinical examinations of such patients. Patients should be contacted from a distance. Thus, clinical elements mentioned in plague tracts were not empirical observation but based on hearsay. Some of this hearsay was consistent, especially that plague buboes developed in plague patients; most often these were visible on the neck because patients were not physically examined.

Not until the Renaissance, in the decades around 1500, was the theory of miasma expanded to include the idea that healthy persons could be infected by touching infected persons or objects contaminated by them with miasma (fomites), the *Fracastoro* miasmatic–contagionistic theory of cross-infection and epidemic spread (*1*–*6*). The basic tenets of miasmatic–contagionistic theory governed the actions and epidemic countermeasures of governments and municipal councils and their medical advisers. This theory explains why measures such as quarantines, sanitary cordons, isolation of persons with and suspected to have plague and with objects used by them, disinfection of houses, and disinfection of textiles were implemented beginning in the early 1500s.

Consequently, through special laws, administrative institutions were created to manage the organization of the sanitary system during plague outbreaks. The most efficient system of prevention and control was established in north-central Italy by the cities of Venice, Genoa, Florence, and Milan during the 14th and 15th centuries (*2*–*4*).

However, in Sardinia, early Modern Age (late 14th–early 15th centuries) health systems were considered mediocre (*7*,*8*). From the arrival of the Black Death in Sardinia (in 1348), plague epidemics required day-by-day organization. No social or political measures were pursued to control and prevent the plague outbreaks.

Despite the lack of organization in the Sardinian health system, in 1455, King Alfonso V of Aragon (1396–1458) established and imposed improvements. The royal ordinance created and imposed the so-called Office of the *Protomedicus* of the Sardinian Reign in Cagliari. Although this institution was already operating in the Catalan–Aragonese region, it represented an innovation for the island. The *Protomedicus*, a person belonging to the upper class and possessing a medical degree, superintended the medical practice and selected persons to certify to perform as physicians. Together with the municipal authorities, the *Protomedicus* coordinated prophylaxis and therapies (*8*).

The Catalan–Aragonese sanitary system of the second half of the 15th century had a Health Guard or Plague Guard, also called *Morber*. A *Morber* was also installed in Sardinia at that time (*8*,*9*). The *Morbers*' task was to watch over the sanitary conditions of the ships docking at the island's harbors by halting the disembarkation of persons with or suspected to have plague and to assist the *Protomedicus* during the plague outbreaks.

During the 15th and 16th centuries, Sardinian literature that focused on the history of medicine was poor and attests to the extreme backwardness of the medical culture and the sanitary structures (*8*). During the 16th and 17th centuries, only a few qualified physicians, selected by the *Protomedicus*, were practicing in Sardinia (*8*). Because of the absence of local universities, upper class students willing to perform medical studies completed their education in universities in Spain or Italy and seldom returned to Sardinia (*10*). Even after the foundation of the Universities of Sassari (1617) and Cagliari (1626), the situation remained almost unchanged because a rigorous faculty of medicine was still lacking (*10*).

The deficient professional background of the Sardinian physicians was reflected in poor communal organization of the sanitary structures. Those limits affected any attempt to prevent and contain the plague outbreaks that lashed the island from the time of the Black Death onward (*8*,*11*,*12*).

We report on the Neapolitan physician Quinto Tiberio Angelerio (*13*,*14*), *Protomedicus* of Alghero, who provided a breakthrough in the fight against the 1582–1583 plague epidemic by introducing novel prophylactic measures. Angelerio's scientific background was influenced by Galen's miasmatic theories and by Fracastoro's contagionistic theories (*2*,*3*). In addition, Angelerio had experience with plague before coming to Alghero. He had practiced in Messina, Sicily, during the 1575–1576 plague epidemic. At that time (1575), the *Protomedicus* of the Sicilian Reign, Giovanni Filippo Ingrassia (1510–1580), was successfully battling the plague outbreak that was spreading in Palermo (*15*).

Ingrassia had introduced useful prevention measures against plague, which included the isolation of persons with and suspected to have plague and of convalescents in 3 different isolation centers; the disinfection of the houses in which plague-related deaths had occurred; and use of dry heat to eliminate the "seeds of contagion" from everyday objects, thus anticipating the concept of modern sterilization (*15*,*16*).

No historical sources provide evidence of direct contact between the 2 physicians. Nonetheless, Angelerio's observations in Sicily formed his notions of how to combat such epidemics (*8*). To stem the spread of the contagion, Angelerio established a set of sanitary and prophylactic instructions that showed strong analogies with those previously adopted by Ingrassia (*8*).

Historical Sources and Demographic Data

Using contemporary documents, we reconstructed the measures introduced by Angelerio and the city government to prevent and control plague epidemics (*17*). The history of the 1582–83 epidemic, which lasted 8 months, is detailed in *Ectypa Pestilentis Status Algheriae Sardiniae* (Instructions on the Alghero, Sardinia, Plague Epidemic) (p. 110) (*17*) (Figure 1). Angelerio wrote and dedicated the booklet, published in 1588 in Cagliari, to the Viceroy De Moncada (*17*,*20*). Two printed versions and a manuscript are extant. The 1588 edition was written in Latin with a 12-page addendum in Catalan entitled *Instructions del Mates Autor* (Instructions from the same author). The second edition, published in Madrid in 1598, was entitled *Epidemiologìa sive Tractatus de Peste* (Epidemiology or Treatise on Plague) and was written exclusively in Castilian (*18*,*20*). A copy of each edition is kept in the University Library of Cagliari, and 1 copy of the Ectypa is preserved at the Alghero's Municipal Library (*19*). The detailed sanitary measures formulated in Ectypa included 57 instructions (Table 1, Appendix, wwwnc.cdc.gov/EID/article/19/9/12-0311-T1.htm). In the Epidemiologìa, the number of instructions was reduced to 30.

The paucity of contemporary documentary records and the discrepancies between their information made a death rate difficult to estimate (*20*–*25*). Death registers were kept only from 1677 onward (*21*–*23*).

The archival data presented below are from notepads belonging to the Archives of the Diocesan Curia of Alghero (ACVA Battesimi) (*21*–*23*) (Table 2). Because of the absence of burial registers for this period and a nearly 4-year gap (November 26, 1581–November 10, 1584) in marriage registers, the demographic reconstruction of the population's profile has to be based only on baptismal registers (*21*,*23*). In 1582, a total of 158 children were baptized (Table 2). When we account for the annual mean of 154 baptisms in the 5 previous years (1577–1581) and the fact that the effects of the plague epidemic on the population can be estimated only for the following year (1583), the monthly distribution of baptisms in 1582 appears to be normal: unaffected by plague (*21*,*23*).

In 1583, the number of baptisms fell by 59.7% to 62. In 1584, the number of baptisms increased sharply to 173, 12.3% above the average for 1577–1581, indicating the immediate onset of recuperative population growth in the wake of the plague epidemic. This growth resulted from a strong increase in marriages and consequent increase in conceptions and births because young adults could easily find jobs and housing vacated because of the plague and, for the same reason, immigration by young adults into the town from the surrounding countryside (*1*,*6*,*21*,*23*–*26*).

The bibliographer and diplomat Toda y Güell (*27*) claimed that the 1582–1583 outbreak caused 6.000 deaths,

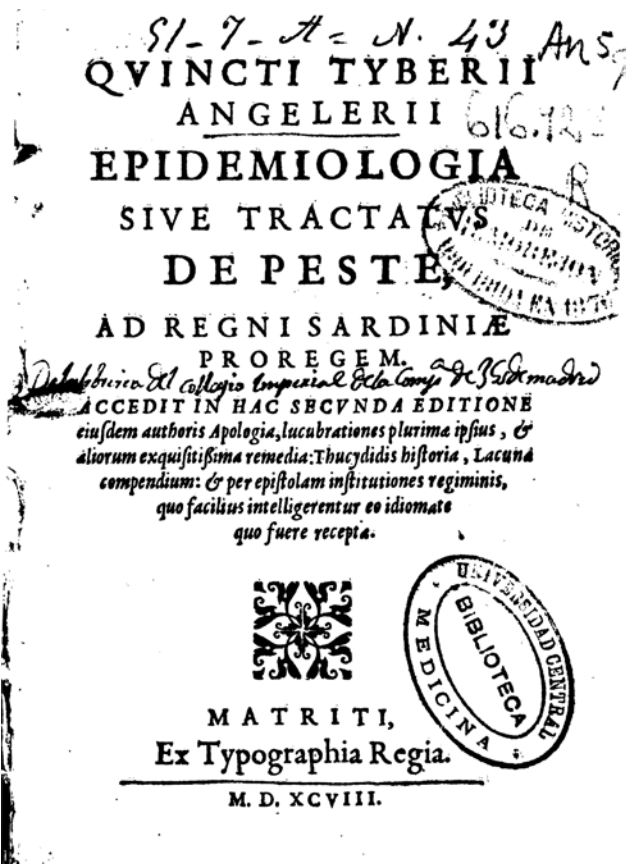
QVINCTI TYBERII
ANGELERII
EPIDEMIOLOGIA
SIVE TRACTATVS
DE PESTE,
AD REGNI SARDINIÆ
PROREGEM.
ACCEDIT IN HAC SECVNDA EDITIONE
eiusdem authoris Apologia, lucubrationes plurimæ ipsius, & aliorum exquisitissima remedia: Thucydidis historia, Lacunæ compendium: & per epistolam institutiones regiminis, quo facilius intelligerentur eo idiomate quo fuere recepta.
MATRITI,
Ex Typographia Regia.
M. D. XCVIII.

Figure 1. Frontispiece of the *Epidemiologìa Sive Tractatus de Peste*.

and only 150 (*cent y sinquanta*) persons survived.According to modern demographists, it is unlikely that Alghero counted 6.000 inhabitants at the end of the 16th century (*19*,*21*,*22*). Data from the 1589 census (*24*) attribute to Alghero 768 fires (each fire was formed by a family of 4.5–5 persons), which correspond to ≈3,400–3,800 inhabitants. Therefore, it appears that the death rate (97.6%) was intentionally augmented by local authorities to obtain a tax reduction (*14*,*21*,*22*).

Findings and Discussion

After the 1528–1529 outbreak, Alghero remained free of plague for ≈60 years (*13*,*14*) (Figure 2). The first new casualty attributed to plague in Alghero was registered on November 19, 1582 (*17*,*19*–*22*). The epidemic lasted 8 months and ended in June. After June 14, 1583, no new cases of contagion were registered (*17*).

The *Protomedicus* Angelerio immediately recognized the clinical manifestation of the infection (inguinal buboes and delirium) in a sailor who had disembarked from a ship docked during 1 night at the beginning of November 1582 (*17*). A sailor on a ship from Barcelona was thought to have been the initial harbinger of the infection (*17*). At that time, however, plague was almost absent from Spain while

ravaging France. More likely the sailor disembarked from a ship coming from Marseille, where the plague had raged since 1581 (*19*). The *Morbers* had not been effective in halting the arrival of the plague (*9*).

After the sailor's death, 2 women, a widow named Cifra and her mother, Grazia, who was assisting her at Alghero's Sant'Antonio Hospital, died in succession. With the help of a priest, Angelerio tried to convince the Bishop, Andrea Baccallar, and the Magistrates to contain the focus of the contagion because he recognized the small punctuate bruises (petecchiae) in the 2 deceased women (*17*,*19*). The Magistrates were indecisive, however, and on the morning of November 20, 1582, Bishop Baccallar asked the Senate to isolate the ill patients. However, the senators did not take Angelerio's report seriously, and Angelerio was accused of having an apocalyptic vision of the future (*19*). Meanwhile, the son of the widow Cifra died from plague. His death was followed by those of a crippled woman, an old woman, and a young daughter of the widow Crippa.

Because Angelerio was unable to persuade the Magistrates, he turned to the Viceroy, don Michele De Moncada (*8*,*17*). Angelerio explained that the forthcoming plague outbreak would have devastating effects on the population. Furthermore, he detailed the rules and sanitary measures needed to contain the epidemic.

Convinced by Angelerio's arguments, the Viceroy De Moncada gave orders to block all commerce from and to Alghero. A triple sanitary cordon was established, and triple barriers were built around Alghero's boundaries. Horse guards checked the city walls (*8*).

The cessation of commerce was taken badly by the inhabitants. Angelerio was loathed by the population, who wanted to lynch him. However, when the contagion spread from the core of the old town to the whole city, Angelerio was finally entrusted with the task of containing the epidemic (*8*,*17*).

Angelerio pioneered the implementation of successful public health measures in 16th-century Sardinia, basing his policies on daily reports of the Alghero population's health conditions and the incidence and location of plague cases. A general public health framework, including laws for plague control, decrees, institutions, and infrastructures was created. A system of basic welfare guaranteed by the city government was also established to satisfy the population needs in terms of medical treatment and food supplies and to implement disinfection of the houses (*25*). The pharmacists had to provide the poorest citizens with the necessary treatments (such as the Armenian bole, a ferruginous, ochreish, red clay used as a therapeutic substance against plague and all types of poisons). A list of the supplied treatments and a list of the citizens had to be kept to distinguish between the poorer and the richer. Richer persons would pay for their treatments, and the city government would pay for paupers (Instruction XXII). The *Morbers* were compelled to completely disinfect the city, house after house. The darkest houses and those lacking aeration had to be whitewashed by painters who had survived the contagion. Bonfires had to be set all around. For the less suspected houses, windows were required to be kept open at all times, perfumes to be sprayed, and all surfaces washed with vinegar (Instructions LIII and XLIX). Movements of people and goods to and from the city were strictly controlled during the epidemics (*17*).

Table 2. Monthly number of births according to the baptismal registers, Sardinia, 1582–1584

Month	No. baptisms, by year 1582	1583	1584
Jan	10	9	7
Feb	12	13	11
Mar	8	5	17
Apr	16	3	15
May	20	2	20
Jun	6	0	11
Jul	12	6	16
Aug	13	6	23
Sep	21	7	20
Oct	15	2	16
Nov	17	4	7
Dec	8	5	10
Total	158	62	173

Angelerio's instructions and measures facilitated interventions and changed the way in which local health officers were selected. The town was divided into 10 wards. Each ward was controlled by a Health Deputy, who was invested with full authority according to the new antiepidemic health laws, and a Plague Guard (Instruction II). The Health Deputies and the *Morbers* gathered twice a day in the so-called "City House" to follow the course of the epidemic and to transmit the information to the Councilors who were assisted by the physicians (Instruction VI) (*17*).

Angelerio's health policy emphasized disease prevention through isolation of persons infected with or suspected to be infected with plague (Instructions IV, V). Persons suspected to have plague were isolated at a center (*tancat*) (Instruction XVIII), whereas plague patients were housed at a *lazaretto* (Instructions XXXI). The main city hospital, the Sant'Antonio, also served as a *lazaretto* to isolate the plague patients. Guards ensured isolation of the above centers (Instruction XX).

Fire had to be set to mattresses, fittings, and furniture from all houses in which cases of plague were registered (Instruction VII). When a person was suspected of having died of plague, the physicians or surgeons had to check the corps to establish whether the deceased person actually had died of plague. If the cause of the death was indeed plague, the victim's relatives carried the corpse in the courtyard or left it outside the door (Instruction X).

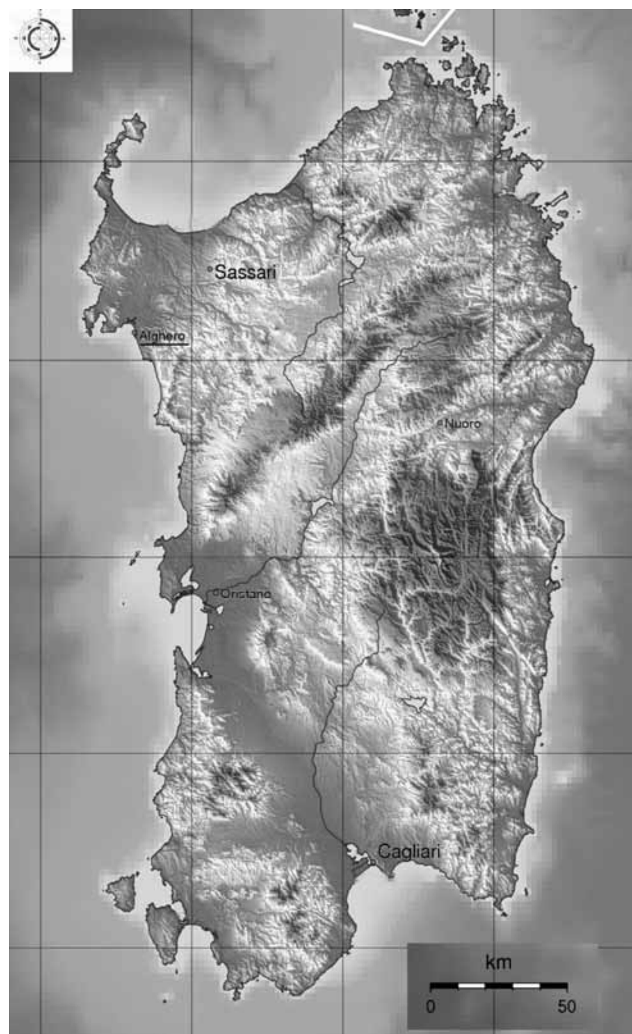

Figure 2. Modern-day Sardinia.

Plague victims were buried in secluded cemeteries within 6 hours after death; burying plague victims inside the churches was strictly forbidden. Long and deep trenches were excavated, and the corpses were covered with lime to not corrupt the air and release mephitic vapors (Instruction XXXII). Grave diggers were selected from among persons who had contracted, and survived, plague during a previous outbreak in another town. They lived separately from the rest of the community and far from the hospital and were not allowed to leave their houses unless a Health Deputy accompanied them (Instruction XII) (*17*).

Moreover, Angelerio introduced a new method for sterilizing clothes, textiles, and objects according to the miasmatic–contagionistic notions. Stoves/ovens similar to those used to cook the flat tiles (*rejolas*) were kept constantly lighted by an underlying fire (Instruction XXXIX). The stove's chamber was filled with the presumed textiles and objects after they had been washed under the *Morbers*' supervision (Instruction XLIV) (*17*).

The causative agent of plague and its vector (rat fleas) were not known scientifically until the end of the 19th century (*28*,*29*). However, Angelerio clearly recognized the role of disinfection in controlling plague with dry heat to eliminate the responsible agents (which he called the *malefica semina*, "bad seeds"). The miasmatic–contagionistic theory implied to Angelerio that miasmatic contagion was present wherever plague patients had been. Therefore, everything they had worn or touched, as well as the place they had stayed, had to be disinfected (*3*,*4*).

Through the introduction of dry heat, both the elimination of the plague bacillus (*Yersinia pestis*) and its vectors (the fleas) were guaranteed (*30*). In addition, the vectors' elimination helped prevent the transfer of infected fleas among citizens. Angelerio's intuition, which led to selection of painters and grave diggers from among persons who had already acquired and survived plague, anticipates the notion that semi-immunity to the bubonic form of plague may develop in long-term recovered patients (*31*).

During the plague outbreak in Sardinia during 1652–1657, Angelerio's instructions were resumed (*20*,*32*). The only 17th-century plague tract used in Sardinia, the *Tratado Universal* (Universal Tract), written by Juan Núnez de Castro in 1648, specifically refers to Angelerio's instructions. Following his instructions, Núnez de Castro ordered the establishment of sanitary cordons, quarantines, isolation centers for persons with and suspected of having plague and for convalescents, and disinfection of clothes and houses; he also ordered the following of the previously detailed rules for mortuary hygiene (*17*,*32*). Núnez de Castro's booklet was reprinted in Cagliari in 1652 by the *Protomedicus* Antonio Galcerín with the following title: *Instruccíon de las Prevenciones que se Hande Disponer en Tiempo de Contagio* (Instruction of the Preventive Measure that Have to be Applied during the Period of Contagion) (*8*). This plague guide became the sole reference for the Sardinian health authorities during the 1652–1657 outbreak that ravaged the island (*8*,*13*,*14*).

In conclusion, Angelerio's observations and well-organized public health services contained the epidemic in the city and halted its spread. His modern prophylactic and hygienic measures represent a successful innovation in the sanitary system of 16th-century Sardinia and in the Mediterranean area and attest to the extraordinary efforts of the city government to prevent the introduction and spread of contagion. The measures he introduced in Sardinia paved the way for subsequent generations of physicians and enabled them to manage plague epidemics more efficiently.

Support was provided by grants from the Fondazione Banco di Sardegna.

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Microsporidial Keratoconjunctivitis after Rugby Tournament, Singapore

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We investigated an outbreak of 47 probable and 6 confirmed cases of microsporidial keratoconjunctivitis involving participants of an international rugby tournament in Singapore in April 2012.The mode of transmission was eye contact with soil. *Vittaforma corneae* was identified in 4 of 6 corneal scrapings and in 1 of 12 soil water samples.

Microsporidia are spore-forming single-cell intracellular parasites, which have recently been shown to be fungi on the basis of phylogenetic analyses (*1*). They are ubiquitous in the environment, and at least 14 species have been implicated in human infections (*2*). Human ocular microsporidiosis first came into prominence as an opportunistic infection in patients with AIDS in the 1980s and, subsequently, in other immunocompromised patients (*3*,*4*). In the 1990s, *Vittaforma corneae* (formerly known as *Nosema corneum*) (*5*) was first described as the cause of corneal infection in an immunocompetent person (*6*) and disseminated infection in an immunocompromised patient (*7*). Since the early 2000s, microsporidial keratoconjunctivitis has been increasingly reported, mostly in Singapore (*8–10*) and India (*11*), among healthy, immunocompetent persons. The infections result predominantly from eye contact with soil or mud in outdoor activities.

On May 18, 2012, the Ministry of Health, Singapore, received a notification from the Centre for Health Protection, Hong Kong, of a suspected outbreak of microsporidial keratoconjunctivitis that was affecting 18 boys in a rugby club who had participated in an international rugby tournament in Singapore on April 21–22, 2012. We report the epidemiology, clinical features, and laboratory findings of the outbreak.

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DOI: http://dxdoi.org/10.3201/eid1909.121464

The Study

After the notification, epidemiologic investigations were undertaken immediately. A medical alert of the outbreak was circulated to all registered medical practitioners in Singapore. Local case-patients identified by clubs and medical practitioners (including ophthalmologists) were interviewed by telephone or email by using a set of questionnaires to obtain relevant clinical and epidemiologic data such as age, sex, nationality, clinical signs and symptoms, date of onset of illness, medical treatment sought, and details of activities at the tournament. Corneal scrapings collected by ophthalmologists were tested by microscopy; modified trichrome staining was used to detect microsporidial sporelike structures. Samples demonstrating these structures were then subjected to DNA extraction and microsporidia-specific PCR sequencing following previously described protocols (*12*). Soil water (water mixed in soil found in muddy fields) samples collected from the tournament venue on May 22, 2012, were tested for microsporidia by the Department of Pathology, Singapore General Hospital. The strategy of serial centrifugation (*13*) was adopted, followed by modified trichrome staining to detect microsporidial sporelike structures. Samples showing these structures also underwent microsporidia-specific PCR sequencing for species identification (*14*).

To investigate possible infections in participants from outside Singapore, we contacted the International Health Regulations National Focal Points of the countries involved and team representatives of foreign rugby clubs. We obtained information on participants in whom symptoms of eye infection developed after the tournament.

The rugby tournament involved 1,594 players of 107 teams from rugby clubs from Singapore, Hong Kong, Malaysia, Australia, and the United Arab Emirates. Tournament participants comprised 1,511 boys and 83 girls; Singapore clubs were represented by 1,122 male players and 69 female players. Two types of rugby were played: touch rugby and full-contact rugby.

A probable case of microsporidial keratoconjunctivitis in a person was defined as follows: $\geq$2 of the following eye signs or symptoms–redness, pain/foreign body sensation, itch, blurred vision, photosensitivity, and/or epiphora–developing from 2 to 30 days after the person participated in the rugby tournament plus a clinical diagnosis by an ophthalmologist using slit lamp biomicroscopy. Biomicroscopy typically revealed the classic microsporidial corneal infection–coarse, multifocal, granular punctuate epithelial keratitis, along with mild follicular or papillary conjunctivitis. The case was classified as confirmed if corneal scrapings were collected and microsporidial spores were shown by microscopy and modified trichrome staining.

Of the 72 local players traced and interviewed, we identified 48 case-patients (46 probable and 2 confirmed)

Table 1. Microsporidial keratoconjunctivitis cases and attack rates among players from 5 participating countries in rugby tournament, Singapore, April 21–22, 2012

Country	Total no. players	No. (%) probable cases	No. (%) confirmed cases	Overall attack rate, no. (%)
Singapore	1,191	47 (3.9)	2 (0.2)	49 (4.1)
Hong Kong	82	0	3 (3.7)	3 (3.7)
Malaysia	281	0	1 (0.4)	1 (0.4)
Australia	19	0	0	0
United Arab Emirates	21	0	0	0
Total	1,594	47 (2.9)	6 (0.4)	53 (3.3)

among the boys and 1 probable case-patient among the girls. Among foreign participants, 4 confirmed cases were identified (Table 1). Besides these affected players, 5 probable cases among Singapore residents were identified, comprising 2 coaches, 2 spectators, and 1 referee. In addition, 6 probable sporadic cases were also notified during the outbreak period; they were identified in persons who were not linked to the tournament but who had participated in other outdoor activities with exposure to mud (Figure).

Forty-six (93.9%) of the 49 affected Singapore players interviewed were children of expatriates. Symptoms developed from April 25 to May 18, 2012. Their ages ranged from 6 years to 17 years (median age, 12 years). The attack rate among full-contact rugby players (5.7%) was significantly higher than that among touch rugby players (0.5%) ($p<0.0001$) (Table 2).

The main presenting ocular signs and symptoms were redness (49/49 patients, 100%), pain/foreign body sensation (41/49 patients, 83.7%), photosensitivity (38/49 patients, 77.6%), blurred vision (37/49 patients, 75.5%), itching (34/49 patients, 69.4%), and epiphora (23/49 patients, 46.9%). Twelve case-patients had bilateral infection. The median incubation period, based on the interval between date of last exposure and onset of illness, was 15 days (range 3–26 days). None of the case-patients was hospitalized. All case-patients responded well to treatment.

Forty-six (93.9%) of the affected players reported having had mud enter their eyes while playing in the tournament. Of these, 43 (87.8%) did not share any personal articles such as towels and handkerchiefs with other players, 12 (24.5%) used the available shower facilities, and 41 (83.7%) indicated that they had washed their faces with water from water hoses or mineral water bottles after each match.

Laboratory analysis of 3 corneal scrapings collected from 2 affected local players and 1 Malaysian player who sought treatment at a private eye center in Singapore revealed microsporidial sporelike structures by microscopy with modified trichrome staining. One corneal scraping was confirmed as *V. corneae* by PCR sequencing with 97% sequence homology to at least 2 published *V. corneae* sequences. The same species was also identified in 3 affected players from Hong Kong rugby clubs (*15*). Sporelike structures consistent with microsporidia were also detected in 12 of 21 soil water samples (average dimensions 2.87 µm × 1.68 µm). *V. corneae* was detected in 1 soil water sample.

Conclusions

We describe a single-source microsporidial keratoconjunctivitis outbreak, to which several factors contributed. First, extreme weather (2 days of heavy rain preceding the

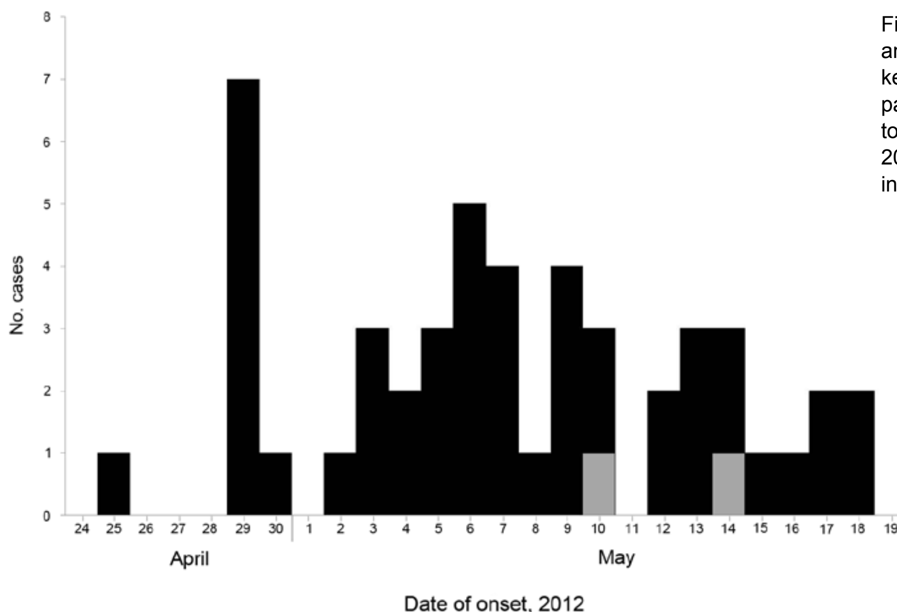

Figure. Dates of onset of eye signs and symptoms of microsporidial keratoconjunctivitis in 49 affected local participants after an international rugby tournament in Singapore, April 21–22, 2012. Black indicates probable cases; gray indicates confirmed cases.

Table 2. Microsporidial keratoconjunctivitis cases and attack rates by type of rugby contact among local players in international rugby tournament, Singapore, April 21–22, 2012

Category	No. players	No. teams	No. (%) probable cases	No. (%) confirmed cases	Overall attack rate, no. (%)
Touch rugby*	369	33	2 (0.5)	0	2 (0.5)
Full-contact rugby†	822	48	45 (5.5)	2 (0.2)	47 (5.7)
Total	1191	81	47 (3.9)	2 (0.2)	49 (4.1)

*For boys 6– 8 years of age and all girls' teams.
†For boys 9–18 years of age.

tournament and on its first day) resulted in the muddy condition of the field. Second, in full-contact rugby, the risk for exposure of the face and eyes to mud and groundwater is high because the defensive players would have to stop the player with the ball by tackling him or her to the ground. Third, the limited washing facilities at the tournament venue resulted in many players having to wash up at home many hours after exposure to mud.

The main limitation of the study is that the majority of the reported cases were not confirmed by laboratory identification of microsporidia. We could not justify obtaining corneal scrapings from the affected players because the participating ophthalmologists became extremely aware of the characteristic signs and symptoms of microsporidial keratoconjunctivitis. In addition, because of the limited amount of clinical materials available for testing for *V. corneae,* no further genetic studies were undertaken to establish their relatedness.

Microsporidial keratoconjunctivitis is an emerging eye infection in Singapore. Public health professionals should be aware that it may be prevalent in other countries when keratoconjunctivitis is considered as a diagnostic possibility.

Acknowledgments

We thank Hwi Kwang Han, Hai Yin Toh, Qing Yuan Pang, and Abdul Rahman bin Osman (all from Surveillance and Response Branch, Communicable Diseases Division, Ministry of Health, Singapore) for their assistance with the field investigation and Sui Sin Goh, Yanhong Cao, and Thye Hoon Lin (all from Department of Pathology, Singapore General Hospital) for technical help with the microbiological investigations.

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Novel Bunyavirus in Domestic and Captive Farmed Animals, Minnesota, USA

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We tested blood samples from domestic and captive farmed animals in Minnesota, USA, to determine exposure to severe fever with thrombocytopenia syndrome virus and Heartland-like virus. We found antibodies against virus nucleoproteins in 10%–18% of samples from cattle, sheep, goats, deer, and elk in 24 Minnesota counties.

Severe fever with thrombocytopenia syndrome (SFTS) is an emerging infectious disease in China, caused by a novel bunyavirus in the genus *Phlebovirus* (*1*). As many as 10,000 SFTS case-patients have been reported since disease emergence in 2009, with fatality rates ranging from 2% to 15% (*1*,*2*), mainly in the eastern provinces of China. SFTS bunyavirus (STFSV) appears to be transmitted by ticks, an unusual difference from other pathogenic phleboviruses, which are transmitted primarily by mosquitoes (*3*). Recently, a new phlebovirus, named the Heartland virus (HLV), was isolated in Missouri from patients with a history of tick bites and signs and symptoms similar to those of SFTS, including high fever and low blood leukocyte and platelet counts (*4*). Phylogenetic analysis showed that HLV is closely related to SFTSV, which suggests that this new phlebovirus could be a serious threat to public health in the United States.

Many bunyaviruses can infect animals (*3*). Little is known about the animal host species that carry HLV or HLV-like bunyaviruses in the United States. Serologic surveys in China found that farm animals, including cattle, goats, and sheep, were infected with SFTSV in disease-endemic areas. In these studies, viral RNA was identified in animal serum specimens, and these isolates shared high sequence homology with isolates from humans (*5*). Strikingly, up to 47% of farm animals in Jiangsu Province, China, had SFTSVs (*4*), indicating that active virus transmission is occurring in the rapidly expanding disease-endemic area. It is critical to identify animal hosts that may be susceptible to, and infected with, HLV or an SFTSV-like virus, and may serve as amplifying hosts that facilitate virus transmission in the United States. To identify animal hosts that may play an essential role in transmission of SFTSV- or HLV-like viruses in the United States, we conducted serologic testing of samples collected from farm animals in Minnesota, USA. Our findings raise the specter of widespread distribution of a novel pathogen among livestock and wildlife that has the potential to be transmitted to humans.

The Study

Blood samples, obtained from several domestic and captive farmed animals of various species, were analyzed at the Minnesota Veterinary Diagnostic Laboratory, College of Veterinary Medicine, University of Minnesota. Samples had been collected from September 8 through October 12, 2012, from cattle, goats, sheep, and elk and white-tailed deer andwere submitted mainly for routine surveillance purposes from 29 Minnesota counties.

No HLV or SFTSV antibody test kits are currently available in the United States. We found that anti-SFTSV nucleoprotein (NP) antibodies cross-react with HLV NP and decided to use SFTSV NP antibody detection kits for detecting antibodies against SFTSV- or HLV-like viruses. A standard ELISA reagent kit, developed by Jiangsu Centers for Disease Control (*6*), was used to detect all subtype antibodies specific to the SFTSV NP, following the providers' instructions. Both positive and negative controls were included, and the results of an assay were considered acceptable when the optical density (OD) of the positive and negative controls were ≥1.50 and ≤0.10, respectively. Samples with an OD value ≥2.1× the mean negative control were considered positive (*6*). Positivity/negativity ratios were calculated for all samples tested, and N represents the mean OD value of negative controls.

Antibodies were detected in serum samples from 64 (15.5%) of 414 cattle, 10 (10.9%) of 92 goats, 6 (12.5%) of 48 sheep, 35 (11.8%) of 296 white-tailed deer, and 7 (18.0%) of 39 elk (17.9%) (Table). Thirty-four of 64 positive samples had positivity/negativity ratios of 4–10, and 11 had ratios ≥10 (Figure 1). Specific antibody titers of these 11 samples ranged from 80 to 1,280 as determined by serial titration. The positive samples came from 24 of 29 counties tested (Figure 2).

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DOI: http://dx.doi.org/10.3201/eid1909.130165

Table Prevalence rates of samples positive for antibodies against SFTSV NP, Minnesota, USA, 2012*

Species	No. tested	No. (%) positive†
Cattle	414	64 (15.5)
Goat	92	10 (10.9)
Sheep	48	6 (12.5)
White-tailed deer	296	35 (11.8)
Elk	39	7 (18.0)
Total	889	122 (13.7)

*SFTSV, severe fever with thrombocytopenia syndrome virus; NP, nucleoprotein; samples were tested by ELISA.
†A sample with a positive/negative ratio ≥2.1 was considered positive.

Conclusions

Our data show that both domestic and captive farmed animals in Minnesota were exposed to SFTSV- or HLV-like virus, as evidenced by the presence of antibodies reactive against the NP of SFTSV at a prevalence ranging from 10% to 18%. Because SFTSV and HLV are closely related (*4*), the viruses detected in this region are most likely HLV or close relatives of HLV. Although NPs of SFTSV and HLV are antigenically cross-reactive, the observed detection rates may be underestimated because the reagents were developed and optimized for SFTSV NP antibodies. A more specific serologic test targeting HLV-specific antigens is under development; it is expected to more accurately assess the prevalence of HLV- or HLV-like virus among animals and humans.

Many arboviruses, including bunyaviruses, are zoonotic pathogens. A high fatality rate has been reported for sheep, goats, cattle, and wildlife infected with Rift Valley fever virus (RVFV) (*7*,*8*), and infections have also led to abortion in 100% of pregnant livestock. Whether the SFTSV- or HLV-like virus detected in this study is pathogenic to animals and can cause disease if transmitted to humans remains to be determined. Our records have shown that all animals that tested positive did not exhibit apparent clinical signs even though infected. Because most samples, including almost all seropositive ones, were sent to the Minnesota Veterinary Diagnostic Laboratory for surveillance purpose, the time of the animal's infection with HLV-like virus is uncertain, but the animals were apparently healthy when sampled. However, we cannot exclude the fact that the animals may have shown clinical signs when infected with the virus.

Vertebrate animals are amplifying hosts for many arboviruses, which have seasonal epidemics (*3*). Distinct strategies are used by various arboviruses for interepidemic virus maintenance. Bunyaviruses, except for hantaviruses, are obligate vector-borne viruses, and their vectors include mosquitoes, ticks, and sandflies. La Crosse virus of the genus *Orthobunyavirus* is transmitted by mosquitoes, and rodents and foxes can be infected as amplifying hosts during seasonal outbreaks (*9*). RVFV is transmitted by mosquitoes as well and can infect a variety of livestock including cattle, goats, and sheep, in which the virus is amplified and transmitted to humans who are in close contact with viremic animals (*10*). Although a bite from an infected mosquito is critical for human infection, humans are more likely to become infected with RVFV through direct contact with viremic animals especially during the process of animal birth or abortion (*11*). RVFV is also confirmed to be highly infectious in aerosols (*12*).

SFTS epidemics in China are seasonal and occur from late March through early November. Infection rates among humans and animals rise in early March, peak in August, and decrease after November (*1*,*2*). The coincidental pattern of human epidemics and animal infections indicates that infected livestock play a critical role as amplifying hosts in SFTS epidemics. In Minnesota, the SFTSV- or HLV-like virus infects a variety of ungulates, both domesticated and wild. We also show that deer and elk (cervids) may be susceptible to this virus in this region. Farmers, hunters, and persons with outdoor lifestyles may

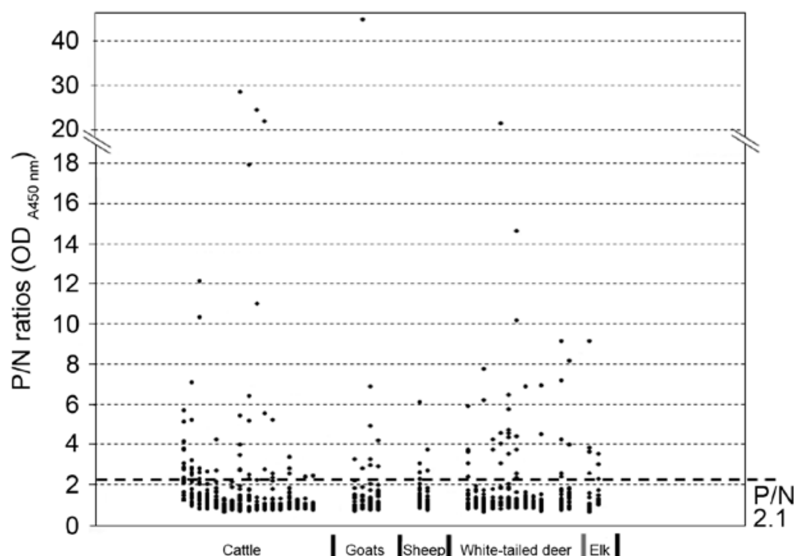

Figure 1. Distribution of positivity (P)/negativity (N) ratios among various animal species tested for antibodies against severe fever with thrombocytopenia syndrome virus nucleoprotein, Minnesota, USA, 2012. N = mean + 3 ´ SD of optical density $(OD)_{450nm}$ values of negative controls; P = OD_{450nm} value of a test sample.

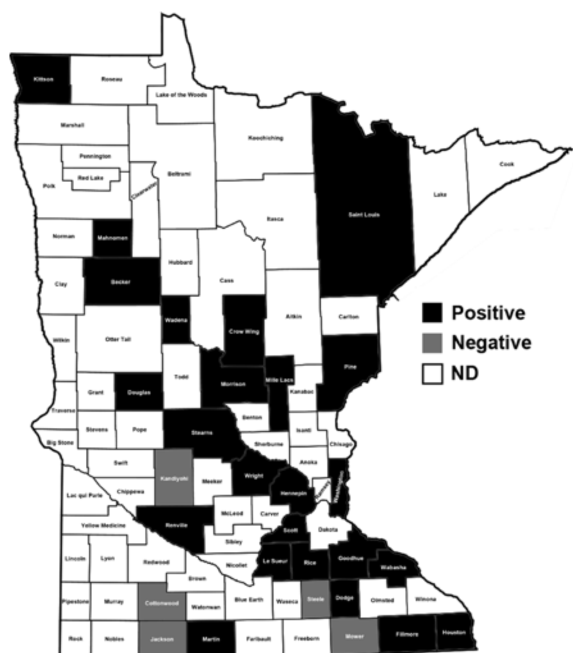

Figure 2. State of Minnesota showing counties. Domestic and captive farmed animals positive for antibodies against severe fever with thrombocytopenia syndrome virus nucleoprotein were found in 24 (black) of 29 counties, 2012. ND, not determined.

become infected when they are bitten by infected ticks. In addition, direct contact with secretions, body liquids, or feces from viremic animals would also put these persons and veterinarians at risk, if HLV- infected animals have substantial amounts of virus in blood and other tissues. The direct contact transmission of SFTSV has been reported in family clusters among persons with no history of tick bites, suggesting that person-to-person transmission may also occur (*13–15*).

Evidence that a novel phlebovirus infects domesticated and captive farmed animals as shown in this study validates the concern that an SFTSV- or HLV-like emerging pathogen could pose a serious public health threat in the United States. Epidemiologic studies with a broader scope need to be conducted to elucidate viral ecology, and effective measures must be adopted to control this virus before it spreads among humans.

Acknowledgments

We thank Sandy Shanks for her expertise in editing the manuscript.

The work was also supported by a Minnesota Rapid Agricultural Response Fund and Grant-in-Aid grant from the Vice President's Office of the University of Minnesota (to Z.X.). Z.X. was also supported by the State Key Laboratory of Pharmaceutical Biotechnology of Nanjing University (KFGW-200902 grant).

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Benznidazole Treatment of Chagasic Encephalitis in Pregnant Woman with AIDS

Margarita Bisio, Jaime Altcheh, Jorge Lattner, Guillermo Moscatelli, Valeria Fink, Juan M. Burgos, Facundo García Bournissen, Alejandro G. Schijman, and Héctor Freilij

We report a case of chagasic meningoencephalitis reactivation in a pregnant woman co-infected with *Trypanosoma cruzi* and HIV that was successfully managed with benznidazole and highly active antiretroviral therapy. Early diagnosis enabled rapid specific treatment that improved the health of the patient and her baby.

Chagas disease, caused by the protozoan *Trypanosoma cruzi*, is transmitted to humans mainly by triatomine bugs (vector-borne route), through blood transfusions, or from mother to child through the placenta (transplacental route) (*1*). In patients co-infected with HIV, Chagas disease reactivation generally occurs in persons with CD4 T-cell counts <200 cells/mm^3 and results in severe meningoencephalitis or myocarditis (*2*). Confirmation of central nervous system (CNS) reactivation requires *T. cruzi* detection in 1) cerebrospinal fluid, 2) brain tissue, or 3) blood, with neurologic manifestations and clinical response to parasiticidal treatment (*2*). CNS reactivation is associated with a high mortality rate, and management consists of combining anti–*T. cruzi* drugs (benznidazole or nifurtimox) with highly active antiretroviral therapy (HAART) to favor immune reconstitution (*2*,*3*).

Migration by persons from Chagas disease–endemic areas to vector-free urban centers, and changes in the epidemiologic profile of HIV, have led to a large overlap in the geographic distribution of the 2 infections (*4*). In fact, the prevalence of *T. cruzi* infection among HIV-seropositive patients from disease-endemic regions was found to be 1.3% in Brazil and 1.9% in Spain (*4*,*5*).

Because safety of benznidazole in pregnancy has not been established (*6*), its use in treating pregnant women is contraindicated (*7*,*8*). Prevalence of vertical transmission of *T. cruzi* infection from immunocompetent women to their fetus varies from 0.1% to 18% among regions (*7*), and such transmission is strongly associated with the maternal blood-parasite load (*7*,*9*). However, patients co-infected with HIV exhibit higher levels of parasitemia and a higher congenital transmission rate (*10*) than those who are not co-infected. Indeed, in our experience, 6 of 7 co-infected pregnant women transmitted *T. cruzi* infection (*11*). We describe a noteworthy case-patient from that series, a woman who experienced reactivation of *T. cruzi* infection during the third trimester of pregnancy but did not transmit the parasite infection, probably because she received, without delay, treatment with benznidazole and HAART.

The Patient

A 33-year-old woman, who had been infected with HIV for 11 years, started receiving lamivudine, zidovudine, and nevirapine at week 26 of pregnancy. At that time, CD4 cell count was 18 cells/mL. She had begun receiving HAART 1 year before pregnancy but abandoned treatment after a few months. Her compliance with treatment, laboratory tests, and follow-up was poor.

At week 32 of pregnancy, she was admitted to the hospital with clinical manifestations of intracranial hypertension and meningoencephalitis (temporospatial disorientation, slurred speech, nausea and vomiting, and hemiparesis). A CNS mass was detected by magnetic resonance imaging (Figure). Results of serologic tests were negative for *Toxoplasma gondii*, *Treponema pallidum*, and hepatitis B and C viruses but positive for *T. cruzi*. Reactivation of Chagas disease was confirmed by a positive microhematocrit measurement (*12*). PCR targeted to *T. cruzi* kinetoplast DNA was positive, and quantitative PCR (qPCR) targeted to satellite *T. cruzi* DNA yielded 308.9 parasite equivalents/mL of blood (*13*). The parasite genotype (discrete typing unit) detected by PCR strategies was identified as TcV (*14*). Most probably, this patient acquired the infection by the vector-borne route in a highly disease-endemic province in Argentina where she had lived as a child.

Although data on benznidazole use in pregnancy are lacking, after an in-depth discussion of potential risks and benefits of the treatment, we concluded that, because of her severe clinical condition, treatment with benznidazole could be beneficial. Within 48 hours of the diagnosis, she received benznidazole (5 mg/kg/day, for 84 days)

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DOI: http://dx.doi.org/10.3201/eid1909.130667

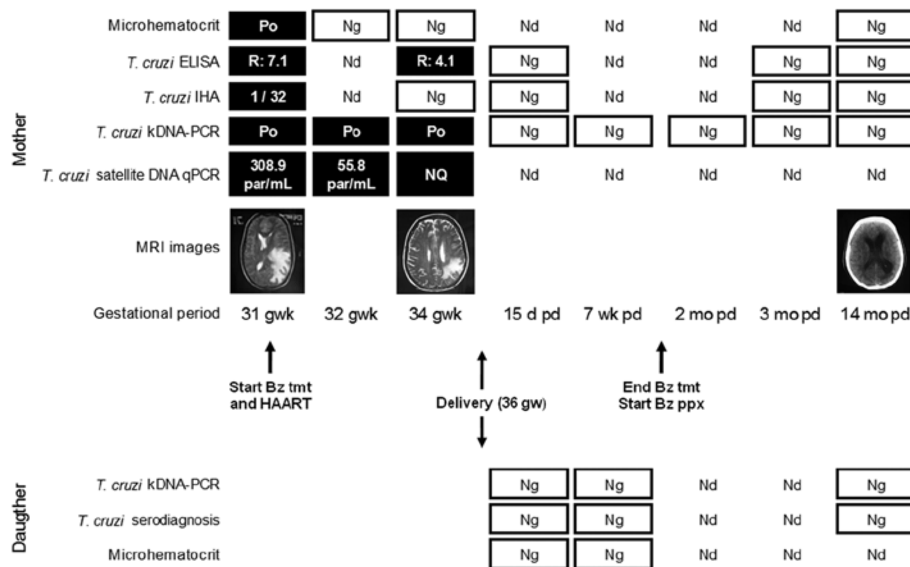

Figure. Diagnosis, treatment, and prophylaxis monitoring of *Trypanosoma cruzi* infection in a pregnant woman and her daughter. The mother, who had chagasic encephalitis and AIDS, was treated with benznidazole (Bz). Results of ELISA were considered positive when R was >1.2); results of IHA were considered positive when titers were >16. Po, positive; Ng, negative; Nd, not determined; IHA, indirect hemagglutination assay; kDNA-PCR, kinetoplast DNA-PCR; qPCR, quantitative PCR (dynamic range of detection: 1–105 parasite equivalents/mL, amplification of efficiency 0.95, R 2 = 0.996); par/mL, parasites/mL of blood; NQ, positive but not quantifiable; MRI, magnetic resonance imaging; gwk, gestational week; pd, postdelivery; tmt, Bz treatment (5 mg/kg/d); HAART, highly active antiretroviral therapy; ppx, Bz prophylaxis (5 mg/kg/3× per week).

without side effects. The patient showed rapid neurologic and clinical improvement. Parasitic loads, determined by qPCR, dropped rapidly (Figure). During treatment, results of the microhematocrit, PCR, and anti–*T. cruzi* serologic testing became negative for the parasite. Magnetic resonance images showed substantial reduction of edema and the mass and reconstitution of the interventricular line without evidence of ventricular dilatation (Figure). Seven weeks after treatment initiation, cerebrospinal fluid was negative for *T. cruzi* by serologic testing, microhematocrit, and PCR.

At week 36 of gestation, the patient gave birth to a girl by elective cesarean section. The baby's birthweight was low for gestational age (1,700 g, <3rd percentile), and her Apgar score was 7/8. The newborn was hospitalized for 32 days. Microhematocrit and PCR results suggested that she had not acquired *T. cruzi* infection. This lack of infection was confirmed by means of serologic testing at 1 year of age, per current Chagas disease guidelines (*12*). Moreover, results of PCR for HIV and p24 antigen tests were negative in 2 different samples, enabling perinatal infection with HIV to be ruled out.

After delivery, the mother continued to receive HAART with benznidazole prophylaxis (5 mg/kg/day 3×/week) for 6 months. A follow-up computed tomographic scan performed 14 months after delivery showed a favorable response. Moreover, serologic, parasitologic, and molecular studies remained negative for *T. cruzi* (Figure). To date, the patient continues to show erratic compliance with follow-up. As a consequence, immunologic recovery has not yet been achieved.

Conclusions

Benznidazole is traditionally considered contraindicated in pregnancy because data that support its safety to the fetus are lacking (*8*). Nevertheless, because of the severe clinical picture of the patient, and the known lower risks of fetal toxicity in the second and third trimesters of pregnancy (*6*), treatment was administered on a compassionate basis. Among patients who have AIDS and Chagas disease reactivation with CNS involvement, early parasiticidal and HAART treatment can improve the poor prognosis (*2*,*3*). Cordova et al. (*2*) reported a mortality rate of 79% in a cohort of 15 patients with co-infection, with a median time of survival of 21 days after hospitalization. However, the researchers associated this high mortality rate with delayed diagnosis, because it took a median of 18 days to recognize the condition. In the patient reported here, the short interval (72 hours) between admission and initiation of benznidazole treatment may have improved the chances of a successful outcome. Moreover, to our knowledge, the patient in this case has longest reported survival time after diagnosis of chagasic reactivation caused by HIV co-infection, with 7 years of follow-up (*15*).

Microhematocrit and PCR successfully demonstrated the presence and elimination of *T. cruzi,* and qPCR enabled the decrease in parasitic load to be monitored. *T. cruzi* TcV detected in peripheral blood was described as prevalent in the population from the Southern Cone (*14*). Notably, negative serologic results were achieved early after treatment, which is only usually seen in immunocompetent patients at the acute phase of disease or in recent congenital infections. The rapid negative seroconversion observed in this

patient is uncommon. The continued seronegative results when her immune system presumably was reconstituted suggests strongly that she had an actual parasitologic cure.

Moreover, the baby's weight at birth was low for gestational age. Various reasons could explain the low birthweight, including the infectious status of the mother or the severe stress she was experiencing at the time of admission. Also, a potential effect of benznidazole for the baby cannot be fully ruled out. Nonetheless, the benefit to both mother and child clearly outweighed the risk. The baby did not acquire either infection.

This case illustrates that parasiticidal treatment may decrease parasitic load and prevent vertical transmission of *T. cruzi*, even in co-infected patients among whom a higher incidence of congenital infection and illness of newborns is observed (*10*). This result reinforces the idea that benznidazole has a role in the prevention of congenital transmission of Chagas disease. Therefore, studies that explore its safety and effectiveness during pregnancy may be warranted.

Acknowledgments

We extend our appreciation to the patient who is the subject of this report and her daughter.

This work was partially supported by grants from the National Council of Science and Technology (CONICET) (PIP 112-20081-02915) to A.G.S. J.A. and H.F. are members of the clinical researcher career of the Government of Buenos Aires. A.G.S., J.M.B., and F.G.B. are members of CONICET researcher's career. M.B. is a research fellow of Bunge y Borne Foundation.

Dr Bisio has a postdoctoral position in the Parasitology and Chagas Disease Department of the Children´s Hospital "Ricardo Gutiérrez," Buenos Aires, Argentina. Her work focuses on improving molecular diagnostic tools and their effect on treatments, evaluation, and control of parasitic diseases.

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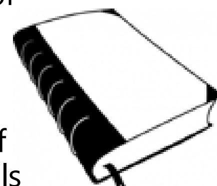
Books, Other Media

Reviews (250-500) words of new books or other media on emerging disease issues are welcome.

Name, publisher, number of pages, other pertinent details should be included.



Mycobacterium chelonae Abscesses Associated with Biomesotherapy, Australia, 2008

Mihaela Ivan, Craig Dancer, Ann P. Koehler, Michaela Hobby, and Chris Lease

An outbreak of skin abscesses occurred in Adelaide, Australia, in association with biomesotherapy, an alternative therapy practice. *Mycobacterium chelonae* was identified in 8 patient and 3 environmental samples. Our findings show *M. chelonae* infection can be associated with alternative therapies when infection-control breaches occur. Tighter regulations of alternative therapy practices are needed.

Biomesotherapy is marketed in Australia as a new therapy that combines homotoxicology, mesotherapy, and acupuncture. Saline solution and homeopathic formulations are injected subcutaneously at specific acupuncture or trigger points, and homeopathic formulations are administered orally during treatment sessions. Biomesotherapy is used for pain management and general well-being.

Mycobacterium chelonae is a rapid-growing mycobacterium that occurs naturally in water sources and produces rare infections in humans. The bacterium can cause pneumonia and skin and ocular lesions, mostly following tissue trauma (*1*). *M. chelonae* is commonly present in tap water; however, it is not reliably removed by filtration or by boiling for short periods. Outbreaks and isolated cases of cutaneous infections caused by *M. chelonae* and other rapid-growing mycobacteria have been described in association with alternative therapies, hospital settings, and spas (*2–8*).

Mycobacterial infections are notifiable in South Australia, a state in the southern central part of Australia. On average, 2 cases of nonpulmonary *M. chelonae* infection are reported in South Australia each year.

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DOI: http://dx.org/10.3201/eid1909.120898

The Study

In June 2008, the Communicable Disease Control Branch of the SA Department of Health received notification of 5 suspected and 1 laboratory-confirmed case of mycobacterial skin infection. All case-patients reported having received biomesotherapy treatment from the same person, Practitioner A.

An investigation team immediately inspected Practitioner A's premises and found poor sanitation and infection-control practices in place. Equipment used for injections at the clinic was seized, and Practitioner A was directed to cease all practices related to injection of clients, including the preparation of formulations. A media release and a public health alert were issued to inform the community about the outbreak.

Case finding was then initiated. A case-patient was defined as a person with "skin lesions compatible with mycobacterial infection at injection site and visit to Practitioner A since January 1, 2008." A list of clients was assembled from Practitioner A's patient records and appointment book, direct information from patients, and physician referrals. To identify possible case-patients and to collect relevant epidemiologic data, we developed a semistructured questionnaire for use during telephone interviews with Practitioner A's clients; 43 clients completed the interview. At the end of interviews, clients were strongly advised to consult a general practitioner for clinical assessment and referral to an infectious diseases physician if they had skin lesions or other concerns.

Through telephone interviews and doctor and laboratory notifications, we identified 27 case-patients, of whom 20 had completed the telephone interview. Abscesses (1–16/case-patient) had developed within days to several weeks after case-patients received biomesotherapy injections from Practitioner A (Figure 1). The mean age of case-patients was 47 years (range 27–77); 17 were female and 20 were male. Thirteen case-patients had visited a physician for their skin lesions, and 18 still had abscesses present at the time of the interview. Twelve case-patients reported that the same needle was used for all injections during a single treatment session. Three case-patients recalled that Practitioner A had washed his/her hands before the procedure; most other case-patients were unsure if this step had been taken.

Twenty clinical samples from 14 case-patients and 36 environmental samples were sent for laboratory testing. The samples were stained (Gram and Ziehl-Neelsen stains) and cultured at 30°C and 35°C on routine agar media in MGIT Mycobacteria Growth Indicator Tube (Becton Dickinson Microbiology Systems, North Ryde, NSW, Australia) broth culture and on Lowenstein-Jensen slopes. The environmental samples were centrifuged and processed as described (*9*). We initially identified organisms by

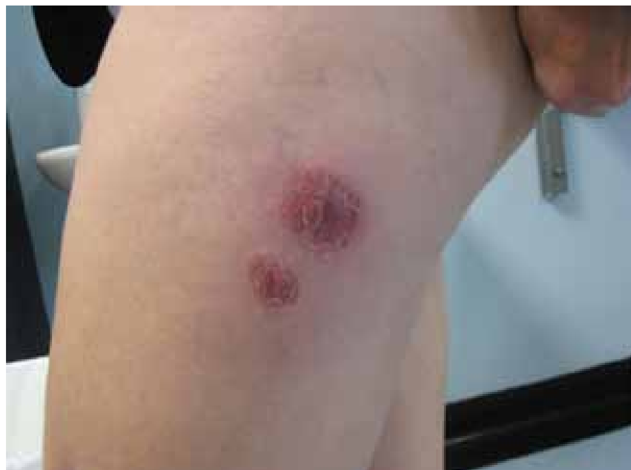

Figure 1. *Mycobacterium chelonae* abscesses associated with biomesotherapy, an alternative therapy practice, Adelaide, South Australia, Australia, 2008. The abscesses are at the biomesotherapy injection site. (Photo courtesy of Erina Gray.)

conventional phenotypic methods (*10*). Molecular identification was performed on all isolates by sequencing 3 regions: 16S rDNA, 16S-23S rRNA internal transcribed spacer, and *rpoB* (*11–13*). Susceptibility testing was performed by using the disk diffusion test and Etest (bioMérieux, Baulkham Hills, NSW, Australia) (*14*).

Histologic examination of clinical samples revealed inflammation and granulomata, and Ziehl-Neelsen staining of samples from 3 case-patients revealed acid-fast bacilli. Phenotypic testing of isolates showed that arylsulfatase activity was positive at day 3 (weakly positive in 1 isolate); *p*-aminosalicylic acid degradation was positive, but the organism was also tolerant to 5% sodium chloride (results uncertain for 1 isolate); and iron uptake was positive, all of which are suggestive of *Mycobacterium abscessus*. Molecular sequencing identified *M. chelonae* with identical genetic profiles in 8 patient samples and 3 therapeutic solutions, including a Coley toxin homeopathic formulation, from Practitioner A's clinic. Isolates were susceptible to clarithromycin, amikacin, tobramycin, and tigecycline but resistant to cefoxitin.

Conclusions

These 27 cases of a rare infection occurred in clients of Practitioner A while no other cases were reported elsewhere in South Australia, representing overwhelming epidemiologic evidence that the source of infection was Practitioner A's clinic (Figure 2). Case-patients had received injections of an *M. chelonae*–contaminated Coley toxin formulation, and the same formulation had been included with colloidal silver in a spray solution that was used to "clean" patients' skin before injections were administered. These 2 uses of the contaminated formulation constitute plausible routes of infection and the source of the outbreak. Practitioner A's procedures demonstrated a profound lack of sterile injection techniques and infection-control practices. Therapeutic formulations were kept for long periods in nonsterile bottles that had been washed in tap water; this could have created favorable conditions for *M. chelonae* to multiply in the resulting biofilm. Sterile saline solution was used for the injections; however, it could have been contaminated before use by Practitioner A's usual procedure of decanting the sterile saline into other containers (e.g., plastic cups) before injection.

Our findings show that *M. chelonae* infection can be associated with alternative therapies if infection-control breaches occur and that tighter regulation of alternative therapy practices is needed. Effective control measures were implemented early in this outbreak; however, the investigation faced many challenges. First, Practitioner A's client records were incomplete, making it difficult to identify and contact all clients. Furthermore, some persons who use alternative therapies distrust mainstream medicine, so many of the clients were reluctant to be interviewed and/or undergo clinical assessment. Practitioner A also advised clients that an inflammatory reaction

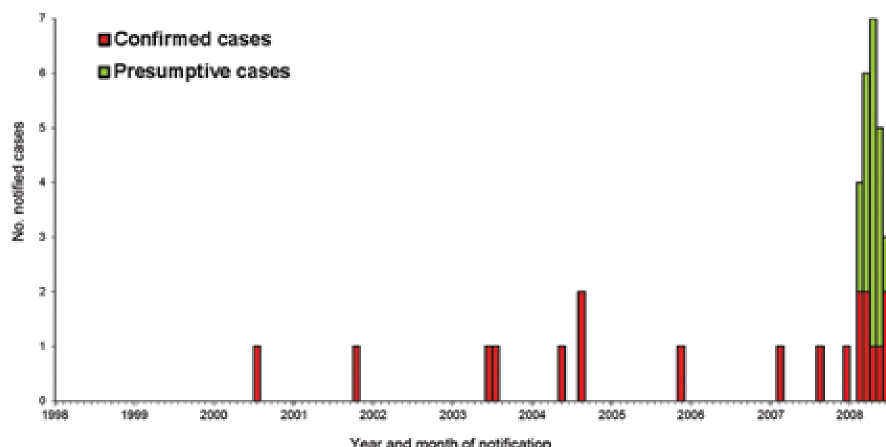

Figure 2. Confirmed and presumptive cases of *Mycobacterium chelonae* infection in South Australia, Australia, by month of onset (January 1998–August 2008). Two presumptive cases from 2008 are not included because onset dates were not known.

(boil) at the inoculation sites was expected as a sign that the biomesotherapy "worked." This advice prevented or delayed many of the case-patients from seeking medical attention when the skin lesions developed.

Second, most interviewees had poor recollection of the date of symptom onset and the materials and techniques used during treatment sessions. Therefore the interviews did not provide reliable evidence regarding the cause of outbreak. Third, mycobacterial infections are rare and difficult to diagnose. In some case, diagnosis, treatment, and reporting of cases were delayed because patients were referred for assessment by a specialist only after multiple courses of antimicrobial drug treatment failed to resolve symptoms. Case-patients who agreed to clinical assessment and treatment represented a challenge for clinicians because they required prolonged therapy and continuous monitoring for antimicrobial drug resistance. Fourth, the investigation and clinical care of case-patients involved substantial costs to the health care system.

Last, in the absence of specific legislation governing the practice of biomesotherapy, the regulatory response to this outbreak was undertaken by using general powers under the Public and Environmental Health Act 1987. This was justifiable on the basis of evidence collected, the risk to the public, and the strong likelihood that the harmful practice would continue because of Practitioner A's lack of insight into his/her role in the outbreak. However the longer term issue of regulating similar practitioners and their practices was also identified as a concern. Outbreaks associated with alternative therapies have sparked calls locally and internationally for the imposition of stringent regulations, including administrative mechanisms requiring registration or licensing of practitioners and mandating minimum standards with regard to premises and procedures.

Dr Ivan is a public health physician and medical epidemiologist. Her research interests are prevention and control of communicable diseases, evidence-based public health, and health-related attitudes and behaviors.

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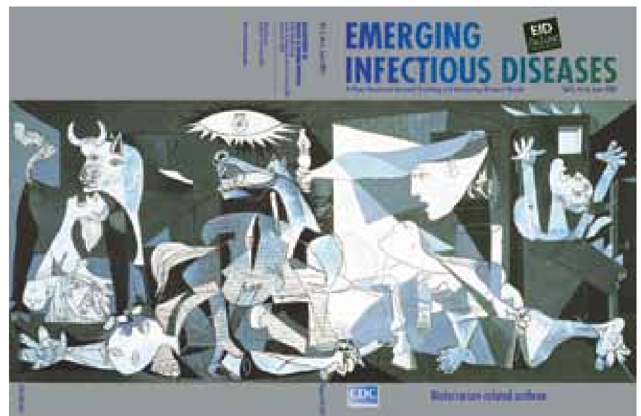

Spread of *Neisseria meningitidis* Serogroup W Clone, China

Haijian Zhou,[1] Wei Liu,[1] Li Xu, Lili Deng, Qiuyun Deng, Jiatong Zhuo, and Zhujun Shao

During February 2011–June 2012, invasive infection with *Neisseria meningitidis* serogroup W was identified in 11 persons in southeastern China. All isolates tested had matching or near-matching pulsed-field gel electrophoresis patterns and belonged to multilocus sequence type 11. The epidemiologic investigation suggested recent transmission of this clonal complex in southeastern China.

Neisseria meningitidis is a major public health threat in many parts of the world, including China. Since 2003, most meningococcal diseases in China have been caused by *N. meningitidis* serogroups A and C; only 3 cases of serogroup W meningococcal disease were reported before 2011 (*1*,*2*). However, during February 2011–June 2012, an increase in invasive disease caused by serogroup W *N. meningitidis* (11 cases total) was seen in southeastern China. To determine if this serogroup is emerging in China, we analyzed strains from 6 of the 11 infected patients reported during 2011–2012, from 16 of their close contacts, and from 3 serogroup W patients reported during 2006–2008.

The Study

Meningococcal disease is reportable in China. *N. meningitidis* isolates, cerebrospinal fluid (CSF), and blood samples from persons with invasive disease are forwarded to the Chinese Centers for Disease Control and Prevention (CDC) for serogroup determination by slide agglutination and/or PCR. Strains are further characterized by use of pulsed-field gel electrophoresis (PFGE) after *Nhe*I restriction enzyme digestion (*3*).

During February 2011–June 2012, we observed an increase in invasive meningococcal disease caused by *N. meningitidis* serogroup W in southeastern China. Of 11 cases total, 9 were diagnosed by strain isolation and 2 by PCR and real-time PCR of CSF samples (Table). Strains isolated from patients 1, 4, and 5 became nonviable during storage in the hospital laboratory. The 6 remaining strains (from patients 2, 3, 7, and 9–11) were submitted to the Chinese CDC along with 16 serotype W strains from close contacts of patients 4, 6, and 8–10. Thus, during 2011–2012, a total of 22 strains were submitted to the Chinese CDC, where they were confirmed as *N. meningitidis* serogroup W by slide agglutination with specific antiserum (Remel, Lenexa, KS, USA). In addition, CSF samples from patients 1, 4–6, and 8 were submitted and confirmed positive for *N. meningitidis* serogroup W by PCR and real-time PCR.

The 22 serogroup W strains from 2011–2012 were analyzed by PFGE; for comparison, 3 strains isolated from patients during 2006–2008 were also analyzed. PFGE patterns were distinguishable for 16 of the 22 strains from 2011–2012. Five strains associated with patient 9 and 1 strain isolated from a close contact of patient 4 had PFGE patterns that differed by 1 and 2 bands, respectively, indicating >94% similarity with the dominant pattern (Figure 1). PFGE patterns for the 3 isolates from 2006–2008 exhibited <90% similarity with those for isolates from 2011–2012, differing by 4–7 bands. All 22 isolates and 5 CSF samples from 2011–2012 were identified as sequence type (ST) 11 and PorA type P1.5,2, identical to the genotype of serotype W isolates associated with outbreaks reported in Saudi Arabia in 2000 and 2001 (*4*,*5*) and Burkina Faso in 2002 (*6*), sporadic cases in other countries (*7*–*9*), and the 3 cases identified in China during 2006–2008 (*2*).

Of the 11 patients with cases reported during 2011–2012, 4 resided in Guangxi Province, 2 in Guangdong Province, and 1 each in Jiangsu, Zhejiang, Anhui, Henan, and Hunan Provinces (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0160-Techapp1.pdf). The median age of patients was 20 years (range 3–46), 9 (81.8%) were male, and all denied recent foreign travel. Three of the 11 patients died of bacteremia. The epidemiologic investigation did not identify any common exposures, social settings, or other connections among the patients. Close contacts of all 11 patients were investigated, and no additional *N. meningitidis* infections were detected. Of the 11 reported cases, 5 occurred in or were associated with Laibin City, Guangxi Province: patients 1, 4, and 5 sought care in Laibin City; patient 3 sought care in Zhejiang Province on April 20, 2012, after having traveled to Zhejiang Province from Laibin City on March 26; and patient 8 sought care in Fangchenggang City, Guangxi Province, 10

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DOI: http://dx.doi.org/10.3201/eid1909.130160

[1]These authors contributed equally to this article.

Table. Clinical and epidemiologic characteristics of patients with ST11 serogroup W meningococcal disease, China, 2011–2012*

Patient ID	Age, y/sex	Date of symptom onset	Province of onset	Patient occupation	Outcome	Method of diagnosis	ST11 W strains from close contacts (no.)
1†	16/M	2011 Feb 12	Guangxi	Student	Survived	Strain isolation/ PCR	No
2	19/M	2011 Apr 1	Jiangsu	Factory worker	Died	Strain isolation	No
3	19/F	2011 Apr 20	Zhejiang	Factory worker	Died	Strain isolation	No
4†	18/M	2011 Apr 27	Guangxi	Student	Survived	Strain isolation/ PCR	Yes (8)
5†	46/M	2011 May 4	Guangxi	Farmer	Survived	Strain isolation/ PCR	No
6†	22/F	2011 Oct 13	Guangdong	Factory worker	Survived	PCR	Yes (1)
7	35/M	2012 Feb 1	Guangdong	Factory worker	Survived	Strain isolation	No
8†	23/M	2012 Feb 2	Guangxi	Factory worker	Survived	PCR	Yes (1)
9	14/M	2012 Feb 14	Anhui	Student	Died	Strain isolation	Yes (4)
10	3/M	2012 Mar 27	Henan	Student	Survived	Strain isolation	Yes (2)
11	9/M	2012 Jun 18	Hunan	Student	Survived	Strain isolation	No

*ID, identification; ST, sequence type.
†Multilocus sequence typing results were obtained from cerebrospinal fluid but not from strains.

days after a close contact (partner) had traveled to Laibin City (online Technical Appendix). The partner of patient 8 was subsequently tested and identified as a carrier of ST11 serogroup W *N. meningitidis*.

A survey of *N. meningitidis* carriage was conducted among the healthy population of Laibin City in September 2011. A total of 1,311 persons 1–45 years of age were investigated, of whom 8.54% (112/1,311 persons) were positive for *N. meningitidis* carriage. Age groups and percentages of infected persons in each age group were 1–6 years (1.4%), 7–12 years (6.0%), 13–15 years (6.7%), 16–20 years (18.5%), and 21–45 years (1.0%). The serogroup for each strain was determined by use of slide agglutination and polyclonal antisera and PCR methods. Of the 112 *N. meningitidis*–positive samples, 20 (17.9%) were ST11 serogroup W, and of those 20 samples, 2, 4, and 14 were from persons 7–12, 13–15, and 16–20 years of age, respectively. All 20 strains exhibited indistinguishable PFGE patterns that matched the dominant pattern of the disease-associated strains. The carriage rate of ST11 serogroup W *N. meningitidis* reached 5.5% (11/200) among 200 students (16–20 years of age) in 1 school.

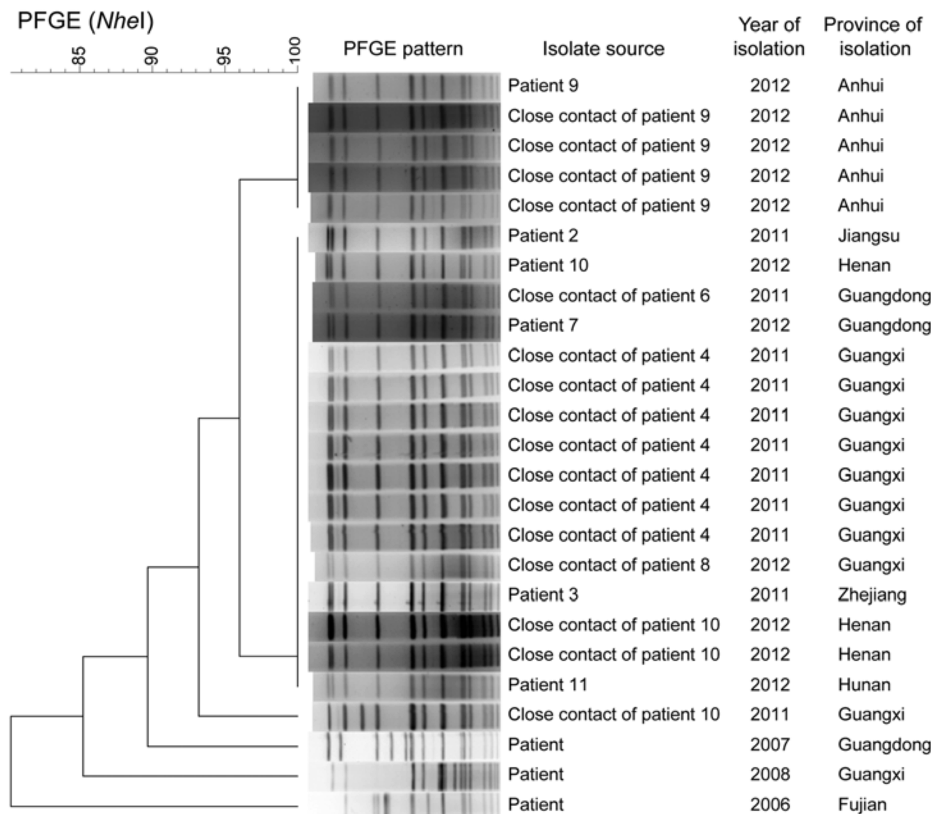

Figure 1. PFGE patterns for 22 *Neisseria meningitidis* serogroup W strains (6 from reported patients, 16 from close contacts) isolated during 2011–2012 and 3 isolated during 2006–2008, China. All isolates were sequence type 11 (determined by multilocus sequence typing) and PorA type P1.5,2. PFGE, pulsed-field gel electrophoresis. Scale bar indicates percent similarity.

Since 2003, the annual incidence of meningococcal disease in China has stayed below 0.2 cases/100,000 population. Surveillance data in China suggest a historical trend for seasonal peaks of meningococcal disease during February–April. This peak season corresponds with the spring dry season in China, a time when tourists are most likely to visit the country, especially southern China. Among the 45 cases of meningococcal disease confirmed during 2011–2012 by meningococcal etiology and PCR methods, 11 (24.4%) were caused by serogroup W *N. meningitidis* (Figure 2); 8 of these 11 cases occurred during February–April. The 3 cases reported during 2006–2008 occurred during May, June, and October, respectively.

Conclusions

The incidence of serogroup W infections reported during February 2011–June 2012 represents a marked increase over that reported during 2005–2010. The emergence and spread of a new *N. meningitidis* serogroup in a region presents a challenge for the prevention and control of meningococcal disease, especially if vaccines used in the region do not cover all serogroups. ST7 serogroup A and ST4821 serogroup C *N. meningitidis* strains were identified as the 2 dominant lineages circulating in China during 2003–2008, causing >90% of meningococcal disease cases (*1*). Meningococcal polysaccharide vaccines A and C have been used in China for routine immunization since the outbreak of *N. meningitidis* serogroup C during 2003–2004. In some African countries, repeated vaccination against *N. meningitidis* serogroups A and C is thought to have led to a selective increase in the incidence of meningococci of other serogroups, thereby resulting in a changed profile of meningococcal disease (*10–12*). Therefore, meningococcal disease caused by *N. meningitidis* strains that belong to serogroups other than A and C, especially those that belong to hyperinvasive lineages, appears to be an emerging problem in China.

The 11 cases of meningococcal disease caused by ST11 serogroup W *N. meningitidis* strains described here had successively emerged in southeastern China; furthermore, ST11 serogroup W meningococci were isolated from close contacts of the patients and from healthy carriers. These observations suggest the possible establishment and spread of a clonal complex of serogroup W meningococci in southeastern China. Carriage and transmission of this strain have led to the emergence of ST11 serogroup W organisms as a cause of endemic meningococcal disease. Further epidemiologic and microbiological surveillance is needed for monitoring of meningococcal diseases caused by serogroup W in southeastern China and preventing the spread of this clone to other regions.

This study was supported by the National Natural Scientific Foundation (no. 81201332) from the Ministry of Science and Technology of the People's Republic of China, grants from the Major State Basic Research Development Program of China (973 Program) (2011CB504900), and the Priority Project on Infectious Disease Control and Prevention (no. 2013ZX10004221 and 2012ZX10004215) from the Ministry of Health and the Ministry of Science and Technology of the People's Republic of China.

Mr Zhou is a microbiologist at National Institute for Communicable Disease Control and Prevention, Chinese CDC, Beijing. His research interests include molecular subtyping of pathogenic bacteria and molecular epidemiology of meningococcal meningitis.

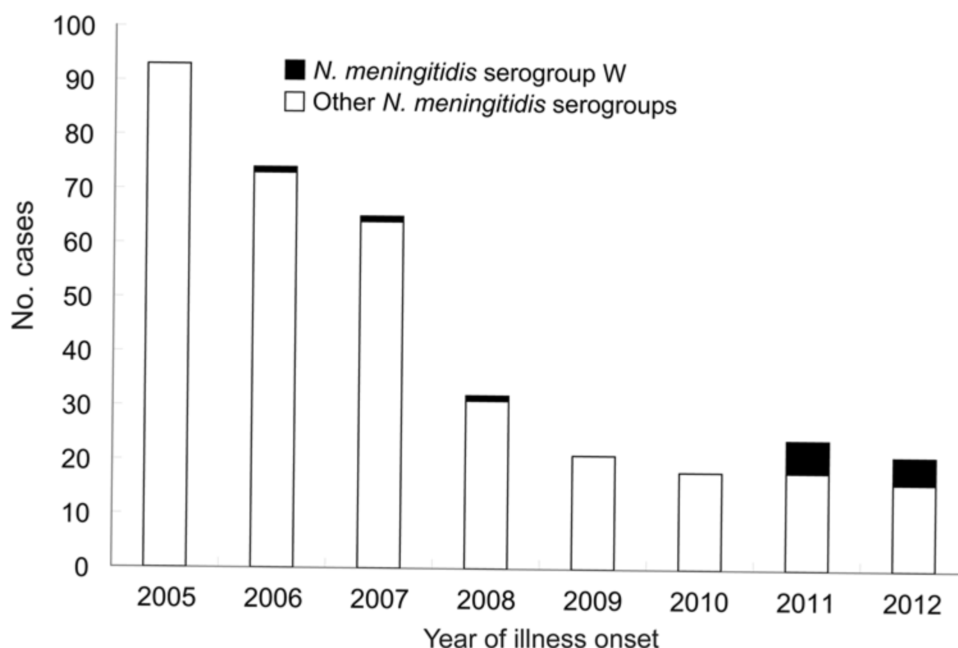

Figure 2. Laboratory-confirmed cases of meningococcal disease, by *Neisseria meningitidis* serogroup and year of symptom onset, China, 2005–2012.

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Human Parainfluenza Virus Type 3 in Wild Nonhuman Primates, Zambia

Michihito Sasaki, Akihiro Ishii, Yasuko Orba, Yuka Thomas, Bernard M. Hang'ombe, Ladslav Moonga, Aaron S. Mweene, Hirohito Ogawa, Ichiro Nakamura, Takashi Kimura, and Hirofumi Sawa

Human parainfluenza virus type 3 (HPIV3) genome was detected in 4 baboons in Zambia. Antibody for HPIV3 was detected in 13 baboons and 6 vervet monkeys in 2 distinct areas in Zambia. Our findings suggest that wild nonhuman primates are susceptible to HPIV3 infection.

Human parainfluenza viruses (HPIVs) (family *Paramyxoviridae*) are major causes of lower respiratory tract infections in infants and elderly persons. HPIVs are second to respiratory syncytial virus as the cause of hospitalizations for lower respiratory tract infections (*1*,*2*) and account for 6.8% of all hospitalizations for fever or acute respiratory illness in children <5 years of age (*3*). Among the 4 serotypes, HPIV3 (genus *Respirovirus*) causes particularly severe disease, including bronchiolitis and pneumonia (*1*–*3*). In addition to young children, HPIV3 poses a threat to the elderly and to immunocompromised adults. HPIV3 infection also causes severe illness leading to death (35%–75% death rate) in patients receiving hematopoietic stem cell transplants (*4*,*5*). Although the virus is distributed worldwide and maintained in human communities, its epidemiology in Africa is unclear.

Nonhuman primates, the closest living relatives of humans, are susceptible to paramyxoviruses that cause respiratory disease in humans. Recently, other researchers reported infections with human respiratory syncytial virus and human metapneumovirus in wild nonhuman primates in Africa (*6*,*7*). Therefore, as a first step in determining the pervasiveness of infection in African wild nonhuman primates, we screened these animals for paramyxovirus in Zambia. The HPIV3 genome was identified by seminested broad-spectrum reverse transcription PCR (RT-PCR). Thereafter, we investigated HPIV3 infection in wild nonhuman primates by using molecular and serologic methods.

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DOI: http://dx.doi.org/10.3201/eid1909.121404

The Study

Baboons and vervet monkeys live side by side with humans in game management areas in Zambia, and this situation often leads to high levels of human–baboon/monkey conflicts. The Zambia Wildlife Authority is mandated by the Zambian government to control the large numbers of these animals. We collected tissues and serum samples from baboons and vervet monkeys killed for pest management purposes with the permission of the Zambia Wildlife Authority (certificate no. 2604). Samples were obtained from 50 yellow baboons (*Papio cynocephalus*) and 50 vervet monkeys (*Chlorocebus pygerythrus*) in the Mfuwe region (13°16′30.2″S, 31°40′00.4″E), Eastern Province, Zambia, in 2009 and from 50 chacma baboons (*P. ursinus*) and 39 vervet monkeys (*C. pygerythrus*) in the Livingstone region (17°50′8.72″S 25°43′ 59.19″E), Southern Province, Zambia, in 2010 and 2011. Sample information is summarized in the Table. The species were identified on the basis of morphologic characters and the mitochondrial cytochrome b (*cytb*) gene sequence. The complete *cytb* gene was amplified from spleen DNA of *Papio* spp. baboons with the primer set papio cytb1F (5′-GATACGAAAAACCATCGCTGT-3′) and papio cytb2R (5′-GCTCCATTTCTGGTTTACAAG-3′), as described (*8*), and from spleen DNA of *Chlorocebus* spp. monkeys with the primer set chlorocebus cytb1F (5′-TGATATGAAAAACCACCGTTGT-3′) and chlorocebus cytb2R (5′-GCTTTCTTTCTGAGTTGTCCTAGG-3′), designed in this study.

Total RNA was extracted from 189 spleen tissue samples by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and screened for paramyxoviruses by seminested broad-spectrum RT-PCR of paramyxovirus polymerase (*L*) genes (*9*). Amplification was carried out with the primers PAR-F1, PAR-F2, and PAR-R. Seminested RT-PCR was positive in only 1 chacma baboon sample. The PCR product (584 bp) was subjected to direct sequence analysis, and the identified paramyxovirus was tentatively named ZMLS/2011. BLAST analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi) indicated that ZMLS/2011 shared 98% nt identity with the HPIV3 *L* gene. To increase the sensitivity of HPIV3 genome detection, we screened all 189 RNA samples by RT-PCR, using the HPIV3 *L* gene–specific primer sets HPIV3 L1F (5′-ATGGGAGAATTCTTCCTCAAGCTC-3′) and HPIV3 L2R (5′-AATGCRGCAACTGATGGATCACC-3′). An HPIV3 genome was detected in 3 (6%) of 50 chacma baboon samples and in 1 (2%) of 50 yellow baboon samples but not from any of the 89 vervet monkey samples. Nucleotide sequences of all 4 amplicons (367 bp) were identical to ZMLS/2011.

Table. Sample information and results of the molecular and serologic analyses of human parainfluenza virus type 3, Zambia

Animal (species)	Sampling location	RT-PCR positive/total*	Western blot positive/total
Yellow baboons (*Papio cynocephalus*)	Mfuwe	1/50	2/50
Vervet monkeys (*Chlorocebus pygerythrus*)	Mfuwe	0/50	3/50
Chacma baboons (*P. ursinus*)	Livingstone	3/50	11/50
Vervet monkeys (*C. pygerythrus*)	Livingstone	0/39	3/39
Total		4/189	19/189

*RT-PCR, reverse transcription PCR.

In an attempt to isolate virus from RT-PCR–positive spleen, Vero cells cultured in minimum essential medium supplemented with trypsin were injected with tissue homogenates; however, after 3 passages, cytopathic effects were not observed. Viral RNA was also not detected in the culture supernatants from the cells.

To confirm and classify ZMLS/2011 as a strain of HPIV3, we amplified and sequenced the genome of ZMLS/2011 using total RNA sample positive for the RT-PCR screening. The obtained ZMLS/2011 sequence (15,298 bp) was deposited in the DDBJ database (GenBank/EMBL/DDBJ entry AB736166). The HPIV3 genome encodes 6 structural proteins, N, P, M, F, HN, and L. All the corresponding open reading frames were found in the ZMLS/2011 genome. A phylogenetic analysis was performed by using MEGA5 and based on the deduced amino acid sequence of the full-length HN protein (*10*). The phylogenetic tree clearly established ZMLS/2011 within the lineage of HPIV3 and distinct from other known parainfluenza viruses related to it (Figure 1). ZMLS/2011 is most closely related to HPIV3 strain Riyadh 149/2009, isolated from a hospitalized child in Saudi Arabia in 2009 (*11*). These results indicated that ZMLS/2011 identified from the chacma baboon is a strain of HPIV3.

Antibodies were detected by a recombinant N protein–based Western blot. Recombinant N protein of ZMLS/2011 was expressed in *Escherichia coli* and purified by histidine tag–based affinity chromatography. The 189 serum specimens were screened by using the Mini-PROTEAN II Multiscreen Apparatus (Bio-Rad, Hercules, CA, USA). Mouse monoclonal HPIV antibody (MAB819; Millipore, Billerica, MA, USA) served as the positive control. Among the serum samples tested, 2 (4%) from 50 yellow baboons, 11 (22%) from 50 chacma baboons, and 6 (7%) from 89 vervet monkeys had HPIV3 antibodies (Table; Figure 2). Positive results were obtained from serum samples collected from

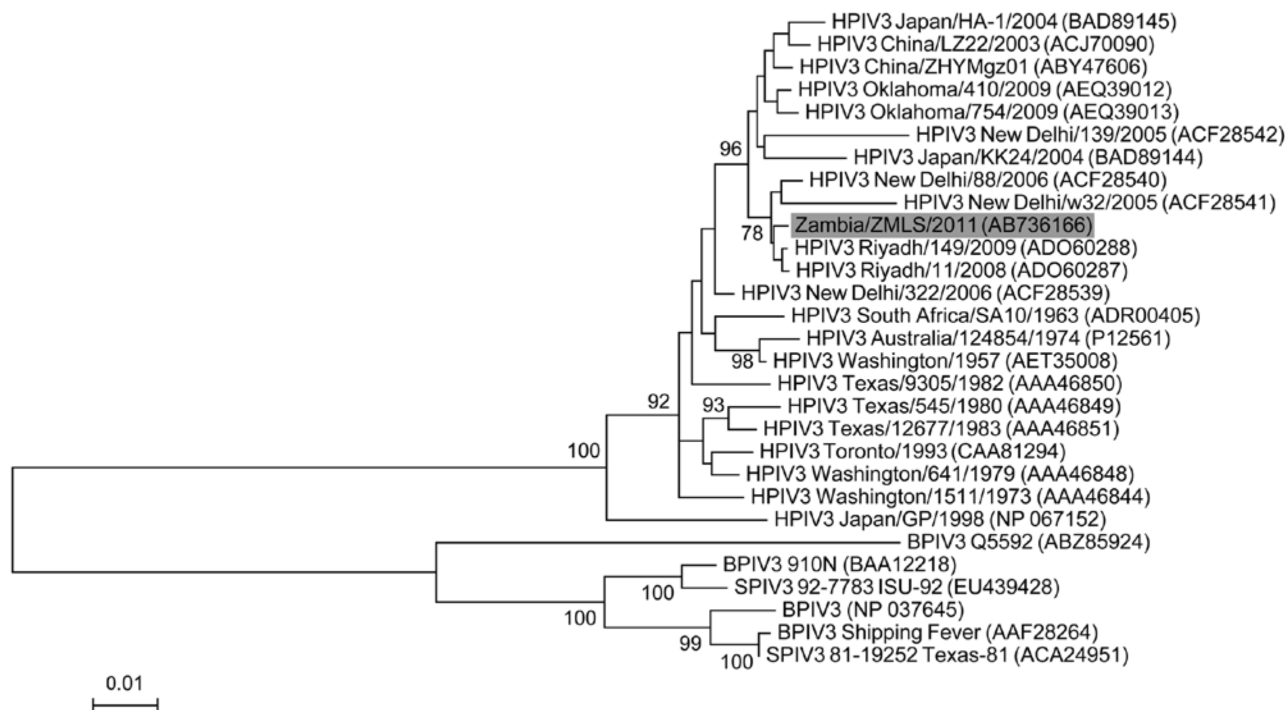

Figure 1. Phylogenetic analysis of the amino acid sequence of the HN protein of human parainfluenza virus type 3 (HPIV3). The phylogenetic tree was constructed on the basis of the deduced amino acid sequence of the full-length HN gene of ZMLS/2011 (gray shading) and known paramyxoviruses. GenBank accession numbers are given in parentheses. Significant bootstrap values (>70%) are shown. Scale bars indicate amino acid substitutions per site. SPIV, simian parainfluenza virus; BPIV, bovine parainfluenza virus.

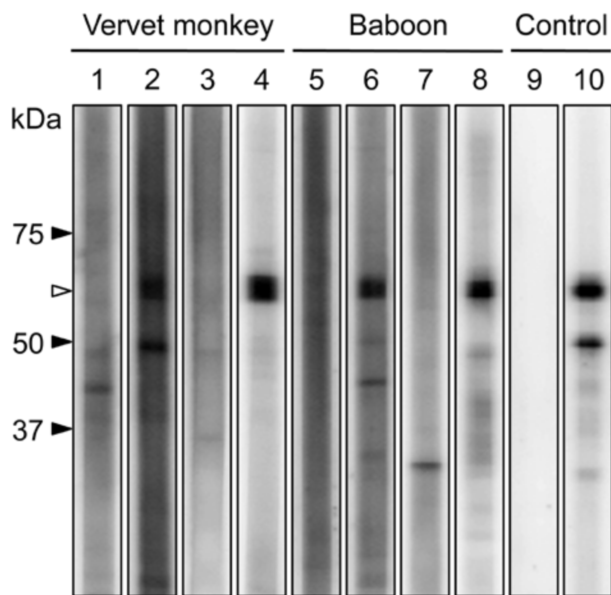

Figure 2. Western blot analysis of purified recombinant N protein from human parainfluenza virus type 3. Western blot analysis was performed by using serum specimens from vervet monkeys (lanes 1–4) and baboons (lanes 5–8) in the Mfuwe (lanes 1, 2, 5, 6) and Livingstone (lanes 3, 4, 7, 8) regions. Results of representative antibody-negative (lanes 1, 3, 5, 7) and antibody-positive (lanes 2, 4, 6, 8) samples are shown. Mock antibody (lane 9) and HPIV monoclonal antibody (lane 10) were used as negative and positive controls, respectively. The putative molecular mass of the recombinant N protein (white arrowhead) is 59 kDa.

animals in the Mfuwe and Livingstone regions. All 4 baboons positive for the HPIV3 genome were negative for HPIV3 antibodies (data not shown), suggesting that, at the time the samples were taken, these HPIV3 antibody–negative baboons might have been in the acute stage of infection, before a detectable immune response had developed.

Conclusions

We identified wild baboons positive for HPIV3 by using molecular and serologic analyses. Seropositive vervet monkeys also were found. These nonhuman primates are widely distributed in Africa (*8*), and their habitats overlap those of humans, mainly in rural areas. A survey performed in Kenya of humans with influenza-like illness and severe acute respiratory illness showed that 9.4% were positive for HPIVs (*12*). However, HPIV3 infection in humans in Africa has been poorly studied, making ZMLS/2011 difficult to compare with an epidemic strain of HPIV3 among humans. The nonhuman primates sampled in this study live side by side with humans. In addition, many tourists visit the Livingstone region and, in some instances, are harmed by the nonhuman primates attempting to grab food carried by humans. Detection of HPIV3 in wild nonhuman primates might be related to these contacts. Further epidemiologic studies of humans and wild nonhuman primates are needed to determine whether HPIV3 is transmitted between humans and wild nonhuman primates.

Serologic evidence of HPIV3 infection was obtained from baboons and vervet monkeys in 2 distinct geographic areas of Zambia, but little is known about HPIV3 infection in wild nonhuman primates. In 1963, simian agent 10 (also known as simian virus 10) was isolated from the mouth of a Samango monkey (*Cercopithecus mitis*) in a laboratory in South Africa (*13*). Complete genome sequence analysis showed simian agent 10 as a strain of HPIV3 (*14*). Experimental infections showed that many nonhuman primates—including chimpanzees; macaques; and squirrel, owl, patas, and rhesus monkeys—are sensitive to HPIV3 infection (*1*,*15*). These previous reports support our finding that wild nonhuman primates are susceptible to HPIV3 infection.

Acknowledgment

We thank the Zambia Wildlife Authority for supporting the HPIV3 research in Zambia.

This study was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology and the Ministry of Health, Labour and Welfare of Japan; the Japan Initiative for Global Research Network of Infectious Diseases; and the Global Center of Excellence Program "Establishment of International Collaboration Centers for Zoonosis Control" (Japan).

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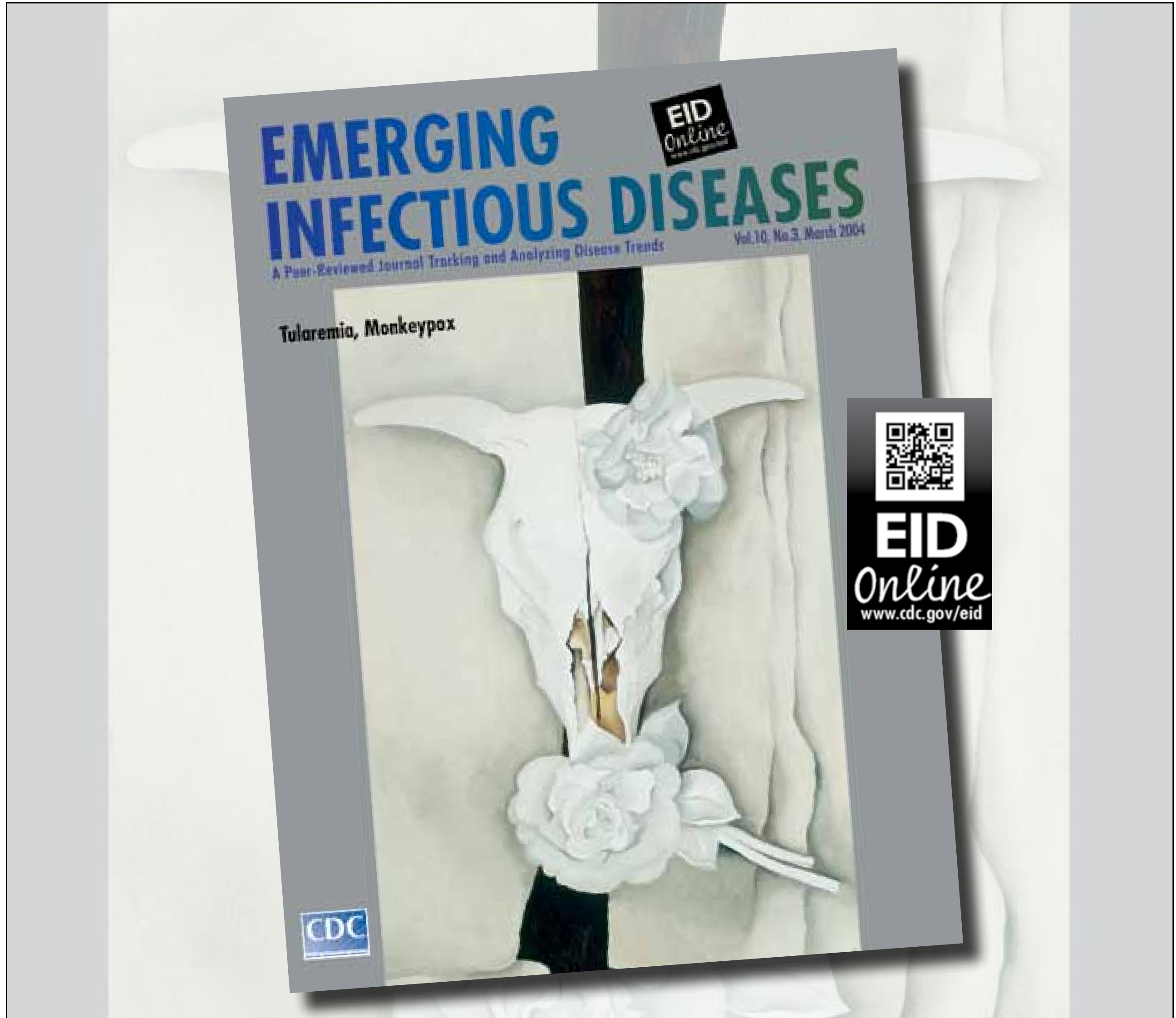

Powassan Meningo-encephalitis, New York, New York, USA

Simon Sung,[1] Alysse G. Wurcel,[1] Susan Whittier, Karen Kulas, Laura D. Kramer, Robin Flam, James Kirkland Roberts, and Simon Tsiouris

Disease caused by Powassan virus (POWV), a tick-borne flavivirus, ranges from asymptomatic to severe neurologic compromise and death. Two cases of POWV meningoencephalitis in New York, USA, highlight diagnostic techniques, neurologic outcomes, and the effect of POWV on communities to which it is endemic.

Powassan virus (POWV), a rare neuroinvasive arbovirus, was first described in 1958 (*1*,*2*). POWV has been isolated from *Ixodes* ticks; implicated hosts include woodchucks, red squirrels, chipmunks, groundhogs, and white-footed mice (*1*–*4*). Symptoms of infection vary from mild myalgia to acute flaccid paralysis and neurologic involvement. In the United States, POWV has been reported in northeastern and north-central states, and incidence is increasing (*3*,*4*). We describe the clinical characteristics and outcomes of 2 patients with POWV in New York, New York, USA.

Case Reports

Case 1

In mid-February 2009, a 22-year-old man with a remote history of Lyme disease (LD) was transferred to Columbia University New York Presbyterian Hospital (CUNYPH) after an extensive work-up for aseptic meningitis. On December 20, 2008, he had flown home to eastern Long Island, New York, from Colorado. Sore throat and influenzaa-like symptoms developed, and he sought care from a local physician in early January. Rapid strep test result was negative, but oral cephalosporin was prescribed. His symptoms improved, and he returned to Colorado. Approximately 2 weeks later, symptoms recurred, along with fever, eye pain, lateral gaze palsy, ataxia, dysarthria, stomach pain, and neck stiffness. After visiting the student health center, he was admitted to a local hospital in early February. Results of blood tests were within normal limits; cerebrospinal fluid (CSF) contained 212 leukocyte/µL (reference 0–5 cells/µL) (95% lymphocytes), 0 erythrocytes/µL (reference 0/µL), protein 55 mg/dL (reference 15–60 mg/dL), and glucose 60 mg/dL (reference 50–80 mg/dL). He received intravenous ceftriaxone and acyclovir, improved slightly, and was discharged after 1 week. He returned to New York and was admitted to a local hospital 1 week after discharge with persistent neurologic signs. Repeat CSF analyses showed 59 leukocytes/µL (100% lymphocytes); given concern about LD, he was placed on ceftriaxone until LD serologic results were negative. All other PCRs and serologic work-ups for infectious agents were negative (online Technical Appendix, Table, wwwnc.cdc.gov/EID/articlepdfs/19/9/12-1846-Techapp1.pdf).

Given limited improvement, he was transferred to CUNYPH for reevaluation. On arrival, neurologic examination revealed decreased alertness, dysarthria, and ataxia. T2-weighted magnetic resonance imaging of the brain showed bilateral caudate and basal ganglia hyperintensities consistent with viral encephalitis (online Technical Appendix, Figure 1, panels A, B). He reported raising chickens, frequently drinking unpasteurized milk, and hiking in a wooded area in Long Island during holiday break, and his college roommate had a recent "mono-like" illness. Results of serologic tests on serum and CSF collected in mid-February showed polyvalent antibodies to POWV (Table). POWV meningoencephalitis was confirmed with >4-fold change in POWV plaque reduction neutralizing test (PRNT) titers in paired serum samples: February titer, 20; March titer, 320. At discharge in late February, symptoms improved with mild residual tremor, but dysarthria persisted. At 1-month follow-up, persistent dysarthria remained, but other symptoms had resolved completely.

Case 2

In early May 2012, a 34-year-old man with a history of genital herpesvirus infection was admitted to a local hospital with lower extremity weakness and altered mental status of unclear etiology. In late April 2012, headache, fever, chills, and bilateral ankle pain had developed. Despite treatment with oral doxycycline for presumptive LD, symptoms persisted; development of bilateral leg weakness, confusion, and diplopia prompted evaluation at the local hospital 1 week after symptom onset. He was afebrile and hemodynamically stable; physical examination revealed decreased alertness, bilateral proximal leg weakness, and absence of neck rigidity. Further questioning

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DOI: http://dx.doi.org/10.3201/eid1909.121846

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Table. Results of initial arboviral serologic testing and POWV RT-PCR on CSF from 2 patients, performed at Wadsworth Center, New York State Department of Health, New York, USA*

Test	Test result, month	
	Patient 1, 2009	Patient 2, 2012
West Nile virus IgM ELISA	Nonreactive, Feb	Nonreactive, May
Eastern equine encephalitis virus IgG IFA	<16, Feb	<16, May
Western equine encephalitis virus IgG IFA	<16, Feb	<16, May
California serogroup IgG IFA	<16, Feb	<16, May
St. Louis encephalitis virus IgG IFA	<16, Feb	<16, May
rPOW-E polyvalent MIA† (titer)	Reactive, Feb	Reactive, May
Powassan PRNT (titer)	+ (20), Feb + (320), Mar	+ (280), early May + (320), late May + (160), early Jun + (10), late Jun
Powassan RT-PCR CSF	Not done	–

*POWV, Powassan virus; RT-PCR, reverse transcription PCR; CSF, cerebrospinal fluid; IFA, immunofluorescent assay; MIA, microsphere immunoassay; PRNT, plaque reduction neutralizing test;; +, positive; –, negative.
†A fluorescent MIA technique using recombinant POWV envelope glycoprotein antigen to detect IgG, IgA, and IgM.

revealed a history of 2 transient rashes (1 each on trunk and arm) 1 week before symptom onset, but they were not visible on physical examination. CSF showed 145 leukocytes/µL (100% lymphocytes), 35 erythrocytes/µL, protein 142 mg/dL, and glucose 39 mg/dL. He received intravenous vancomycin, ceftriaxone, and acyclovir. T2-weighted magnetic resonance imaging of the brain showed hyperintensities in bilateral temporal lobes (online Technical Appendix, Figure 2). Ceftriaxone was discontinued after the LD test result was negative. Results of all other infectious disease tests were negative (online Technical Appendix, Table).

Because of worsening symptoms, the patient was transferred to CUNYPH 3 days later. Repeat CSF analysis showed 78 leukocytes/µL with lymphocyte predominance. The patient resided in rural New York, had a remote history of tick bites, and had recently disposed of a bird nest. His wife worked as a daycare assistant, and he had 2 healthy children <5 years of age.

Serum was sent to the New York State Department of Health, and testing for POWV was requested after the family reported that, 2 years earlier, a neighborhood child 2 years was infected with POWV. POWV reverse transcription PCR in CSF sent from his previous hospital was negative; however, polyvalent antibody to POWV was detected in serum collected in early May. POWV infection was confirmed by >4-fold change in serum POWV PRNT titers in paired serum samples: early May, 1,280; late May, 320; early June, 160; and late June, 10. Serologic results for other arboviral diseases were negative (Table). On day 10 of hospitalization, his mental status returned to baseline, and he began to walk with a rolling walker. He was discharged on day 11 to an acute-care rehabilitation facility. Two weeks after discharge, the patient reported major improvement in leg strength and took a few steps unassisted. His symptoms relapsed 1 month after hospitalization, prompting admission for a trial of intravenous steroid therapy (1,000 mg methylprednisolone for 3 days). Repeat work-up for encephalitis was negative for all viral, bacterial, and fungal diseases, except positive for POWV antibodies. His symptoms improved, and he walked assisted by a rolling walker. Six months after the initial admission, he had improved substantially with outpatient physical therapy; after 10 months, he returned to work despite residual leg weakness .

Conclusions

In 2011, the Centers for Disease Control and Prevention confirmed 16 cases of POWV infection—a substantial increase from previous years but most likely an underestimate because underdiagnosis is probably common (*4–6*). New York State alone reported 12 POWV neuroinvasive cases during 2001–2010 (*5*). Although most cases reported in the literature occurred during June–September, tick feeding and transmission of POWV can extend into the milder periods of spring, autumn, and even winter. The clinical characteristics, course of disease, and residual neurologic deficits vary (*3*,*7–11*).

As with other arboviral infections, POWV diagnosis is complicated and requires knowledge of and access to diagnostic tests (*12*). Physicians in areas to which POWV is endemic should be aware of POWV and request serologic testing on serum or CSF samples from the state or other laboratories capable of performing POWV serologic tests. CSF PCR for POWV may be negative, especially if patients seek care later in their clinical course, and PRNT may be useful, especially when clinical suspicion is high. Although increasing PRNT titers suggest acute infection, we cannot determine the significance of the change in titers over time. A cohort study with long-term follow-up of POWV-infected patients with careful documentation of serology and radiologic images would help with prognostication; identify risk factors for more severe disease; and measure the effect of interventions, such as corticosteroid therapy.

Because no vaccines or effective antiviral agents exist for POWV, physicians in disease-endemic areas should advise their patients to take precautions to prevent tick bites,

including wearing light-colored clothes with pants tucked into socks, using tick repellent, and carefully inspecting themselves and their pets for ticks after spending time outdoors (*13*). In addition, physicians should be aware of the existence, epidemiology, prognosis, diagnosis, and complications of POWV infection because this disease is likely to increase in areas to which it is endemic.

Acknowledgments

We thank the patients and their families for their proactive attitudes toward diagnosis, treatment, and education of other doctors and families in the community about POWV. We also thank Ian Dupuis for conducting the PRNTs. Finally, we thank the Arbovirus Laboratory, Diagnostic Immunology Laboratory, and the Viral Encephalitis Laboratory, Wadsworth Center, New York State Department of Health; for performing real-time PCRs on CSF for human herpesviruses, adenovirus, enterovirus, and POWV.

Dr Sung is a pathology resident at Columbia University/New York Presbyterian Hospital. His research interests include neuroinvasive diseases and brain tumors.

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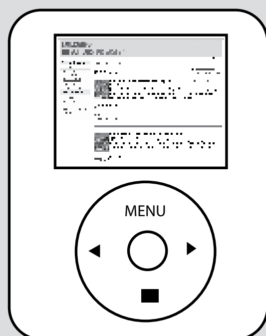

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Serogroup W135 Meningococcal Disease, The Gambia, 2012

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In 2012, an outbreak of *Neisseria meningitidis* serogroup W135 occurred in The Gambia. The attack rate was highest among young children. The associated risk factors were male sex, contact with meningitis patients, and difficult breathing. Enhanced surveillance facilitates early epidemic detection, and multiserogroup conjugate vaccine could reduce meningococcal epidemics in The Gambia.

Meningococcal disease is endemic to the African "meningitis belt"; outbreaks occur regularly (*1*,*2*). *Neisseria meningitidis* serogroup A causes most (80%) cases. However, during 2002–2003, serogroup W135 caused a major epidemic in Burkina Faso (attack rate [AR] 251 cases/100,000 population) (*3*). Thereafter, the incidence of serogroup W135 was low, with isolated cases and a small-scale outbreak in the meningitis belt (*4*,*5*). In 2010, serogroup A conjugate vaccine was introduced into the African meningitis belt and substantially reduced the incidence of meningitis (*6*).

In The Gambia, only 6 serogroup W135 cases were identified during 1990–1995; the most recent case had been reported in 1995 (*7*). In 2012, a large epidemic of serogroup W135 occurred throughout the meningitis belt, including The Gambia (*1*). Most risk factors identified in the meningitis belt concern serogroup A (*8*,*9*), and risk factors for serogroup W135 are little studied. Therefore, we report the investigation of this epidemic and the related risk factors.

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DOI: http://dx.doi.org/10.3201/eid1909.130077

The Study

The Gambian Ministry of Health and the Medical Research Council Unit, The Gambia, investigated a serogroup W135 epidemic that occurred during February–June 2012 in the Central River Region (CRR) and Upper River Region (URR). Since 2008, surveillance of invasive bacterial diseases has been ongoing in Bansang Hospital in CRR and Basse Health Centre in URR (*10*). The peripheral health centers refer severely ill patients to these health facilities. Three approaches were used to recruit persons with suspected cases of serogroup W135: enhanced prospective surveillance in Bansang Hospital and Basse Health Centre, retrospective case identification from hospital records, and visits to households with confirmed case-patients serogroup W135 to identify other suspected cases. A suspected case was defined as a history of acute onset of fever and any of the following: altered consciousness, inability to eat, neck stiffness, seizures, petechial rash, or bulging anterior fontanel in a child <2 years of age. Cerebrospinal fluid (CSF) and/or blood samples were collected from hospitalized persons with suspected serogroup W135. A confirmed case was a suspected case in which serogroup W135 was identified by culture and/or an antigen-specific test. The alert threshold was defined as ≥5 meningitis cases per 100,000 persons per week; the epidemic threshold was ≥10 cases (*11*).

The investigation team administered 1 dose of ciprofloxacin to each close contact of confirmed case-patients and provided health information to raise awareness. At the end of the epidemic, The Gambian government deployed the tetravalent meningococcal polysaccharide vaccine.

CSF and blood samples were cultured for bacteria in BACTEC Medium (Becton Dickinson, Franklin Lakes, NJ, USA) and tested for serogrouping by latex agglutination by using BACTEC and Ramel (Thermo Fisher Scientific, Waltham, MA, USA) test kits. Antimicrobial drug susceptibility was tested.

We conducted a matched case–control (ratio 1:1) study to identify risk factors. Healthy controls were matched by age and village with confirmed case-patients, including those who died. Demographic, socioeconomic, and exposure (within 14 days before illness onset) data were collected by using a structured questionnaire. Risk factors were analyzed by conducting bivariate matched and multivariate conditional logistic regression analyses. The Joint Gambia Government/Medical Research Council Ethics Committee approved the study. All study participants or legal guardians provided written informed consent.

During February 1–June 25, 2012, a total of 469 suspected cases were identified, and 114 were confirmed to be

Table 1. Confirmed and suspected cases of *Neisseria meningitidis* serogroup W135, CRR and URR, The Gambia, February 1–June 25, 2012*

Health region/case-patient age group, y	Cases, no. (%)	Deaths, no. (%)	2011 population†	Cases/100,000 population
CRR				
<1	62 (20)	8 (13)	4,216	1,470
1–4	138 (45)	4 (3)	29,470	468
5–14	70 (23)	2 (3)	66,545	105
≥15	37 (12)	4 (11)	115,995	32
Total	307 (100)	18 (6)	216,227	142
URR				
<1	47 (29)	10 (21)	4,086	1,150
1–4	70 (43)	5 (7)	27,564	254
5–14	36 (22)	2 (6)	64,014	56
≥15	9 (6)	1 (11)	111,663	8
Total	162 (100)	18 (11)	207,327	78
CRR and URR				
<1	109 (23)	18	8,302	1,312
1–4	208 (44)	9	57,034	364
5–14	106 (23)	4 (4)	130,560	81
≥15	46 (10)	5 (11)	227,658	20
Total	469 (100)	36 (8)	423,554	111

*CRR, Central River Region; URR, Upper River Region.
†Estimated on the basis of 2003 census.

serogroup W135. Thirty-one were co-primary or secondary cases in confirmed case-patients' households. Most (67%) suspected case-patients were <5 years of age, and 56% of cases occurred in male patients. The overall case-fatality rate was 8%.

The overall AR was 111 cases per 100,000 persons but was much higher among younger children (Table 1). The epidemic threshold was exceeded in the last week of February and continued until April for persons of all ages and until June for children <5 years of age (Figure 1, panels A, B). Among children <5 years of age, the peak AR attained 83 and 47 cases per 100,000 persons per week in CRR and URR, respectively (Figure 1, panel B). The epidemic peaked during the high temperature/driest months (March–May) and ended abruptly after the first rainfalls in June (Figure 2).

The most common signs and symptoms among the 113 confirmed serogroup W135 case-patients were weakness (96%), irritability (88%), neck stiffness (81%), and inability to eat (80%). Bulging fontanelle (74%), altered mental status (73%), and seizures (65%) occurred in a slightly lower proportion of case-patients.

Blood and/or CSF samples were collected from 301 (69%) of 438 hospitalized suspected case-patients, of which almost half (138) were positive for bacterial pathogens. Serogroup W135 was the major pathogen (114 [83%] of 138); followed by *Streptococcus pneumoniae* (13%) and *Staphylococcus aureus* (2%). Common antibacterial drugs used for meningitis were tested on 92 (81%) serogroup W135 isolates, which were susceptible to most of them (ampicillin, 100%; chloramphenicol, 99%; ciprofloxacin, 99%; penicillin, 95%; and tetracycline, 92%) but not to erythromycin (59% susceptible) and trimethoprim/sulfamethoxazole (100% resistant).

We enrolled 106 confirmed case–control pairs. Risk factors identified in the univariate analysis were male sex, students, >4 children 1–5 years of age in the household, contact with a meningitis case-patient, preceding history of respiratory illness (nasal discharge, difficult breathing), and itchy eyes (Table 2, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0077-T1.htm). In the multivariate analysis, male sex (odds ratio [OR] 1.9; 95% CI 1.0–3.7), contact with meningitis case-patients (OR 4.8; 95% CI 1.3–17.8), difficult breathing (OR 6.8; 95% CI 1.4–33.4), and itchy eyes (OR 4.4; 95% CI 1.3–14.4) remained significantly associated with cases.

Conclusions

Before the current cases, the most recent sporadic cases in The Gambia were reported in the early 1990s. These cases were part of a larger epidemic in the meningitis belt with a comparable predominance of serogroup W135 followed by *S. pneumoniae* (*1*). After introduction of MenAfriVac (serogroup A conjugate vaccine), incidence and epidemics caused by serogroup A decreased substantially in the meningitis belt (*6*,*12*). The reemergence of epidemic serogroup W135 in this region requires a strategy for surveillance, epidemic detection and control, and revised vaccination policy.

Serogroup A outbreaks usually affect children >5 years of age and young adults (*5*,*13*,*14*). However, two thirds of the serogroup W135 cases occurred in children <5 years of age, for whom the AR was 5-fold higher than it was for older age groups, similar to the characteristics of

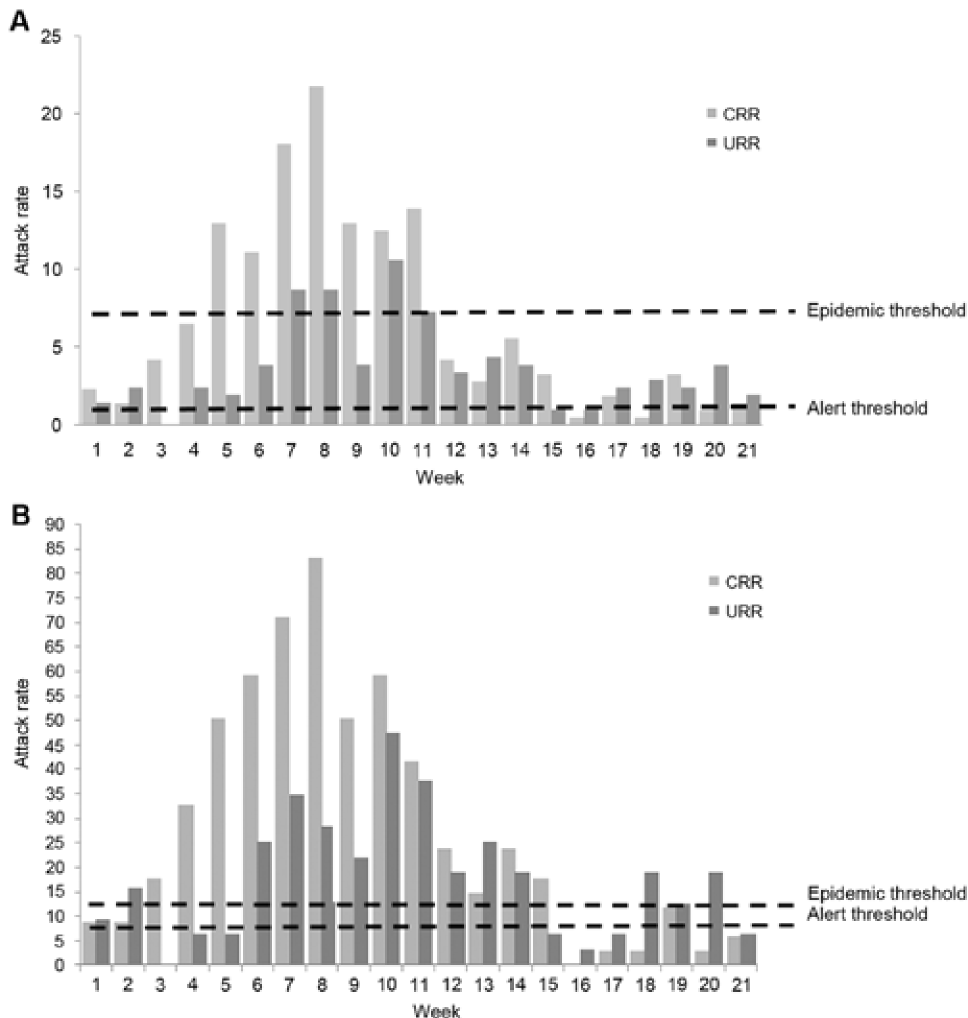

Figure 1. Outbreak of *Neisseria meningitidis* serogroup W135, Central River and Upper River Regions, The Gambia, February 1–June 25, 2012. A) Attack rate per 100,000 persons per week. B) Attack rate per 100,000 children <5 years of age per week. Light gray bars, Central River Region; dark gray bars, Upper River Region. Alert threshold corresponds to an attack rate of 5; epidemic threshhold, to an attack rate of 10.

the serogroups W135 outbreaks in Burkina Faso (2002–2003) and Niger (2010) (*3*,*5*). Therefore, the current operational definition of alert and epidemic thresholds, drawn mostly from serogroup A data, should be revised because merging the AR for all age groups may delay, or result in nondetection of, a serogroup W135 epidemic in younger age groups.

Signs and symptoms of concurrent respiratory illness were more prevalent among case-patients than controls;, itchy eyes and difficult breathing were associated with disease. The temporal sequence of these signs relative to the occurrence of meningococcal disease was not determined, and whether these factors facilitated the invasion of serogroup W135 carried in the nasopharynx or whether these symptoms were part of the initial serogroup W135 infection before onset of severe disease is unclear. Contact with confirmed serogroup W135 case-patients was a strong risk factor. These results are consistent with information available for the other serogroups and with the route of serogroup W135 transmission through droplet infection (*15*).

Our findings suggest that isolation of case-patients and prophylactic treatment of contacts may reduce transmission of meningococcal disease during epidemics. Enhanced surveillance for meningitis is recommended for early detection of epidemics. The occurrence of this large serogroup W135 outbreak suggests that multiserogroup conjugate vaccine should be deployed for control and prevention.

Acknowledgment

We thank the communities of the CRR and URR of The Gambia, the staff of the government health facilities, the Regional Health Teams, and the Ministry of Health for participating and supporting outbreak investigation. We also thank Lady Chilel Sanyang and Jarrah Manneh for laboratory testing; Golam Sarwar and Sarra Baldeh for organizing the data; and Edrissa Sabally and Yerro Bah for coordinating the field team and data collection. We thank Pa Cheboh Saine for the logistical support.

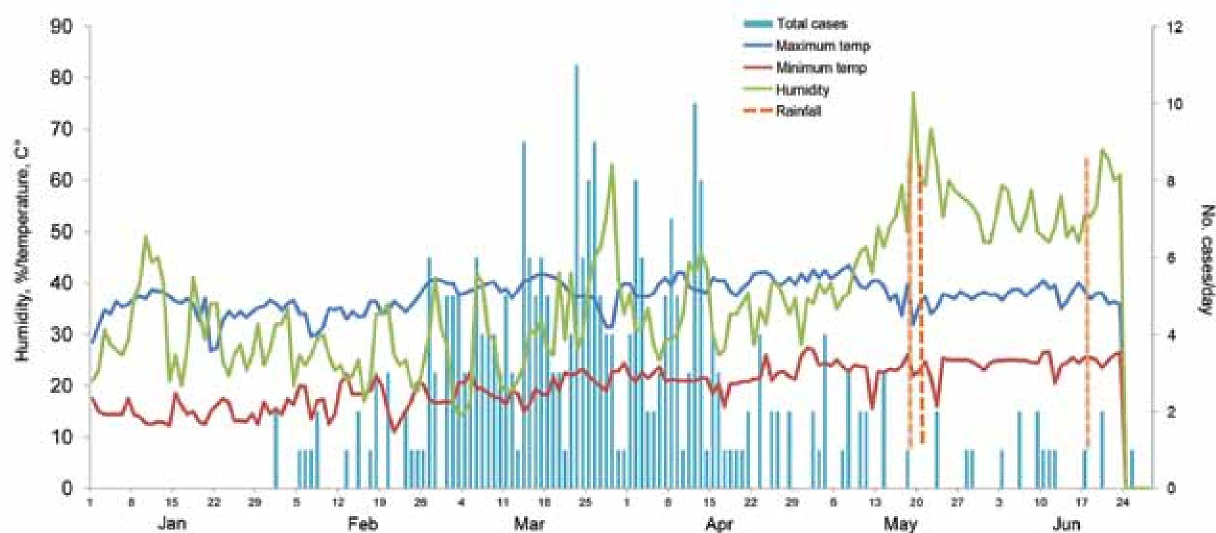

Figure 2. Number of epidemic cases of *Neisseria meningitidis* serogroup W135 in relation to time, temperature, humidity, and rainfall, Central River Region, The Gambia, February 1–June 25, 2012. A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/09/13-0077-F2.htm).

The study was supported by the Medical Research Council. The Bill & Melinda Gates Foundation funded the prospective bacterial disease surveillance.

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Novel Cyclovirus in Human Cerebrospinal Fluid, Malawi, 2010–2011

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To identify unknown human viruses, we analyzed serum and cerebrospinal fluid samples from patients with unexplained paraplegia from Malawi by using viral metagenomics. A novel cyclovirus species was identified and subsequently found in 15% and 10% of serum and cerebrospinal fluid samples, respectively. These data expand our knowledge of cyclovirus diversity and tropism.

The list of diseases caused by viral pathogens is ever changing and growing (*1*). Breakthroughs in the field of metagenomics had far-reaching effects on the identification of emerging viral pathogens and on the recognition that an increasing number of diseases that were once attributed to unknown causes are actually caused by infectious agents (*1*). Paraplegia is an impairment of motor or sensory functions of the lower extremities. Although it can be caused by spinal cord injury, nontraumatic paraplegia also should be considered, particularly in a tropical environment; tuberculosis and schistosomiasis may play a role, but in many cases, no firm diagnosis can be made (*2*). In this study, cerebrospinal fluid (CSF) and serum samples were obtained from 58 patients from Malawi who had paraplegia of unknown etiology and were studied for the presence of known or unknown viruses by using a metagenomics approach.

The Study

During 2010–2011, we enrolled 58 adults who sought care at Queen Elizabeth Central Hospital in Blantyre, Malawi, for unexplained paraplegia in this study. All procedures were performed in compliance with relevant laws and institutional guidelines. The study was approved by the College of Medicine Research and Ethics Committee (P.99/00/92).

Serum and CSF samples obtained from 12 paraplegia patients were available for virus discovery studies by using random PCR amplification with next-generation sequencing with a 454 GS Junior Instrument (Roche, Indianapolis, IN, USA) (*3*,*4*). More than 234,000 trimmed reads were assembled by using de novo assembly in CLC Genomics Workbench 4.5.1 (CLC Bio, Aarhus, Denmark) and analyzed according to nucleotide and translated nucleotide BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Classification of sequences based on the taxonomic origin of the best-hit sequence was performed by using MEGAN 4.40.4 (*5*), using E-value cutoffs of 0.001 and 10^{-10} for BLASTn and BLASTx searches, respectively.

Most of the sequences were of eukaryotic or bacterial origin or did not have hits to nucleotide or amino acid sequences in GenBank in agreement with previous viral metagenomic studies (*6*). Serum samples from all patients were positive for viruses from the family *Anelloviridae* in serum. Anellovirus infections are acquired during early childhood, when the virus establishes a chronic productive infection with long-lasting detectable viremia (*7*). Two of these patients were positive for anelloviruses in the CSF, which has been described (*7*). In 2 patients, hepatitis B virus sequences were detected. Hepatitis B virus infection occurs worldwide but is most prevalent in Southeast Asia and sub-Saharan Africa, with reported prevalence rates of 3%–26% (*8*). CSF from 2 other patients showed evidence of HIV infection in CSF samples (Table). None of these viral infections seem to explain the paraplegia.

A cyclovirus genome was obtained by 454-sequencing from serum of patient VS5700009. Cycloviruses (family *Circoviridae*, genus *Cyclovirus*) have been detected in human and chimpanzee feces and tissues of farm animals, bats, and dragonflies (*9*–*12*). They are nonenveloped viruses with a single-stranded circular DNA genome of ≈2 kb (*13*). The genome contains 2 major inversely arranged open reading frames (ORFs) encoding the putative replication-associated protein (Rep) and capsid protein (Cap). A potential stem–loop structure with a conserved nonanucleotide motif located between the 5′-ends of these 2 ORFs is required to initiate the replication of the viral genome (*13*).

The genome organization of human cyclovirus VS5700009 (GenBank accession no. KC771281) resembles that of human cycloviruses TN18 and TN25 (*11*). The Rep ORF of human cyclovirus VS5700009 was interrupted by a 96-bp intron with a splice donor site (GT)

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DOI: http://dx.doi.org/10.3201/eid1909.130404

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Table. Viruses detected in serum and CSF samples from patients with paraplegia in a study of novel human cyclovirus, Malawi, 2010–2011*

	Next-generation sequence		Cyclovirus	
Patient no.	Serum	CSF	Serum	CSF
VS5700001	Anelloviridae			
VS5700002	Anelloviridae			+
VS5700003	Anelloviridae			
VS5700004	Anelloviridae, HBV			
VS5700005	Anelloviridae			
VS5700006	Anelloviridae	HIV-1		
VS5700007	Anelloviridae			
VS5700008	Anelloviridae, HBV	HIV-1, Anelloviridae		
VS5700009	Anelloviridae, Cyclovirus		+	+
VS5700010	Anelloviridae	Anelloviridae	+	
VS5700011	Anelloviridae			
VS5700012	Anelloviridae			
VS5700021	NA	NA	+	
VS5700022	NA	NA	NA	+
VS5700025	NA	NA	+	
VS5700029	NA	NA	+	
VS5700031	NA	NA	+	
VS5700040	NA	NA	+	NA
VS5700042	NA	NA	+	NA
VS5700044	NA	NA		+

*CSF, cerebrospinal fluid; +, positive; HBV, hepatitis B virus; NA, not available. Blank cells indicate negative results.

and splice acceptor site (AG). The 3′ intergenic region between the Rep and Cap ORFs was only 7 bp long. The cyclovirus stem–loop structure with the conserved nonamer sequence (5′-TAATACTAT-3′) in the 5′ intergenic region was observed, as were 3 other potential ORFs (Figure, panel A, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0404-F1.htm). These latter ORFs are not conserved among cycloviruses and show low partial homology to bacterial enzymes, NAD-dependent DNA ligase of *Psychrobacter* (ORF3), transketolase of *Sinorhizobium* (ORF4), and dTDP-D-glucose 4,6-dehydratase of *Actinomyces* (ORF5). The complete Rep protein of human cyclovirus VS5700009 and representative strains of cycloviruses were used for phylogenetic analysis (Figure, panel B, Appendix). Multiple alignments were created by using ClustalX (2.0.10) (*14*). Phylogenetic analyses were conducted with MEGA5 (*15*). The human cyclovirus VS5700009 Rep protein was most closely related to the Rep proteins of human cycloviruses TN18 and TN25 (≈75% aa identity) (*11*). Similar relations, but with much lower amino acid identities, were observed in Cap proteins (≈37% identity; data not shown). Cycloviruses belong to the same species when sharing >85% aa identity in the Rep region (*11*). Thus, human cyclovirus VS5700009 represents a new cyclovirus species. Most of the closest relatives of human cyclovirus VS5700009 (which include TN18 and TN25) detected in feces from children with nonpolio acute flaccid paralysis (*11*), a condition related to paraplegia, which may indicate that TN25/VS5700009-like viruses may be more pathogenic than other cycloviruses (Figure, panel B, Appendix).

To determine the prevalence of human cyclovirus VS5700009 in the serum and CSF samples of the 58 patients, a VS5700009-specific PCR was performed. Total nucleic acid was extracted from an aliquot (≈100 μl) of serum and CSF samples by using the Magnapure LC total nucleic acid isolation kit and the MagNAPure LC isolation station (Roche). A genomic area, corresponding to nt 373–752 in human cyclovirus VS5700009, was amplified by nested PCR, with primers VS711 (5′-CGAGCGCA-CATTGAGAAAG-3′) and VS712 (5′-CCATCCCAC-CATTCTCCTC-3′) by using Amplitaq gold DNA polymerase (Roche). Negative water controls were taken along. Eight (15%) of 54 serum samples and 4 (10%) of 40 CSF samples from paraplegia patients were human cyclovirus positive (Table). For several amplicons, the cyclovirus nature was confirmed by Sanger sequencing, and the amplicons showed 75%–99% identity to human cyclovirus VS5700009 (Figure, panel C, Appendix). Only in patient VS5700009 were both CSF and serum samples positive for human cyclovirus.

Conclusions

Our results indicate that cycloviruses are commonly found in serum and CSF of paraplegia patients from Malawi. Diverse cycloviruses have been discovered in human and chimpanzee fecal samples and in muscle tissue of farm animals, such as cows, sheep, goats, and chickens (*11*). Cycloviruses have been suggested to cause human enteric infections and were not derived from consumed food because the human and animal cycloviruses showed limited genetic overlap (*11*). Our data indicate that cycloviruses may cause systemic infections and are present in multiple organ compartments in humans. Whether cycloviruses play a role in development of paraplegia remains to be determined; this study lacks a control group of healthy persons, and the relatively high virus prevalence in persons with paraplegia may also reflect high overall prevalence in healthy persons. In addition, the apparent interleaved evolution of human and animal cycloviruses suggests the potential for frequent cross-species exposure and zoonotic transmission.

Our observations expand the knowledge of cycloviruses in humans and show how epidemiologic baseline information on virus host range and tropism in animals (*11*) may indicate the presence of similar viruses in different organ systems of humans. To clarify the epidemiology and pathogenicity of cycloviruses in humans, additional surveillance should be conducted, especially because the prevalence and diversity of human cycloviruses is relatively high (*11*; this study), cross-species transmission of cycloviruses seems plausible (*10*), and closely related cyclovirus species may be pathogenic.

This work was partially funded by the European Community's Seventh Framework Program 8 (FP7/2007–2013) under the project "European Management Platform for Emerging and Reemerging Infectious Disease Entities" European Commission agreement no. 223498; and by Virgo Consortium.

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Gastroenteritis Outbreak Associated with Unpasteurized Tempeh, North Carolina, USA

Stephanie E. Griese, Aaron T. Fleischauer, Jennifer K. MacFarquhar, Zackary Moore, Cris Harrelson, Anita Valiani, Sue Ellen Morrison, David Sweat, Jean-Marie Maillard, Denise Griffin, Debra Springer, Matthew Mikoleit, Anna E. Newton, Brendan Jackson, Thai-An Nguyen, Stacey Bosch, and Megan Davies

During an investigation of an outbreak of gastroenteritis caused by *Salmonella enterica* serovar Paratyphi B variant L(+) tartrate(+), we identified unpasteurized tempeh as a novel food vehicle and *Rhizopus* spp. starter culture as the source of the contamination. Safe handling of uncooked, unpasteurized tempeh should be emphasized for prevention of foodborne illnesses.

Infections with *Salmonella* spp., a leading cause of hospitalizations and death among persons with foodborne illness in the United States, are most often associated with contaminated poultry or eggs (*1*,*2*). *S. enterica* serovar Paratyphi B variant L(+) tartrate(+) (formerly *Salmonella* var. Java) accounted for 1.1% of *Salmonella* infections reported to the Centers for Disease Control and Prevention (CDC) in 2009 (*3*). We investigated an outbreak of gastroenteritis caused by *S. enterica* ser. Paratyphi B var. L(+) tartrate(+) in North Carolina, USA, and found that the infections were associated with contaminated *Rhizopus* spp. starter culture and unpasteurized tempeh, a meat substitute, as a novel food vehicle.

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DOI: http://dx.doi.org/10.3201/eid1909.130334

The Study

On March 30, 2012, a local health department notified the North Carolina Division of Public Health (NCDPH) of 5 persons who had laboratory-confirmed infection with *S. enterica* ser. Paratyphi B var. L(+) tartrate(+) and 3 epidemiologically linked persons who also had gastroenteritis. All 8 ill persons ate or worked at the same restaurant in Buncombe County, North Carolina; 5 (63%) were food handlers. Patient interviews did not identify a common source or vehicle for the infection. On April 24, NCDPH was notified of 10 additional persons with laboratory-confirmed *S. enterica* ser. Paratyphi B var. L(+) tartrate(+) infection; all had visited or resided in Buncombe County during the infection's incubation period.

Pulsed-field gel electrophoresis (PFGE) patterns of isolates from all 15 laboratory-confirmed case-patients were indistinguishable and represented a pattern not previously reported to the national database of enteric PFGE patterns, PulseNet, coordinated by CDC (www.cdc.gov/pulsenet; outbreak strain identification *Xba*1 JKXX01.1228). NCDPH initiated an investigation to determine the extent of the outbreak, identify the transmission source, and implement control measures.

A confirmed case was defined as laboratory identification of the outbreak strain from a person's clinical specimen, regardless of illness onset date or exposure location. A probable case was defined as gastroenteritis in a person epidemiologically linked to a confirmed case. Passive reporting was enhanced through media reports and provider alerts. Active case finding was performed by hospital-based public health epidemiologists. Patients were interviewed by using the standard NCDPH salmonellosis reporting form to assess clinical symptoms; travel history; and food, water, and animal exposures.

A total of 89 cases (87 confirmed, 2 probable) were identified among residents of 5 states; illness onset dates were February 29–May 8, 2012 (Figure 1). Of the 89 case-patients, 81 were residents of North Carolina; 80 reported travel to or residence in Buncombe County during the incubation period. Ten self-identified as food service workers, 2 as health care providers, and 39 as students of or visitors to University A, located in Buncombe County. All 86 patients for whom data were available experienced diarrhea ($\geq$3 loose stools in a 24-hour period; median duration 7 days, range 2–24); 30 (37%) of 82 reported bloody diarrhea (denominator reflects the number of case-patients who responded to the question). Eighty-three patients sought medical care; 8 were hospitalized; and none died (Table).

Consumption of vegetarian cuisine was commonly reported by case-patients. Because meat substitutes (e.g., tofu and tempeh) were not included on the standard reporting form, an outbreak-specific questionnaire was designed to gather more detailed exposure history. The first 50 patients

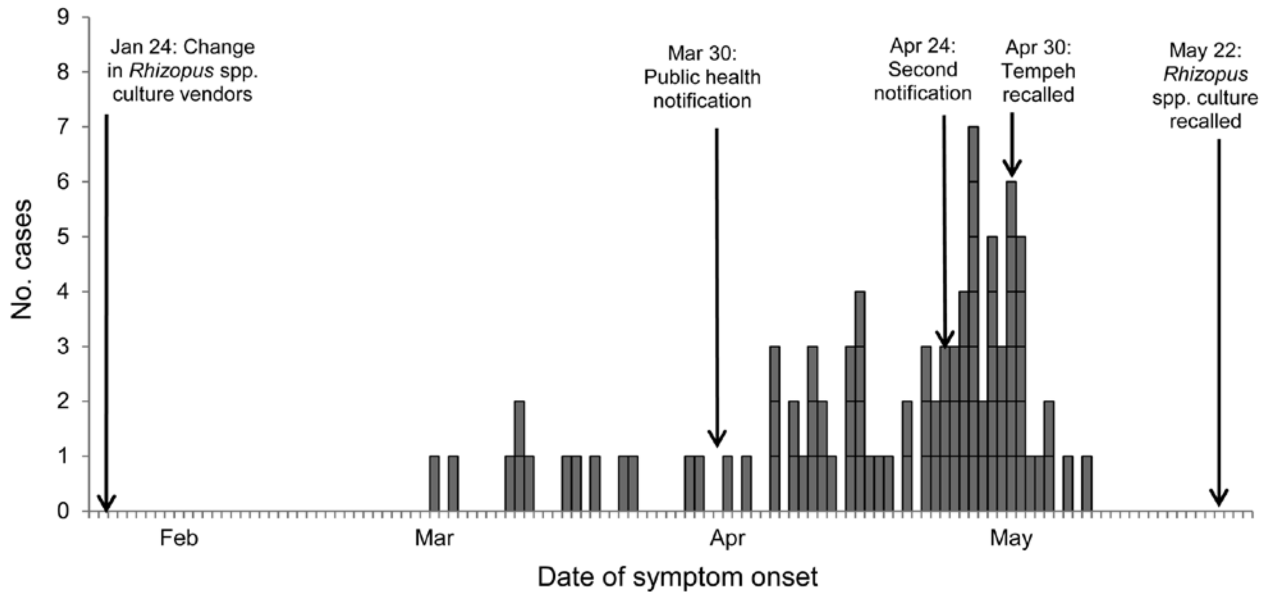

Figure 1. Cases of *Salmonella enterica* serovar Paratyphi B variant L(+) tartrate(+) gastroenteritis, by date of symptom onset, North Carolina, USA, February 29–May 8, 2012. For comparison, the date that the manufacturer of Brand A tempeh changed *Rhizopus* spp. starter culture vendors and dates of public notifications and recalls are indicated.

identified received this questionnaire; 41 (82%) responded. Of these, 18 (44%) indicated that they had eaten tempeh, a fermented bean product that is usually pasteurized and cooked before consumption. Of these 18 patients, 12 had eaten tempeh at a restaurant, 4 had eaten it at University A, and 2 had sampled it at a grocery store. Two of the 18 persons who consumed tempeh also regularly handled it at a restaurant.

Table. Characteristics of 89 case-patients identified during investigation of infections with *Salmonella enterica* serovar Paratyphi B variant L(+) tartrate(+), North Carolina, USA, February 29–May 8, 2012*

Characteristic	No. (%) patients
Case classification	
Confirmed	87 (98)
Probable	2 (2)
Sex	
F	45 (51)
M	44 (49)
Patient age, y	
Range	4–79
Median	26
Mean	30
Signs or symptoms	
Diarrhea, n = 86	86 (100)
Abdominal cramps, n = 82	70 (85)
Fever, n = 84	69 (82)
Vomiting, n = 84	33 (39)
Bloody diarrhea, n = 82	30 (37)
Treatment and outcomes	
Sought medical care	83 (93)
Hospitalized	8 (12)
Died	0 (0)

*Values are no. (%) except as indicated. n values indicate number of case-patients who responded to question.

Site visits were conducted at the 3 restaurants most frequently identified in patient interviews (12%–40% of patient reports). Interviews with managerial staff and observation of food preparation identified multiple opportunities for cross-contamination, including preparation of uncooked, unpasteurized tempeh on the same surfaces used to prepare ready-to-eat (RTE) foods; failure to perform handwashing after handling uncooked tempeh; and bare-hand contact with RTE foods.

On April 26, the North Carolina Department of Agriculture and Consumer Services (NCDA&CS) notified NCDPH that salmonellae had been presumptively identified from samples of Brand A tempeh, which had been collected for routine food product testing before this outbreak was reported. Brand A produced unpasteurized tempeh in Buncombe County and distributed it to 34 restaurants in North Carolina. Additional distribution sites included grocery stores in several southeastern states and University A's cafeteria. All 41 case-patients who completed the outbreak-specific questionnaire had eaten at a restaurant or venue that served Brand A tempeh. No cases were linked to grocery stores outside North Carolina.

NCDA&CS and NCDPH visited Brand A tempeh's production facility to interview staff, review tempeh production, and obtain food samples. Of 6 employees, none reported recent illness or international travel. Production of the tempeh began in October 2009 and involved combining beans (e.g., soybeans, black beans, or black-eyed peas), vinegar, and *Rhizopus* spp. starter culture. The starter culture was added after cooking; the

bean product was then fermented until it formed a dense cake, and the unpasteurized product was packaged, frozen, and shipped.

Product testing revealed that Brand A tempeh was contaminated with *S. enterica* ser. Paratyphi B var. L(+) tartrate(+) that had a PFGE pattern matching the outbreak strain. *Salmonella* spp. were not recovered from raw soybeans or black-eyed peas, black beans were unavailable for sampling, and vinegar was not tested, but the outbreak strain was identified in opened and unopened bags of *Rhizopus* spp. starter culture (Figure 2).

The makers of Brand A tempeh switched *Rhizopus* spp. vendors in January 2012 and began using the new culture on January 24. The new *Rhizopus* spp. culture was produced in Indonesia and distributed internationally. Brand A tempeh was recalled voluntarily on April 30, 2012 (*4*). The *Rhizopus* spp. culture was recalled voluntarily, domestically and internationally, on May 22, 2012 (*5*).

Conclusions

An outbreak of 89 cases of gastroenteritis related to infection with *S. enterica* ser. Paratyphi B var. L(+) tartrate(+) occurred in North Carolina during February–May 2012. The outbreak source was a *Rhizopus* spp. culture used in Brand A tempeh, which then acted as a novel vehicle for spreading salmonellae to consumers, probably through cross-contamination of RTE foods.

NCDPH confirmed the association between illness and Brand A tempeh through patient interviews and laboratory testing. The contaminated starter culture was distributed internationally; it is unclear why cases related to other tempeh brands did not occur, but a hypothesis is that, unlike other commercial tempeh products, Brand A tempeh is unpasteurized, and thus pathogens remained in the finished product.

The role of cross-contamination in foodborne outbreaks is well established (*6–11*). Bacteria can be transferred from surfaces to food products hours after surface contamination (*6*,*7*,*9*). RTE foods typically do not include a heating or cooking step to kill pathogens; consequently, raw vegetables and salads are commonly associated with foodborne outbreaks caused by cross-contamination (*7*,*10–12*). In this outbreak, all case-patients who responded to an outbreak-specific questionnaire reported eating at a venue that served Brand A tempeh. Although fewer than half recalled eating or handling tempeh, other case-patients might have been exposed during handling or consumption of cross-contaminated RTE foods.

Control measures addressing bare-hand contact with RTE foods, sanitation of food contact surfaces, and separation of raw and RTE foods were provided to restaurants that received Brand A tempeh and to the local Independent Restaurant Association. Correct handling of raw, unpasteurized tempeh was emphasized. Although tempeh can be part of a healthy diet (*13*), public health considerations should focus on safe handling of unpasteurized tempeh to prevent illness.

Acknowledgments

We thank the Buncombe County Health Department, especially Gibbie Harris, Jennifer Mullendore, Ellis Vaughan, Susan Creede, David Mease, Marc Fowler, and Gaylen Ehrlichman, for their excellent management of a prolonged outbreak; our liaisons with the North Carolina Department of Agriculture and Consumer Services, Joan Sims, Janna Spruill, and Jim Melvin,

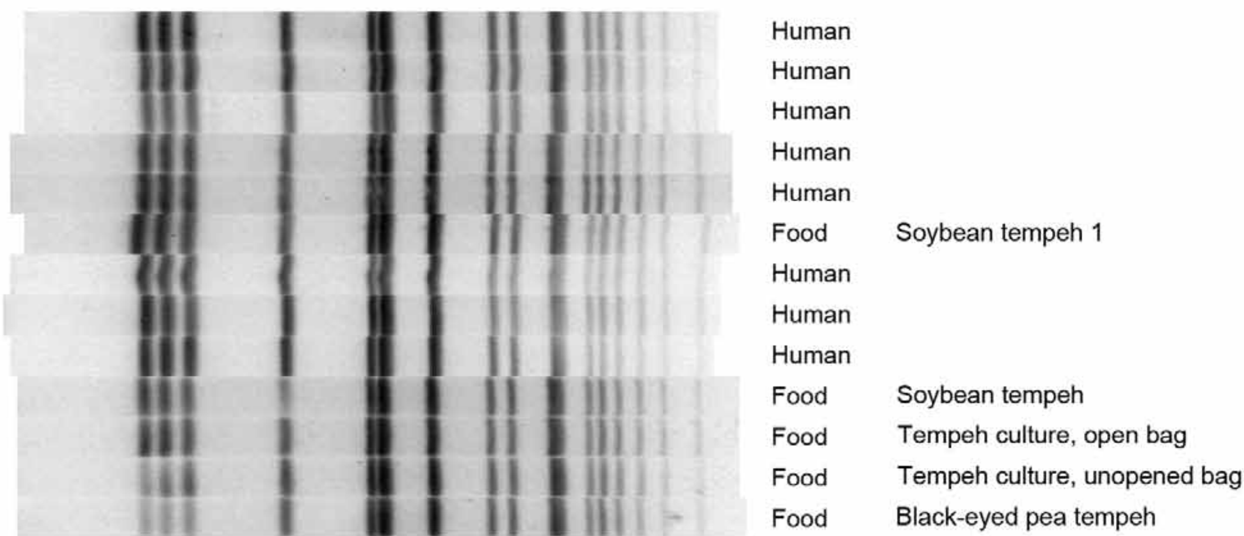

Figure 2. Pulse-field gel electrophoresis dendrogram showing *Xba1* enzyme band patterns for 8 case-patients, tempeh, and *Rhizopus* spp. starter culture associated with outbreak of *Salmonella enterica* serovar Paratyphi B variant L(+) tartrate(+) gastroenteritis, by date of symptom onset, North Carolina, USA, 2012.

for their collaboration; and the North Carolina Division of Public Health, particularly Evelyn Foust, Julie Casani, Lorri Taylor, Kathy Dail, Lana Deyneka, Nicole Lee, Susan Thompson, Carl Williams, Phyllis Rocco, Sandy Pace, Rob Pace, Samuel Merritt, Shermalyn Greene, Cami Hartley, Robbie Hall, Christy Spratt, and Kim Pruess, for their tireless efforts during an ongoing public health investigation. We are especially indebted to the network of hospital-based public health epidemiologists and the local health department communicable disease nurses who helped investigate each case.

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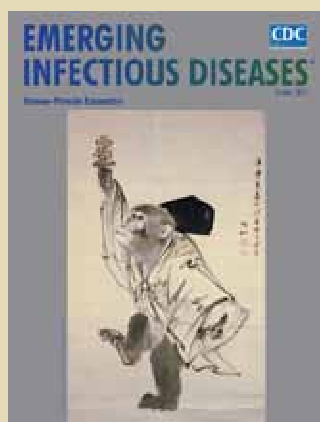

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West Nile Virus RNA in Tissues from Donor Associated with Transmission to Organ Transplant Recipients

Dianna M. Blau, Ingrid B. Rabe, Julu Bhatnagar, Rachel Civen, Kavita K. Trivedi, Dominique Rollin, Susan N. Hocevar, Matthew Kuehnert, J. Erin Staples, Sherif R. Zaki, Marc Fischer, and the West Nile Virus Transplant-Associated Transmission Investigation Team[1]

We identified West Nile virus (WNV) RNA in skin, fat, muscle, tendon, and bone marrow from a deceased donor associated with WNV transmission through solid organ transplantation. WNV could not be cultured from the RNA-positive tissues. Further studies are needed to determine if WNV can be transmitted from postmortem tissues.

West Nile virus (WNV), a mosquito-borne flavivirus, was detected in North America in 1999 and has since become endemic to the United States, where it causes annual seasonal outbreaks. An estimated 70%–80% of human WNV infections are asymptomatic (*1*). Most symptomatic persons experience acute systemic febrile illness; West Nile neurologic disease develops in <1% of infected persons but has a case-fatality rate of 9% (*2*).

Most WNV infections are acquired through bites from infected mosquitoes. However, the virus can also be transmitted by transfusion of infected blood products or by solid organ transplantation (*3*,*4*). In 6 clusters of organ transplant–transmitted WNV infections reported to public health agencies in the United States, 12 (75%) of 16 recipients were infected (*5*). Encephalitis developed in 9 (75%) of those recipients; 4 of those 9 died.

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DOI: http://dx.doi.org/10.3201/eid1909.130365

WNV transmission through tissue transplantation (i.e., skin, muscle, or connective tissues) has not been identified, and the risk for transmission by this route is not known. We evaluated tissues collected from a deceased donor who was associated with transmission of WNV through solid organ transplantation to determine if WNV RNA, viral antigen, or infectious viral particles could be detected in postmortem tissues.

The Study

In 2011, the Centers for Disease Control and Prevention (CDC) assisted state and local health departments in an investigation of a cluster of WNV disease transmitted through solid organ transplantation (*6*). The adult male donor had a history of cerebral palsy, seizures, and blindness. He was cared for at home and had outdoor exposure in a county with known WNV activity. In late summer, he had acute onset of fever and lethargy; 2 days after symptom onset, a urinary tract infection was diagnosed, and he received oral antimicrobial drugs. The following day, he suffered cardiopulmonary arrest. After resuscitation, he remained unresponsive, and an electroencephalogram showed no cortical activity. After consent was obtained, solid organs (i.e., kidneys, lungs, and liver) and tissues (i.e., skin, fat, muscle, tendon, and bone) were procured 9 days after his illness onset. Corneas, heart valves, and vascular tissue were not procured. The donor's organs were transplanted into 4 recipients; none of the donor tissues were transplanted.

After WNV infection was detected in 1 of the organ recipients 10 days after transplantation, the donor's stored clinical samples (i.e., serum and spleen/lymph node homogenate) were retrospectively tested for WNV; this testing occurred within 5 weeks after transplantation. The donor's serum sample was positive for WNV IgM, IgG, and neutralizing antibodies by serologic testing but negative for WNV RNA by nucleic acid amplification testing. WNV RNA was detected in spleen/lymph node homogenate. Subsequently, all 4 organ donor recipients were tested and had positive results for WNV RNA. Two of the recipients died of WNV infection.

Five weeks after the donor's death, frozen spleen/lymph node homogenate from the donor that had been used for human leukocyte antigen testing was sent from the

[1]Members of the West Nile Virus Transplant-Associated Transmission Investigation Team: Holenarasipur R. Vikram, David Mulligan, (Mayo Clinic, Phoenix); Gundeep Dhillon (Stanford University School of Medicine); Drew J. Winston, Johnny C. Hong (University of California Los Angeles Medical Center); Thomas Mone (OneLegacy); Marek J. Nowicki (Mendez National Institute of Transplantation); Joel Osborne, Pamela Rawlins (Musculoskeletal Transplant Foundation); and Selam A. Tecle (Arizona Department of Health Services).

transplant center to CDC, and initial WNV PCR testing was performed as part of the transplant-transmission investigation (*7*). Eight weeks after the donor's death, skin samples that had been treated in cryopreservative solution containing an antibiotic and unprocessed fat, muscle, tendon, and bone samples, all of which had been stored frozen at -70°C at a tissue bank, were transferred to CDC. At CDC, the tissues remained frozen at –20°C to -70°C in individual double-wrapping and plastic bags and were handled and tested separately to reduce the risk for cross-contamination.

RNA was extracted from each 3–5 mm section of tissue by using a phenol-chloroform extraction method as described (*8*), with the following modifications. Tissue was homogenized in Buffer RLT (QIAGEN, Valencia, CA, USA) and digested with proteinase K for 15 min at 55°C; an additional 24-h digestion at 40°C was used for bone. RNA quality was ensured by amplification of housekeeping genes. RNA samples were tested by using WNV-specific reverse transcription PCR (RT-PCR) targeting the nonstructural protein 1 (NS1), capsid, and premembrane genes (*9*). Positive PCR amplicons were sequenced for confirmation. To assess for infectious virus, we injected homogenates from tissues positive by RT-PCR into Vero E6 cells; for cells with cytopathic effect, we confirmed the presence of WNV by RT-PCR, immunofluorescence, and electron microscopy. Immunohistochemical (IHC) staining for WNV was performed on RNA-positive tissues (*10*). RT-PCR was performed 25–26 weeks after the specimens were collected from the donor; virus culture and IHC staining were performed 50 weeks after specimens were collected.

WNV RNA was detected in samples from the spleen/lymph node, skin, and fat associated with the tibia bone, as well as 1 of 2 muscle specimens, 1 of 4 tendon specimens, and 1 of 2 bone marrow specimens (Table). Cytopathic effect was noted only in Vero cells injected with the spleen/lymph node homogenate; these cells were positive for WNV by RT-PCR, immunofluorescence, and electron microscopy. Cytopathic effect was not observed in Vero cells injected with skin, fat, muscle, tendon, or bone marrow. Results of IHC staining of skin, fat, muscle, and bone marrow samples were negative for WNV antigens.

Conclusions

We identified WNV RNA in spleen/lymph node homogenate, skin, fat, muscle, tendon, and bone marrow samples obtained postmortem from a donor associated with transmission of WNV through solid organ transplantation. WNV was isolated from the spleen/lymph node homogenate, indicating infectious virus. However, infectious virus could not be cultured, and WNV antigens were not identified by IHC staining from any of the WNV RNA-positive tissues.

Data on the detection of WNV in postmortem organs or tissues are limited. In a study published in 1954, a total of 95 patients with terminal cancer were injected intramuscularly with WNV (*11*). Among 14 patients who died within 1 month after inoculation, virus was isolated postmortem from solid organs in 11 patients and, in 1 patient each, from skin, muscle, or connective tissue. In a more recent study of 6 patients with fatal mosquito-borne WNV encephalitis, WNV RNA or antigens were variably detected in solid organ samples from all patients, and WNV antigens were identified in skin samples from 1 patient (*12*). However, 4 (67%) of these patients were severely immunocompromised transplant recipients; of the 2 immunocompetent patients, 1 had WNV RNA in brain, spleen, and kidney samples and 1 had WNV antigens only in brain samples. WNV has also been cultured from an antemortem skin biopsy sample from a patient with rare hemorrhagic manifestations of disease (*9*).

This study has several limitations. The findings are from a single donor, and thus their generalizability is uncertain. The tissues were stored frozen for almost 1 year before culture, which may have decreased the ability to isolate viable WNV, although virus was isolated from the spleen/lymph node homogenate. Several tissues that are commonly transplanted with minimal processing (e.g., corneas, heart valves, and vascular grafts) and that have been implicated in recent transmission of other viruses, such as hepatitis B and C (*13*,*14*), were not procured or tested in our study. Finally, although the specimens were stored,

Table. WNV in tissues from solid organ donor associated with WNV transmission to solid organ transplant recipients*

	Tests performed†		
Specimens tested	RT-PCR	Cell culture	IHC staining
Spleen/lymph node homogenate	+	+	NT
Tissues			
Skin	+	–	–
Fat, tibia	+	–	–
Tendon			
Gracilis	+	–	NT
Achilles	–	NT	NT
Semitendinosus	–	NT	NT
Tibialis	–	NT	NT
Muscle			
Attached to tibia	+	–	–
Adjacent to gracilis tendon	–	–	NT
Bone marrow			
Femur	+	–	–
Pelvis	–	NT	NT
Bone, pelvic cortical	‡	NT	NT

*WNV, West Nile virus; RT-PCR, reverse transcription PCR; IHC, immunohistochemical; +, positive; NT, not tested; –, negative.
†For spleen/lymph node homogenate, fat, tendon, muscle, bone marrow, and bone, a single sample was tested for WNV RNA by RT-PCR. If positive, a second specimen was tested by RT-PCR, inoculated into Vero E6 cells, and evaluated by IHC. For skin, after initial WNV RT-PCR was positive, 3 subsequent samples were tested for WNV RNA by RT-PCR, inoculated into Vero E6 cells, and evaluated by IHC.
‡Sample inadequate for testing.

handled, and tested individually, false-positive results or cross-contamination cannot be completely ruled out.

Although WNV RNA was detected in unprocessed tissues obtained from the organ donor, the absence of viral antigen by IHC staining and failure to culture infectious virus from skin, muscle, and tendon suggests that the risk for WNV transmission may be lower for transplantation of these tissues than for transplantation of solid organs. Further studies are needed to determine if infectious WNV can be recovered from and possibly transmitted by transplantation of postmortem tissues and, if so, to assess the period of risk and whether tissue processing would mitigate the risk (*15*).

Acknowledgments

We thank Jana Ritter for assistance with tissue dissection and Amanda Panella for transfer and handling of the tissue specimens. We also acknowledge all of the tremendous work of the American Association of Tissue Banks Ad Hoc WNV Studies Focus Group.

Dr Blau is a medical epidemiologist in the Infectious Diseases Pathology Branch at CDC. Her research interests include unexplained deaths and pathogenesis of infectious diseases, especially those with a viral etiology.

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R292K Substitution and Drug Susceptibility of Influenza A(H7N9) Viruses

Katrina Sleeman,[1] Zhu Guo,[1] John Barnes, Michael Shaw, James Stevens, and Larisa V. Gubareva

Neuraminidase inhibitors are the only licensed antiviral medications available to treat avian influenza A(H7N9) virus infections in humans. According to a neuraminidase inhibition assay, an R292K substitution reduced antiviral efficacy of inhibitors, especially oseltamivir, and decreased viral fitness in cell culture. Monitoring emergence of R292K-carrying viruses using a pH-modified neuraminidase inhibition assay should be considered.

The recent emergence of an avian influenza A(H7N9) virus causing human infections in China (*1–2*) is of global concern. Most patients infected during this outbreak have experienced severe disease and required hospitalization; the mortality rate is 21% (*3*). Although epidemiologic investigations have revealed no evidence of sustained human-to-human transmission (*4*), suspected limited human-to-human transmission has been reported (*3*).

As with any emergent influenza virus, it is critical to assess the susceptibility of the influenza A(H7N9) outbreak virus to antiviral drugs, which are the first line of defense before an effective vaccine becomes available. Two classes of antiviral drugs are approved for management of influenza A infections, neuraminidase (NA) inhibitors (NAIs) and matrix 2 protein (M2) blockers (adamantanes). The outbreak viruses carry the established adamantane resistance marker, an S31N substitution in the M2 protein (*2*), leaving NAIs as the only licensed treatment option. Among the 4 NAIs, oseltamivir and zanamivir are approved in many countries; peramivir has been approved in Japan, South Korea, and China; and laninamivir is approved only in Japan. In contrast to those for adamantanes, genetic markers of resistance to NAIs are often subtype specific and drug specific (*5*). Therefore, monitoring drug susceptibility of the influenza A(H7N9) viruses requires testing in phenotypic assays using all available NAIs.

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DOI: http://dx.doi.org/10.3201/eid1909.130724

The Study

Our aim was to assess NAI susceptibility of 2 influenza A(H7N9) outbreak virus isolates provided by the Chinese Center for Disease Control and Prevention. The influenza A/Anhui/1/2013 isolate was recovered from an untreated patient and contained no notable NAI-resistance markers in the NA gene. When tested in the NA inhibition (NI) assay (*6*), the virus yielded subnanomolar IC_{50}s (concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%) with all 4 NAIs, similar to results for the drug-sensitive seasonal influenza A viruses used as controls (Table 1). The second isolate, influenza A/Shanghai/1/2013, was collected from a patient who had received 2 doses of oseltamivir; the isolate was reported to contain an NA substitution, R292K (*2*). R292K is known to alter NAI susceptibility in viruses of N2 (*7*) and N9 (*8*) subtypes. However, A/Shanghai/1/2013 virus was reported to be susceptible to both oseltamivir and zanamivir on the basis of NI assay data (*2*). To clarify the effect of R292K on NAI susceptibility of influenza A(H7N9) viruses, the A/Shanghai/1/2013 egg-grown isolate (E1) was received and tested at the US Centers for Disease Control and Prevention by using the NI assay (*6*). Our data showed full susceptibility of A/Shanghai/1/2013 virus to oseltamivir (Table 1), an observation consistent with a previous report (*2*). Analysis of the E1 isolate by pyrosequencing assay (*9*) revealed a polymorphism at NA residue 292, containing arginine (23%) and lysine (77%; Table 1). Further analysis of the E1 isolate by PacBio deep sequencing confirmed that 77% of the virus population possessed the lysine 292 variant (Table 1).

The inability to detect changes in oseltamivir IC_{50} despite the presence of R292K raised 2 questions: are conventional NI assays sufficiently sensitive to detect oseltamivir resistance caused by R292K, and is R292K truly a marker of oseltamivir resistance when it is present in these A(H7N9) outbreak viruses? We hypothesized that failure to detect the oseltamivir-resistant population by using the NI assay may stem from substantially reduced activity of the R292K variant NA. Previous studies have shown that the optimal pH for R292K enzyme activity is ≈5.3 (*7*), whereas the conventional NI assay uses a buffer at pH 6.5. We retested A/Shanghai/1/2013 (E1) by using the NI assay under the lower pH condition. The E1 isolate exhibited a higher oseltamivir IC_{50} (643 nmol/L vs. 0.6 nmol/L; Table 2) than that determined by the conventional assay, a finding consistent with our hypothesis. IC_{50}s of A/Anhui/1/2013 and reference viruses were either unchanged or found to increase slightly at the lower pH (Table 2).

As part of further investigation of the role of R292K in altering NAI susceptibility, recombinant NA proteins

[1]These authors contributed equally to this article.

Table 1. Susceptibility of influenza viruses to neuraminidase inhibitors, according to NI assay*

			AA at	%	IC_{50} nmol/L (-fold)			
Sample type	Subtype	Virus name (passage)	292†	K292	Oseltamivir	Zanamivir	Peramivir	Laninamivir
Virus isolate	H7N9	A/Anhui/1/2013 (E2/S1)	R		0.17 (1)	0.33 (1)	0.06 (1)	0.46 (1)
		A/Shanghai/1/2013 (E1)	R and K	77	0.59 (3)	NT	NT	NT
Recombinant NA	H7N9	A/Anhui1/2013	R		0.25 (1)	0.52 (2)	0.07 (1)	0.60 (1)
			K	100	5153 (>1,000)	28.05 (54)	127.60 (>1,000)	17.53 (29)
		A/Shanghai/1/2013	R		0.25 (1)	0.46 (1)	0.06 (1)	0.44 (1)
			K	100	4987 (>1,000)	23.46 (51)	101.89 (>1,000)	15.35 (35)
Reference virus	H3N2	Oseltamivir-sensitive A/Washington/01/2007	–		0.07 (1)	0.23 (1)	0.08 (1)	0.29 (1)
		Oseltamivir-resistant A/Bethesda/956/2006	K	100	3974 (>1,000)	6.83 (30)	16.27 (203)	2.51 (9)
	H1N1‡	Oseltamivir-sensitive A/California/12/2012	–		0.19 (1)	0.16 (1)	0.06 (1)	0.17 (1)
		Oseltamivir-resistant A/Texas/23/2012	–		157.25 (828)	0.20 (1)	15.81 (264)	0.26 (2)

*NI, neuraminidase inhibition; AA, amino acid; IC_{50}, concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%; E, passage in eggs; S, passage in MDCK-SIAT1 cells (/ separates passage before and after arrival to CDC); NA, neuraminidase; NT, not tested; RT-PCR, reverse transcription PCR.
†AA position: R292K (N2 numbering); R294K (straight full-length N9 numbering). Single-nucleotide polymorphism analysis was performed by using the pyrosequencing assay (RT-PCR primers: N9-F731-Bio, 5’-CT GGA CCT GCA GAC ACA AGA ATA-3’, N9-R926, 5’-TGT GTC ATT GCT ACT GGG TCT ATC-3’; sequencing primer: N9–292/294-R889-seq, 5’-TAT TTG AGC CCT GCC-3’) and confirmed by deep sequencing. Pac bio RS sequencing library was constructed by using a 701-bp RT-PCR amplicon generated by RT-PCR (N9NA-F204,5’- CAACATCCAAATGGAAGAGAGAA-3’; N9NA-R903 5’-TGTGTCATTGCTACTGGGTCTATC-3’). A single v3 SMRT cell was used for each library, and data were collected on 2 × 55 min movies. Only circular consensus sequencing reads were used in the analysis. Subpopulation detection was analyzed by using CLC Genomics Workbench version 6.01 (CLC Bio, Aarhus, Denmark). Isolates were tested in the NI assay by using the NA-Fluor kit (6). Fold change in IC_{50}- compared with drug-sensitive subtype-specific control. IC_{50} values represent the average taken from at least 4 replicates, with the exception of A/Shanghai/1/2013 (E1), because of insufficient sample volume. Oseltamivir-susceptible and oseltamivir-resistant reference viruses were used as controls in NI assays. Oseltamivir refers to oseltamivir carboxylate. Reference virus A/Texas/23/2012 contains H275Y oseltamivir-resistance conferring neuraminidase substitution.
‡Pandemic influenza A(H1N1) 2009 virus.

(rNAs) of A/Shanghai/1/2013 isolate and A/Anhui/1/2013 isolate were expressed in insect cells by using a transient expression system. The rNAs were tested with 4 NAIs in conventional and pH-modified NI assays (Tables 1, 2). Irrespective of the assay and N9 backbone used, oseltamivir showed an inhibitory effect on the R292K rNAs activity only at concentrations >1,000 nmol/L. The R292K rNAs also showed increased IC_{50}s for peramivir, zanamivir, and laninamivir (Tables 1, 2), consistent with previous findings (*5*). IC_{50}s of the NAIs for the rNAs lacking R292K were comparable with those for the A/Anhui/1/2013 virus.

The NA activity of the rNAs was tested at multiple pH points in MES buffer supplemented with 4 mmol/L $CaCl_2$. Activity of the R292K rNA peaked at pH 5.1 and increased by 5-fold compared with that measured under conventional assay conditions (pH 6.5). Conversely, the NA activity of rNA lacking this change was almost unchanged across the pH range tested (pH 4.9–6.9). These findings indicate that the R292K virus population could be concealed because of its reduced enzymatic activity under conventional assay conditions. NI assays with rNA proteins can clarify the extent of NAI sensitivity for each virus mutant and should be considered when analyzing heterogeneous virus populations with suspected NAI resistance.

To interpret NI assay results, criteria from the World Health Organization Antiviral Working Group were applied (*10*), and comparative differences in IC_{50}s (which defines inhibition as normal [<10], reduced [10–100] or highly reduced [>100]) were determined by using a subtype-specific reference. The A/Shanghai/1/2013 (E1) isolate exhibited highly reduced inhibition by oseltamivir at pH 5.1. On the basis of data obtained by using rNAs, the R292K conferred highly reduced inhibition by peramivir, in addition to oseltamivir, and reduced inhibition by zanamivir and laninamivir (Tables 1,2).

Conclusions

R292 is a highly conserved amino acid across all NA subtypes, and together with 2 other highly conserved residues (R118 and R371), it forms an arginine triad in the enzyme active site (*5*). R292K is a rare substitution and to date has only been reported in viruses collected from patients treated with oseltamivir (*2,5*). In addition to A/Shanghai/1/2013 isolate, there is evidence of additional influenza A(H7N9) isolates with the R292K substitution (*11*). In this study, propagation of A/Shanghai/1/2013 (E1) isolate in eggs and in MDCK-SIAT1 cells resulted in reversion to wild-type (23% Arg in E1 to 100% in E1/S3), confirming results of previous studies with N2 subtype viruses (*12*). Therefore, fitness of the A/Shanghai/1/2013 R292K virus is probably compromised when replication occurs in the absence of an NAI. However, propagation of the E1 isolate in the presence of oseltamivir (100 nmol/L) resulted in enrichment of the R292K population (from 77% to 100%), demonstrating a growth advantage over the wild-type.

Table 2. Susceptibility to neuraminidase inhibitors according to modified NI assay with pH 5.1*

Sample type	Subtype	Virus name (passage)	AA at 292†	% K292	IC_{50} nmol/L (fold)			
					Oseltamivir	Zanamivir	Peramivir	Laninamivir
Virus Isolate	H7N9	A/Anhui/1/2013 (E2/S1)	R		0.90 (1)	1.00 (1)	0.12 (1)	1.29 (1)
		A/Shanghai/1/2013 (E1)	R and K	77	642.78 (714)	NT	NT	NT
Recombinant NA	H7N9	A/Anhui1/2013	R		0.53 (1)	0.85 (1)	0.09 (1)	0.92 (1)
			K	100	9,078 (>1,000)	54.18 (56)	89.70 (814)	30.50 (25)
		A/Shanghai/1/2013	R		1.08 (1)	1.45 (1)	0.19 (1)	1.90 (1)
			K	100	8,351 (>1,000)	53.36 (53)	77.01 (642)	29.72 (23)
Reference virus	H3N2	Oseltamivir-sensitive A/Washington/01/2007	–		0.68 (1)	0.45 (1)	0.10 (1)	0.86 (1)
		Oseltamivir-resistant A/Bethesda/956/2006	K	100	6,083 (>1,000)	14.57 (32)	8.58 (86)	5.65 (7)
	H1N1‡	Oseltamivir-sensitive A/California/12/2012	–		0.74 (1)	0.23 (1)	0.07 (1)	0.21 (1)
		Oseltamivir-resistant A/Texas/23/2012	–		364.74 (493)	0.31 (1)	30.17 (431)	0.42 (2)

*NI, neuraminidase inhibition; AA, amino acid; IC_{50}, concentration of neuraminidase inhibitor required to reduce enzyme activity by 50%; E, passage in eggs; S, passage in MDCK-SIAT1 cells (/ separates passage before and after arrival to CDC); NA, neuraminidase; NT, not tested; RT-PCR, reverse transcription PCR.
†AA position: R292K (N2 numbering); R294K (straight full-length N9 numbering). Single-nucleotide polymorphism analysis was performed by using the pyrosequencing assay (RT-PCR primers: N9-F731-Bio, 5'-CT GGA CCT GCA GAC ACA AGA ATA-3', N9-R926, 5'-TGT GTC ATT GCT ACT GGG TCT ATC-3'; sequencing primer: N9–292/294-R889-seq, 5'-TAT TTG AGC CCT GCC-3') and confirmed by deep sequencing. Pac bio RS sequencing library was constructed by using a 701bp RT-PCR amplicon generated by RT-PCR (N9NA-F204, 5'-CAACATCCAAATGGAAGAGAGAA-3'; N9NA-R903 5'-TGTGTCATTGCTACTGGGTCTATC-3'). A single v3 SMRT cell was used for each library and data was collected on 2 × 55 min movies. Only circular consensus sequencing reads were used in the analysis. Subpopulation detection was analyzed by using CLC Genomics Workbench version 6.01 (CLC Bio, Aarhus, Denmark). Isolates were tested in the NI assay by using the NA-Fluor kit (6). Fold change in IC_{50} compared with drug-sensitive subtype-specific control. IC_{50} values represent the average taken from at least 4 replicates, with the exception of A/Shanghai/1/2013 (E1), due to insufficient sample volume. Oseltamivir-susceptible and oseltamivir-resistant reference viruses were used as controls in NI assays. Oseltamivir refers to oseltamivir carboxylate. Reference virus A/Texas/23/2012 contains H275Y oseltamivir,resistance conferring neuraminidase substitution.
‡Pandemic influenza A(H1N1) 2009 virus.

Replication of the E1 isolate in the presence of any NAI in cell culture might lead to enrichment with R292K, because even a small growth advantage would reduce the proportion of the wild type. The efficacy of NAIs in clinical management of influenza (H7N9) infection remains unknown and may be compromised to a certain extent when R292K is present. Animal model studies are needed to aid in the understanding of clinical relevance of R292K. Reduction of NA activity caused by R292K may detrimentally affect transmission of the virus, as indicated by an R292K influenza A(H3N2) virus that showed reduced infectivity in mice (*13–14*) and ferrets (*12–13,15*) and was not transmitted among ferrets (*12,15*). The data reported here demonstrate the continued importance of monitoring drug susceptibility in emergent influenza viruses and highlight the challenges involved in laboratory assessment of NAI drug susceptibility testing.

Acknowledgments

We thank the Chinese Center for Disease Control and Prevention for sharing the influenza A/Anhui/1/2013 A(H7N9) virus and members of the US Centers for Disease Control and Prevention Influenza Division for their contributions.

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Macrolide-Resistant *Mycoplasma pneumoniae* in Humans, Ontario, Canada, 2010–2011

AliReza Eshaghi, Nader Memari, Patrick Tang, Romy Olsha, David J. Farrell, Donald E. Low, Jonathan B. Gubbay, and Samir N. Patel

Antimicrobial drug resistance rates for *Mycoplasma pneumoniae* was determined in clinical specimens and isolates obtained during 2011–2012 in Ontario, Canada. Of 91 *M. pneumoniae* drug-resistant specimens, 11 (12.1%) carried nucleotide mutations associated with macrolide resistance in the 23S rRNA gene. None of the *M. pneumoniae* specimens were resistant to fluoroquinolones or tetracyclines.

Mycoplasma pneumoniae is a major cause of community-acquired pneumonia among children and adults (*1*). Macrolides are recommended for treatment of *M. pneumoniae* pneumonia (*1*).

High rates of macrolide-resistant *M. pneumoniae* have been reported in China (>90%) and Japan (87.1%) (*2,3*). In Europe, reports of macrolide resistance have ranged from 3% in Germany to 9.8% in France (*4,5*). In the United States, 8.2% of *M. pneumonia*–positive specimens identified during 2007–2010 were resistant to macrolides (*6*). *M. pneumoniae* confer macrolide resistance primarily as a result of nucleotide substitutions at specific positions in the V domain of the 23S rRNA gene. Mutations at nt 2063 (A2063T/G), 2064 (A2064G), and 2617 (C2617A/G) have been shown to be associated with increased MICs to macrolides, including erythromycin, azithromycin, and clarithromycin (*2,3,7,8*). Use of macrolides to treat macrolide-resistant *M. pneumoniae* result in lower effectiveness and increased clinical severity compared with macrolide-susceptible *M. pneumoniae (9)*. In contrast to macrolides, resistance to quinolones or tetracyclines among clinical isolates of *M. pneumoniae* has not been reported, although development of such resistance after use of increased concentrations of fluoroquinolones or doxycycline has been demonstrated in in vitro settings (*10,11*).

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DOI: http://dx.doi.org/10.3201/eid1909.121466

The Public Health Ontario Laboratory, which is the reference microbiology laboratory for the province of Ontario, provides molecular testing for detection of *M. pneumoniae* for hospitalized and ambulatory patients. In August 2011, the positivity rate for specimens with *M. pneumoniae* increased to 9.3% and peaked in December 2011 to 17.5%. During the same time, increased numbers of cases of *M. pneumoniae* were reported throughout Europe. In response to the increased positivity rate and lack of data for Canada on macrolide resistance in *M. pneumoniae*, we investigated antimicrobial drug susceptibility profiles of *M. pneumoniae* detected during February 2010–January 2012 by using molecular methods. In addition, available *M. pneumoniae* isolates were characterized by sequencing the P1 gene to determine the prevalence of circulating types in Ontario, Canada (*12,13*).

The Study

During February 1, 2010–January 31, 2012, a total of 2,898 respiratory specimens were tested for *M. pneumoniae* and *Chlamydophila pneumoniae* by using a multiplex testing real-time assay (ProPneumo-1 Assay; Gen-Probe Inc., San Diego, CA, USA). A total of 96 specimens were positive for *M. pneumoniae*, and 16 specimens were positive for *C. pneumoniae.* Among *M. pneumoniae*–positive specimens, 67 (70%) and 29 (30%) were from the upper and lower respiratory tract, respectively. Six (6.0%) specimens were collected from children < 4 years of age, 48 (50%) from persons 5–20 years of age, 19 (20%) from persons 21–40 years of age, 19 (20%) from persons 41–60 years of age, and 23 (24%) from persons >65 years of age. All *M. pneumoniae*–PCR positive specimens were cultured and 42 (44%) of the 96 primary specimens yielded positive isolates.

Nested PCR amplification and DNA sequencing of the partial 23S rRNA gene were performed to detect mutations at nucleotide positions 2063, 2064, 2067, 2617 in the 23S rRNA gene, which are associated with macrolide resistance (*2,8*). In addition to macrolide resistance, molecular determinants of fluoroquinolones (*gyrA* and *parC*) and tetracycline (16S rRNA) resistance were also analyzed (*10,11*).

For macrolide resistance, 91 (95%) of 96 specimens were amplified and analyzed for mutations. Mutations that have been associated with macrolide resistance were found in 11 (12.1%) of the 91 specimens (Table 1). Of the 11 isolates with a mutant genotype, 10 (90.9%) contained a mutation at nucleotide position 2063 (A2063G), and 2 (18.2%) specimens had a mutation at position 2064 (A2064G). In 4 isolates, a mixed population of wild type and mutant at position 2063 were identified on sequence chromatograms.

Table 1. Macrolide-resistant *Mycoplasma pneumoniae* identified in Ontario, Canada, 2010–2011*

Patient	ID no.	Age, y/sex	Specimen collection date	Specimen source	Substitution in 23s rRNA			
					A2063	A2064	A2067	C2617
1	H72992–11	43/F	2011 Aug 8	SPT	A/G	A/G	A	C
2	C706158–11	10/M	2011 Aug 25	NP	A/G	A	A	C
3	K35611–11	38/F	2011 Sep 1	NP	G	A	A	C
4	C751048–11	44/F	2011 Sep 8	BAL	G	A	A	C
5	P54752–11	42/M	2011 Oct 6	NP	G	A	A	C
6	P54912–11	12/M	2011 Oct 13	NP	A/G	A	A	C
7	M29279–11	3/F	2011 Dec 9	NP	G	A	A	C
8	N223472–11	5/M	2011 Dec 14	BAL	G	A	A	C
9	N223473–11	5/M	2011 Dec 14	BAL	A	G	A	C
10	C34899–12	10/F	2012 Jan 20	NP	G	A	A	C
11	C63502–12	37/F	2012 Jan 23	BW	A/G	A	A	C

*ID, idenitifcation; SPT, sputum; NP, nasopharyngeal swab; BAL, bronchoavelor lavage; BW, bronchial washing.

One specimen had wild type and co-mutations at positions 2063 and 2064. None of the specimens contained any mutations at positions A2067 or C2617.

In addition to macrolide resistance, molecular determinants of fluoroquinolone and tetracycline resistance in *M. pneumoniae* were examined. A previous report showed that substitutions at position 99 (83 for *Escherichia coli*) of *gyrA* and positions 81, 83, and 87 (78, 80, and 84 for *E. coli*) of *parC* were associated with fluoroquinolone resistance (*10*). In our study, none of the isolates contained any mutations that have been associated with fluoroquinolones resistance. Similarly, amplification and sequencing of 16S rRNA gene regions encompassing the tetracycline binding site did not show any mutations at positions 968 (T968C) and 1193 (G1193A), which have been shown to be associated with tetracycline resistance among *M. pneumoniae* (*11*).

Typing of *M. pneumonia*e isolates (42/96) by amplification and Sanger sequencing of almost the entire P1 adhesion gene was performed by using primer pairs ADH1/2, ADH3/4, and ADH2BF/R, which amplify 3 fragments of ≈2,280, 2,580 and 767 bp, respectively (*13*). Sequencing reactions were performed in both directions by using the Big Dye Terminator Cycle Sequencing Ready Reaction DNA Sequencing Kit in an ABI 3730 or 3750 automated sequencer (Applied Biosystems, Foster City, CA, USA).

P1 gene sequencing identified 6–11 variable number tandem repeat AGT sequences. Using sequence typing and comparing homology of nucleotide and amino acid sequences to reference sequences, we found that 16 (38%) isolates belonged to type 1 and shared high homology at nucleotide (99.7%–99.9%) and amino acid (99.5%–99.9%) levels. Seven of the P1 type 1 isolates had a point mutation in E179K, and 3 of these isolates also had a second mutation Q1232E compared with the type 1 reference strain (M129). Twenty-six (62%) isolates were characterized as type 2 and were differentiated within 3 variants (Table 2). Among 5 resistant isolates that were typed, 3 belonged to type 1 and 2 belonged to type 2.

Conclusions

In this study, 12.1% of *M. pneumoniae*–positive specimens contained mutations that are associated with macrolide resistance, and most (90.9%) specimens had a mutation at nt 2063. This finding is not surprising because this mutation has been shown to be predominant among macrolide-resistant *M. pneumoniae* and has been associated with high-level resistance (erythromycin MIC >64 mg/L) (*2,3,14*). None of the specimens contained any mutations at positions 2067 or 2617 because mutations at these positions are rare. Previous studies have shown reduced efficacy rate of macrolides for treating infections with *M. pneumoniae* isolates containing 3 mutations (*7,9*).

Typing of the P1 gene showed no clear association between macrolide-resistant isolates and specific subtype

Table 2. Typing of *Mycoplasma pneumoniae* isolates by P1 adhesion gene, Ontario, Canada, 2010–2011*

P1 gene type	No. isolates	Variant	Reference	GenBank accession no.†	Nucleotide homology, %	Amino acid homology, %	Nonsynonymous mutations compared with reference (no. isolates)	No. VTR AGT sequences	No. drug-resistant isolates
1	16	NA	M129	U00089.2	99.7–99.9	99.5–99.9	E179K (7), D559N (1), Q1232E (4)	7–11	3
2	8	NA	Mp1842	AF290002.1	99.6–99.9	99.5–99.8	S841L, E962D, I1302V	7–11	1
	3	2a	Mpn309	AP012303.1	99.8–99.9	99.5–99.6	Y210N (1), T837N (3)	6	0
	8	2b	T-103	AB691539.1	99.5–99.9	99.5–99.9	S1516P: 6 isolates had a deletion at 1244-QTNS-1247	7–9	1
	7	2c	P033	JN048891.1	99.8–100	99.7–100	V1411E	7–9	0

*VTR, variable tandem repeat. NA, not applicable (strains M129 and Mp1842 are considered prototypes representative for types 1 and 2).
†The sequences of the P1 gene generated in this study have been deposited in GenBank (accession nos. KF154740–KF154759).

because 3 of the 5 resistant isolates belonged to type 1 and the remaining 2 belonged to type 2; there was no evidence of clonality. This finding is consistent with reports in which macrolide-resistant *M. pneumoniae* isolates of both types were found (*2,5,8*). However, according to a recent study in China, where macrolide-resistant *M. pneumoniae* is highly prevalent (>90%), most isolates were type 1 (*15*).

None of the *M. pneumoniae* isolates contained any *gyrA* gene mutations associated with fluoroquinolone resistance or any 16S rRNA gene mutations associated with tetracycline resistance. These findings are consistent with those of a study in which none of the isolates were resistant to fluoroquinolones or minocycline (*2*).

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Hepatitis E Virus Genotype 4, Nanjing, China, 2001–2011

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During 2001–2011, hepatitis E virus (HEV) was found in the blood of patients in Nanjing, China. All HEV-positive patients had virus genotype 4; subgenotype 4a was predominant. The effective population of HEV in Nanjing increased in ≈1980 and continued until ≈2003 when it plateaued.

Hepatitis E virus (HEV) is a positive-sense, single-stranded RNA virus of the family *Hepeviridae*. Strains of HEV infecting swine, boars, deer, mongooses, rabbits, and humans belong to the genus *Hepevirus* (*1*). Hepeviruses are divided into 4 genotypes that cause sporadic and epidemic outbreaks of hepatitis E in humans. Epidemic outbreaks occur primarily in developing countries in Asia and Africa, and sporadic outbreaks occur worldwide (*2*,*3*). A study showed that HEV causes autochthonous hepatitis E in industrialized countries (*4*).

Genotypes 1 and 2 are transmitted from human to human and cause epidemic and sporadic outbreaks (*3*,*5*). Genotypes 3 and 4 are transmitted to humans zoonotically (*6*) and cause primarily sporadic cases of hepatitis E. The dominant genotype of HEV in China is genotype 4 (*5*,*7*,*8*). The purpose of this study was to analyze the prevalence and genotypes of HEV among infected patients in Nanjing, China.

The Study

A total of 15,910 patients admitted to the Second Hospital in Nanjing, Jiangsu Province, China, because of suspected acute viral hepatitis during January 1, 2001–April 30, 2011, were evaluated by physicians to determine whether they were infected with HEV. Informed consent was obtained from all patients participating in this study. This study was conducted in accordance with national ethics regulation and was approved by the research ethics committee of Southeast University in Nanjing. Because several physicians evaluated the patients, a variety of clinical symptoms were used to diagnose viral hepatitis. Patients were tested for HEV by using serologic analysis. A total of 813 patients who were positive for IgM against HEV were tested for HEV RNA.

ELISAs for antibodies against HEV (Wan Tai Biologic Pharmacy Enterprise Co. Ltd, Beijing, China) were performed according to the manufacturer's instructions. In-house sandwich enzyme immunoassays were used according to Dong et al. (*9*).

HEV primers were designed to amplify a 691-bp segment from open reading frame 2 genotypes 1–4 (Table 1). RNA extraction and cDNA sequencing were performed as described (*10*). Sequences identified have been deposited in GenBank under accession nos. JX997438–JX997647).

A total of 210 sequences were aligned by using ClustalX (*11*) and trimmed at their termini to remove gaps by using BioEdit version 7.0.5.3 (*12*). All sequences (n = 178) without a collection date were removed from the dataset. This sequence set was analyzed to estimate the time to the most recent common ancestor and to generate a skyline plot. These sequences align with open reading frame 2 sequence at nt 6454–6966 (GenBank accession no. AJ272108).

Sequencing confirmed that all 210 HEV RNA–positive patients were infected with HEV genotype 4. This exclusivity probably reflects the recent trend from infection by genotype 1 toward infection by genotype 4, and the dominance of genotype 4 in Nanjing is similar to that in other regions of China (*7*–*9*,*13*). Although the year of collection for serum specimens was recorded during 2006–2011, before 2006, specific years of collection were not recorded. Records show there were 11 HEV RNA–positive patients at the hospital during 2001–2003 and 16 HEV RNA–patients during 2004–2005. During 2006–2010, the increase in the number of HEV RNA–positive patients was nearly linear. The total number of acute hepatitis E cases during 2011 could not be determined because patients were examined only through the end of April 2011 (Figure 1).

Subgenotypes were determined by creating a neighbor-joining phylogenetic tree from all patient sequences and reference sequences obtained from GenBank: EF077630 for subgenotype 4a, EU676172 for 4b, AB193177 for 4c, GU361892 for 4d, AY723745 for 4e, AB220974 for 4f, and AB108537 for 4g by using ClustalX (*11*). Subgenotypes detected were 4a, 4b, 4c, 4d, 4g, and 3 sequences that appear to belong to an undescribed subgenotype and are most similar to GenBank sequences AB369690, AB521805, AB521806, DQ450072, and EF570133. The predominant

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DOI: http://dx.doi.org/10.3201/eid1909.130013

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Table 1. Primers used for RNA extraction and cDNA synthesis of hepatitis E, virus, Nanjing, China*

Primer	Sequence, 5′→3′	Type
JM2	CCG ACA GAA TTG ATT TCG TCG GC	EF
JM36	CAT YTT AAG RCG CTG MAG CTC AGC	ER
JM3	TYG TCT CRG CCA ATG GCG AGC	IF
JM35	CGR CAY TCM GGG CAR AAR TCA TC	IR

*E, external; F, forward; R, reverse; I, internal.

subgenotype was 4a (50.2%) (Table 2). Subgenotypes 4b, 4c, 4d, and 4g had prevalences of 5.4%, 9.3%, 32.7% and 1.0%, respectively. The unknown subgenotype had a prevalence of 1.5%.

To examine whether there was a change in the prevalence in any of the 3 most abundant subgenotypes (4a, 4c, and 4d) detected, we conducted χ^2 analyses. Numbers of subgenotype sequences found for each subgenotype were compared with every other subgenotype in adjacent time intervals. In addition, numbers of subgenotypes were compared between the first time period (2001–2003) and 2010 and 2011. The p values for all comparisons were >0.11 except for comparison between subgenotypes 4a and 4c from 2008 with those from 2009 ($p = 0.04$). Values for subgenotypes 4b, 4g, and the unknown subgenotype were all within 2 SD of their respective means (Table 2). This finding indicates that there were no major differences in subgenotype prevalence over time.

BEAST version 1.71 (*14*) was used to estimate the time to the most recent common ancestor and to create a skyline plot as described (*13*) but with use of the Kimura 3-parameter substitution model. Calculation of the time to the most recent common ancestor for sequences with confirmed dates of collection estimated that the most recent common ancestor for these sequences existed 102.5 years ago (range 69.1–141.4 years ago). A skyline plot created from the dated sequences suggests that the effective population of HEV in Nanjing increased slightly more than 1 log. This increase started in ≈1980 and continued until ≈2003, when the effective population of HEV genotype 4 in Nanjing reached a plateau (Figure 2).

Conclusions

The current findings are different from those reported by Purdy and Khudyakov (*13*), which showed an increase in the effective population of HEV genotype 4 in China

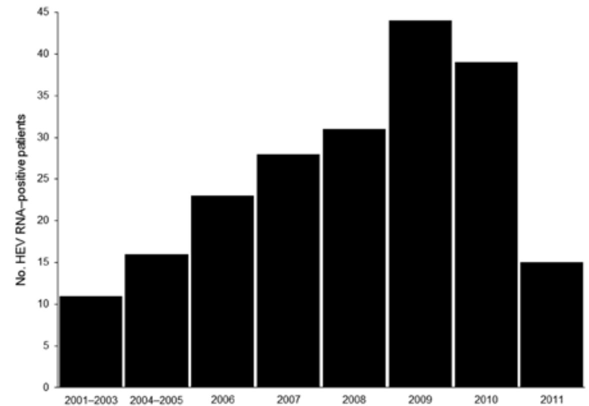

Figure 1. Hepatitis E virus (HEV) RNA–positive patients at Affiliated Second Hospital, Nanjing, China. Data for 2011 are for the first 4 months of the year only.

from ≈1900 through ≈1940 when the effective population plateaued. Although both plots show an increase in the effective population that plateaus, the periods for these changes differed.

There are 3 potential causes for these differences. First, because the earlier plot (*13*) was constructed with fewer sequences, sampling error may be a factor. Second, the earlier plot nonetheless used longer sequences with many more segregating sites. Third, the earlier plot included sequences originating from a larger geographic area. The second and third reasons could potentially result in more sites with substitutions, resulting in higher sequence diversity, which could extend time estimates further into the past. Because of these 3 disparate factors, we cannot ascertain which plot would be more accurate or whether both plots are correct within their specific contexts. Nevertheless, the common feature of both plots is that the effective population of HEV genotype 4 has increased and subsequently reached a plateau.

These data suggest that HEV genotype 4 has undergone a relatively recent expansion in the late twentieth century in Nanjing. The most dominant subgenotype is 4a, and subgenotype distribution has not changed over the past decade. Therefore, a stable population of genotype 4 HEV has been established in the Nanjing region of China. This finding is consistent with the trend observed in other areas within China in which decreases in the prevalence of HEV genotype

Table 2. Distribution of hepatitis E virus subgenotypes, Nanjing, China 2001–2011

Subgenotype	2001–2003	2004–2005	2006	2007	2008	2009	2010	2011*	No. (%)	Mean ± SD
4a	6	11	11	11	16	24	17	6	103 (50.2)	12.9 ± 6.0
4b	0	0	2	1	1	4	2	1	11 (5.4)	1.4 ± 1.3
4c	2	1	0	4	6	1	4	1	19 (9.3)	2.4 ± 2.1
4d	3	4	9	9	8	12	16	6	67 (32.7)	8.4 ± 4.2
4g	0	0	0	1	1	0	0	0	2 (1.0)	0.3 ± 0.5
4?†	0	0	0	1	0	1	0	1	3 (1.5)	0.4 ± 0.5

*Sequences from 2011 are for the first 4 months of the year.
†Putative new subgenotype.

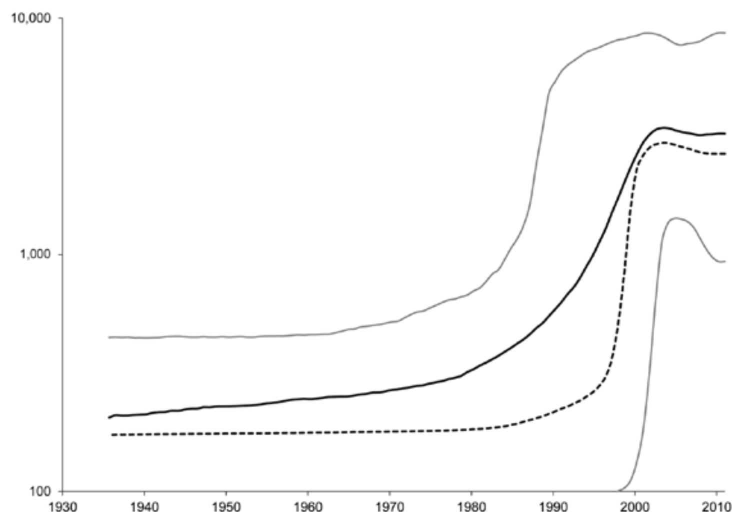

Figure 2. Effective populations of hepatitis E virus, Nanjing, China, calculated from sequences with known years of collection. Solid black line, mean effective population; dashed black line, median effective population; gray lines, the upper and lower limits of the 95% highest posterior density. N_e is the effective population with HEV, and t is the generation time of the virus in years.

1 have been accompanied by increases in the prevalence of genotype 4 (*5*,*7*,*8*). Whether this replacement of genotype 1 by genotype 4 reflects changes in the environment, changes in mode of transmission, or introduction of HEV genotype 4 to animal reservoirs to which humans have been exposed requires further studies. Such investigations are particularly urgent given the continuing increase in China of the prevalence of acute hepatitis E associated with HEV genotype 4 (*15*).

Acknowledgments

We thank Chong-Gee Teo and several reviewers for helpful suggestions and edits for this paper.

This study was supported by the National High Technology Research and Development Program (863 Program 2006AA02A235) of China, the Natural Science Foundation (BK2006098), and the Science and Technology Support Program (BE2009664) of Jiangsu Province, China.

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Highly Pathogenic Avian Influenza A(H7N3) Virus in Poultry Workers, Mexico, 2012

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We identified 2 poultry workers with conjunctivitis caused by highly pathogenic avian influenza A(H7N3) viruses in Jalisco, Mexico. Genomic and antigenic analyses of 1 isolate indicated relatedness to poultry and wild bird subtype H7N3 viruses from North America. This isolate had a multibasic cleavage site that might have been derived from recombination with host rRNA.

Although wild birds might be infected with influenza A(H7) viruses, outbreaks among poultry are rare. Human infection with influenza A(H7) virus is rare but has been documented after direct contact with infected birds (*1*). Conjunctivitis or upper respiratory tract symptoms developed in patients infected with this virus, and outcomes ranged from mild disease to death (*1*,*2*). In North America, 6 persons infected with influenza A(H7) virus have been reported; all patients recovered (*2*–*6*). We report the cases of 2 poultry workers with conjunctivitis caused by highly pathogenic avian influenza (HPAI) A(H7N3) viruses during poultry-related outbreaks in Jalisco, Mexico (*5*).

The Study

In June 2012, outbreaks of (HPAI) A(H7N3) virus in poultry on farms throughout Jalisco State were reported by the National Service for Health, Safety, and Food Quality in Mexico (*7*,*8*). A 32-year-old poultry worker who reported irritation in her left eye was examined at a clinic in Jalisco on July 7. Bilateral conjunctival swab specimens were collected and sent to the Institute for Epidemiologic Diagnosis and Reference (InDRE) in Mexico City, where H7 subtype virus infection was confirmed by real-time reverse transcription PCR (RT-PCR). HPAI A(H7N3) virus had been suspected because the patient collected eggs on a farm that had had HPAI A (H7N3) virus infection among poultry. The Mexican International Health Regulation authority reported the case to the World Health Organization on July 19.

Several days later, a 52-year-old man, who was related to the first patient and worked on the same farm, visited a local clinic and reported conjunctivitis. Conjunctival swab specimens from this patient were also positive for H7 subtype virus infection by real-time RT-PCR. Both patients were treated symptomatically and recovered without sequelae (*5*). We describe characteristics of the virus isolated from the 32-year-old woman.

Conjunctival swab specimens were placed in virus transport medium and shipped to InDRE for diagnostic testing. RNA from clinical samples was extracted by using the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's protocol. Samples were subjected to real-time RT-PCR by using an H7 hemagglutinin (HA) gene–specific assay. Viruses were isolated from RT-PCR–positive clinical samples collected from each eye by inoculating embryonated chicken eggs and incubating them for 48 h before harvest of allantoic fluid. Isolates were sent to the Centers for Disease Control and Prevention (Atlanta, GA, USA), where virus was reisolated in embryonated chicken eggs for further characterization.

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DOI: http://dx.doi.org/10.3201/eid1909.130087

[1]These authors contributed equally to this article.

[2]Deceased.

Group	Virus	Sequence (HA0 cleavage ↓ before RG)
HPAI	A/seal/Mass/1/1980	PENPKKEHPSAGKDPKKTGGPIYRRTRG
	A/turkey/Oregon/1971	PENPKTSLSPLYPGRTTDLQVPTA--RG
	A/chicken/Chile/2002	PENPKTCSPLSRCRET----------RG
	A/chicken/Jalisco/CPA1/2012	PENPK[DRKSRHRRT]------------RG
	A/Mexico/InDRE7218/2012	PENPK[DRKSRHRRT]------------RG
	A/chicken/BC/2004	PENPKQAYQKRMT-------------RG
	A/Canada/rv504/2004	PENPKQAYQKRMT-------------RG
	A/Canada/rv444/2004	PENPKQAYQKQMT-------------RG
	A/chicken/SK/HR-00011/2007	PENPKTTKPRPR--------------RG
	A/chicken/Germany/01/1979	PEIPKKKKKK----------------RG
	A/chicken/Italy/5074/1999	PEIPKGSRVR----------------RG
	A/chicken/Victoria/1976	PEIPKKREK-----------------RG
	A/starling/Victoria/1985	PEIPKKREK-----------------RG
	A/fowl/Dobson/1927	PELPKKRRK-----------------RG
	A/chicken/Germany/R28/2003	PEIPKRRR------------------RG
	A/chicken/Belgium/06600/2003	PEIPKRRR------------------RG
	A/Netherlands/127/2003	PEIPKRRR------------------RG
	A/chicken/Netherlands/2586/2003	PEIPKRRR------------------RG
	A/goose/Leipzig/137/8/1979	PEIPKRKK------------------RG
	A/chicken/Victoria/1/1992	PEIPKKKK------------------RG
	A/chicken/Queensland/667/1995	PEIPRKRK------------------RG
	A/turkey/England/1963	PETPKRRR------------------RG
	A/chicken/Murree/NARC-01/1995	PETPKRRK------------------RG
LPAI	A/New York/107/2003	PEKPKP--------------------RG
	A/turkey/Virginia/4529/2002	PEKPKP--------------------RG
	A/env/New York/19495-1/2006	PEKPKP--------------------RG
	A/chicken/NY/3572/1998	PENPKP--------------------RG
	A/chicken/Chile/184240-2/2002	PEKPKT--------------------RG

Figure 2. Multibasic cleavage sites of highly pathogenic avian influenza (HPAI) A(H7N3) virus isolated from a poultry worker with conjunctivitis in Jalisco State, Mexico, July 2012, and other influenza viruses. Box indicates novel amino acid cleavage site sequence motif. HA, hemagglutinin; LPAI, low pathogenicity avian influenza. Hyphens indicate gaps in the sequence alignments whereby 1 sequence has an insertion of amino acids relative to shorter sequences.

Nucleotide sequences of 8 influenza A gene segments from a virus isolate were generated by semiconductor next-generation sequencing with Ion PGM (Life Technologies, Carlsbad, CA, USA) and MBTuni12 and MBTuni13 primers as described (*9*) at InDRE/Instituto Nacional de Enfermedades Respiratori and by RT-PCR of overlapping fragments of each gene by using H7N3 subtype and avian influenza virus–specific primers at the Centers for Disease Control and Prevention. Sequences were aligned and phylogenetic trees were constructed from each gene alignment by using a neighbor-joining approach implemented in MEGA5 (www.megasoftware.net/) with 1,000 bootstrap replicates.

Genomic sequences confirmed that the conjunctivitis was caused by infection with an HPAI A(H7N3) virus closely related to HPAI A(H7N3) viruses collected during poultry outbreaks in Jalisco State (Figure 1, Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0087-F1.htm). The full genome of 1 isolate was deposited in GenBank under accession no. CY125725–32. Like reported avian A(H7N3) virus sequences from Jalisco, the human isolate had a multibasic cleavage site indicative of an HPAI A virus (*7*) (Figure 2). Genetic similarity of nucleotides at the cleavage site suggested that this region was inserted into the H7 HA gene at the site of HA0 protein cleavage by nonhomologous recombination of host rRNA from an unknown source (*7*). Comparison of this protein sequence motif with other HPAI and low pathogenicity avian influenza (LPAI) H7 viruses showed that this sequence indicated a novel cleavage site not observed in influenza A virus HA gene sequences (Figure 2). However, multiple arginine amino acids in this motif would be predicted to result in a highly pathogenic phenotype in chickens.

Phylogenetic trees of HA and neuraminidase (NA) genes indicated high similarity of HPAI A(H7N3) viruses detected in Mexico and LPAI viruses collected from wild birds and poultry in North America (Figure 1). HA genes clustered with LPAI A(H7N9) viruses from turkeys, geese, and guinea fowl in the United States during 2011 (*10*). The N3 NA genes grouped with LPAI viruses of various subtypes, clustering most closely with viruses collected from wild birds in the midwestern United States in 2009. Internal genes also clustered with LPAI viruses from various subtypes collected primarily in California in 2010 (online Technical Appendix, wwwnc.cdc.gov/EID/article/19/9/13-0087-Techapp1.pdf). An exception was the polymerase acidic gene, which was most closely related to an H11N9 subtype virus from Mississippi.

HA and other protein gene alignments were assessed for putative markers of virulence, mammalian adaptation, receptor-binding specificity, and antiviral drug resistance. Besides the multibasic cleavage site, the virus had typical avian consensus amino acid residues in the HA protein at positions involved in preferential receptor binding to avian sialic acid receptors (amino acids Q226 and G228 by H3 numbering). Avian consensus sequences at other

Table. Hemagglutination inhibition titers of North American and Eurasian lineage influenza A (H7) viruses from wild birds, poultry, and humans*

Antigen	Virus and titer								
Reference	CN/444	CN/504	MX/7218	GS/NE	TK/VA	NY/107	TK/MN	NL/219	DK/VN
A/Canada/RV444/2004 H7N3	**80**	320	80	80	80	80	80	160	10
A/Canada/RV504/2004 H7N3	160	**320**	160	160	80	80	160	160	20
A/Mexico/INDRE7218/2012 H7N3	160	320	**160**	160	80	80	160	160	20
A/GS/Nebraska/17097–4/2011 H7N9	160	320	160	**160**	80	160	80	160	20
A/TK/Virginia/4529/2002 H7N2	160	320	160	160	**320**	1,280	20	80	20
A/New York/107/2003 H7N2	160	320	80	160	160	**160**	10	80	20
A/TK/Minnesota/0141354/2009 H7N9	40	80	40	80	20	20	**80**	40	5
A/Netherlands/219/2003 H7N7	20	40	10	40	20	5	40	**160**	10
A/DK/Vietnam/NCVD-197/2009 H7N3	80	160	80	20	5	10	20	40	**80**
Test									
A/Canada/RV444/2004 x PR8 (H7N3)	160	320	80	160	80	80	80	160	20
A/GF/Nebraska/17096–1/2011 (H7N9)	320	640	320	640	160	320	640	320	40
A/CK/Arkansas/10/2008 (H7N3)	160	320	80	160	80	80	320	80	20

*Homologous titers of reference antigen to serum samples are indicated in **boldface**. CN, Canada; MX, Mexico; GS, goose; TK, turkey; NY, New York; NL, The Netherlands; INDRE, Institute for Epidemiologic Diagnosis and Reference; DK, duck; GF, guinea fowl; CK, chicken.

motifs/amino acid positions in proteins of interest were identified, suggesting that the virus had not accumulated described mammalian host adaptive mutations or known virulence markers.

Antigenic characterization was performed by using a panel of ferret antiserum in hemagglutination inhibition (HI) tests with turkey erythrocytes as described (*11*). The HI assay demonstrated relatedness of HPAI A(H7N3) virus with other H7 subtype viruses from North America and a high level of cross-reactivity with the current H7 World Health Organization prepandemic vaccine candidate, A/Canada/rv444/2004, and other North American and Eurasian lineage H7 viruses (Table). Antiserum against HPAI A(H7N3) virus was cross-reactive with North American and Eurasian lineage H7 subtype viruses but showed higher levels of heterologous cross-reactivity with recent H7 viruses collected in the United States and a greater reduction in heterologous titers against Eurasian lineage H7 viruses. Although there were several amino acid differences compared with older North American H7 HA1 protein sequences (27–32 changes), only 5 changes were identified when compared with A/Canada/rv444/2004 virus, indicating a high degree of genetic conservation among this group of H7 viruses.

To determine the drug concentration required to inhibit 50% of NA activity, we preformed a functional neuraminidase inhibition (NAI) assay. A fluorescent NAI test was conducted as described (*12*). Oseltamivir-sensitive H1N1 subtype virus (A/Texas/36/91) and its oseltamivir-resistant counterpart with mutation H274Y (N2 numbering) were included as controls. NAI assays showed that the virus was sensitive to neuraminidase inhibitors (zanamivir and oseltamivir). No putative markers of antiviral drug resistance were identified in either NA or matrix genes.

Conclusions

Emergence of a novel (HPAI) A(H7N3) virus is a reminder of the devastating effect this virus can have on poultry industries and its potential for interspecies transmission. The finding that the HA cleavage site of this virus was probably a result of nonhomologous recombination, as described for other avian influenza A(H7) virus outbreaks, underscores the potential for emergence of HPAI H7 viruses (*13*,*14*). Established mammalian models of ocular infection with H7 subtype influenza A viruses associated with human conjunctivitis demonstrated that these viruses replicated efficiently in eye and respiratory tract tissues (*15*).

Although further studies are needed to investigate in vivo transmissibility of this virus, direct transmission of this virus from infected poultry to humans remains a threat and warrants use of personal protective equipment (including goggles for eye protection) and monitoring persons at risk to prevent additional cases in humans. Health authorities should consider avian influenza A virus infection in patients who have conjunctivitis or influenza-like illness and contact with poultry in areas with known avian influenza outbreaks.

Acknowledgment

We thank the originating and submitting laboratories for providing sequences from the Global Initiative On Sharing All Influenza Data EpiFluTM database, which were used in this analysis.

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EMERGING INFECTIOUS DISEASES

Outbreak of Chikungunya Virus Infection, Vanimo, Papua New Guinea

Paul F. Horwood, Lisa J. Reimer, Rosheila Dagina, Melinda Susapu, Grace Bande, Michelle Katusele, Gussy Koimbu, Stella Jimmy, Berry Ropa, Peter M. Siba, and Boris I. Pavlin

In June 2012, health authorities in Papua New Guinea detected an increase in febrile illnesses in Vanimo. Chikungunya virus of the Eastern/Central/Southern African genotype harboring the E1:A226V mutation was identified. This ongoing outbreak has spread to ≥8 other provinces and has had a harmful effect on public health.

Chikungunya virus (CHIKV) is a mosquito-transmitted virus of the family *Togaviridae* and genus *Alphavirus*. CHIKV can be classified into 3 distinct genotypes: Asian, Eastern/Central/Southern African (ECSA), and Western African. The usual vectors for CHIKV are *Aedes aegypti* mosquitoes; *Ae. albopictus* mosquitoes are a potential secondary vector. Human infection with CHIKV results in illness characterized by high fever, severe polyarthralgia, headache, maculopapular rash, fatigue, nausea, and vomiting. CHIKV has recently been responsible for explosive outbreaks of disease in the Indian Ocean region (*1*) and southern India (*2*).

Before June 2012, chikungunya had not been reported in Papua New Guinea. The recent increase in reported outbreaks of chikungunya in Papua New Guinea has coincided with the appearance of a variant strain of CHIKV that has a mutation from alanine to valine at amino acid position 226 in the envelope 1 (E1) glycoprotein gene. This mutation enables CHIKV strains to more efficiently replicate in the salivary gland of *Ae. albopictus* mosquitoes, thus enhancing the role of this vector in transmission of virus to susceptible human hosts (*3*). We report molecular detection, epidemiologic and entomologic investigations, and viral genetic characterization for an outbreak of chikungunya in Papua New Guinea.

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DOI: http://dx.doi.org/10.3201/eid1909.130130

The Study

In late June 2012, an increase in cases of prolonged fever for ≥3 days was reported from the Vanimo General Hospital in Vanimo, Sandaun Province. The illness was characterized by high fever (temperature >40°C), arthralgia, emesis, and severe nausea. In most patients, fever subsided within 24–72 hours and patients were discharged after abatement of initial signs and symptoms. However, many patients returned within a few days reporting lingering arthralgia and severe pruritus. On the basis of clinical characteristics, several arboviruses were immediately suspected as causes of the illness.

Serum samples were collected from 86 patients with acute fever during September–October 2012. Samples were screened for CHIKV by using a reported real-time reverse transcription PCR (*4*), and 31 (36%) were positive for CHIKV. Logistic regression showed that no clinical findings were predictive of laboratory confirmation of CHIKV. Partial E1 genes for 3 CHIKV strains from Papua New Guinea (VN033–KC524770, VN064–KC524771, and VN083–KC524772) were sequenced (*4*). The Papua New Guinea strains had high levels of nucleotide (99.9%) and amino acid (100%) identity; only 2 synonymous sequence polymorphisms were observed between the 3 strains. Sequence alignment and phylogenetic analysis of E1 sequences showed that the Papua New Guinea outbreak was caused by CHIKV from the ECSA genotype (Figure 1). All 3 CHIKV strains had the E1:A226V mutation.

During June 25–November 25, a total of 1,590 suspected cases of chikungunya were recorded at Vanimo General Hospital (Figure 2). Detailed data were collected for 98 patients (54 female patients; $p = 0.31$). The age range of the patients was 2–60 years (median age 24 years). The most common signs and symptoms were fever and arthralgia (100%; these symptoms constituted the case definition), headache (83%), cough (31%), and nausea (26%).

In November 2012, an entomologic survey was conducted at 49 households in Vanimo and surrounding settlements. All potential indoor and outdoor breeding containers were recorded and container, household, and Breteau indices were calculated (Table) on the basis of the presence of aedine larvae. A portion of larvae from each collection was reared to adults for morphologic identification, and 100% (n = 137) were identified as *Ae. albopictus* mosquitoes.

Adult mosquitoes were collected over 10 days by using the Biogents-Sentinel odor-baited trap (Biogents AG, Regensberg, Germany). All 155 mosquitoes collected were identified morphologically to species; 154 were identified as *Ae. albopictus* mosquitoes, and 1 was identified as an *Ae. scutellaris* mosquito. Mosquitoes were separated by sex

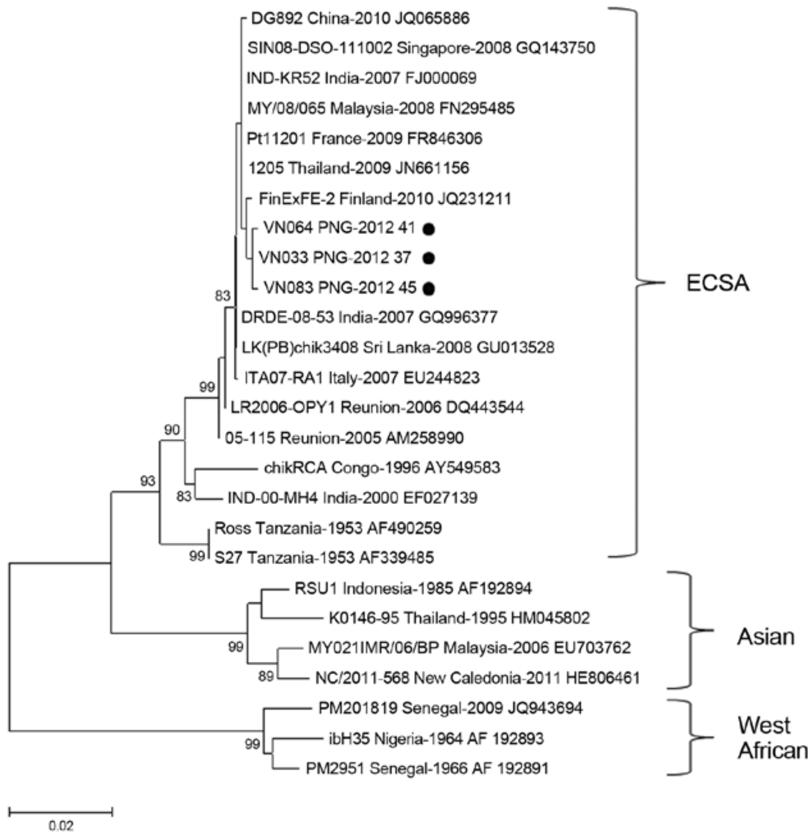

Figure 1. Phylogenetic analysis of a 924-nt fragment of partial envelope 1 glycoprotein gene sequences of chikungunya virus strains isolated in Vanino, Papua New Guinea (PNG) and global strains. The tree was constructed by using bootstrap analysis (1,000 replicates) and the neighbor-joining method with the Kimura 2-parameter method for nucleotide data analysis. Values along branches are bootstrap percentages. Black circles indicate strains isolated in this study. ECSA, Eastern/Central/Southern African. Scale bar indicates nucleotide substitutions per site.

and location and combined in pools of ≤10 for detection of CHIKV by use of real-time reverse transcription PCR. All mosquito pools were negative for CHIKV.

Conclusions

The outbreak of chikungunya in Vanimo is still ongoing. There are grave concerns that the outbreak will spread throughout the country; as of this writing, confirmed or suspected cases have been reported in 8 other provinces (Madang, West New Britain, East New Britain, New Ireland, Eastern Highlands, Oro, Chimbu, and Morobe) in Papua New Guinea.

Although no previous outbreaks of chikungunya in Papua New Guinea have been documented, a study con-

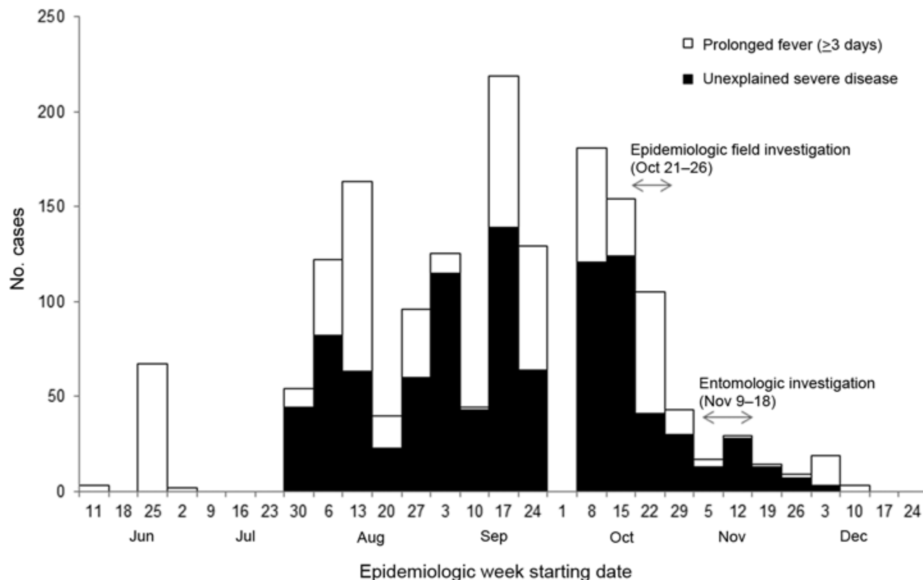

Figure 2. Syndromic cases (n = 1,565) reported from Vanimo General Hospital, Papua New Guinea, July–October 2012. Two of the 8 syndromes under surveillance were increased during the chikungunya outbreak. It is not known why no cases were recorded during the week of October 1.

Table. Descriptive data for entomologic survey for chikungunya virus, Vanino, Papua New Guinea

Characteristic	Value
No. houses surveyed	49
No. containers with water	898
No. mosquito-positive containers	159
No. mosquito-positive outdoor containers (%)	157 (99)
No. mosquito-positive refuse containers (%)	117 (74)
House index, % (range)	76 (43–100)
Container index, % (range)	18 (10–50)
Breteau index (range)	324 (150–729)

ducted in the mid-1970s reported that the seroprevalence of CHIKV was ≤30% in some regions of the country (*5*). A similar study at that time also reported a high seroprevalence of CHIKV throughout Indonesia and Southeast Asia (*6*). These studies suggest that there may have been a widespread outbreak of chikungunya in the region during preceding years. However, the antigenic cross-reactivity of arboviruses is widely recognized and cannot be ruled out as a cause of the high seropositivity to CHIKV. The broad age distribution of persons in the outbreak reported here suggests a lack of neutralizing antibodies against CHIKV, at least in the Vanimo area.

The recent evolution of ECSA strains with the E1:A226V mutation has resulted in several outbreaks of chikungunya in widely distributed geographic regions. The emergence of these strains seems to be a case of convergent evolution with adaption to *Ae. albopictus* mosquitoes occurring during outbreaks in India and the Indian Ocean region (*7*). Analysis of E1 genes in this study suggests that virus strains in Papua New Guinea are closely clustered with mutant strains that evolved in India and subsequently spread to other countries, such as Malaysia, Singapore, Sri Lanka, Thailand, and Italy. However, full-genome analysis is required to establish the relationship between strains from Papua New Guinea and other regions.

Neighborhoods in Vanimo had a high Breteau index of 324, which indicated high transmission potential for CHIKV (*8*) and dengue virus (*9*). *Ae. albopictus* mosquitoes were found breeding in many types of containers found outdoors and in vases found indoors. Most infested containers were water storage vessels, such as old metal drums and buckets without proper coverings; in some instances, covered vessels were also infested. Old tires were a highly used breeding site, and gutters were observed to be densely populated.

Because of the absence of a reticulated water system in Vanimo and the general ubiquity of artificial containers (water storage and refuse), our observations indicated that opportunities were rife for breeding of *Aedes* spp. mosquitoes. These breeding opportunities existed despite the fact that Sandaun Provincial Health authorities initiated multiple cleanup campaigns before our survey (although no other vector control measures were implemented).

Because of logistic constraints, the entomologic survey was conducted during the decrease in the outbreak and was designed to identify the dominant vector and opportunities for control. We were unable to positively implicate *Ae. albopictus* mosquitoes as the primary vector. However, the predominance of this mosquito throughout the outbreak region (*10*,*11*) and its role in CHIKV transmission in other regions (*12–14*) highlights the need for targeted vector control to curtail the spread of disease.

Acknowledgment

We thank John Aaskov for providing control samples.

This study was supported by the World Health Organization and a Partnership in Health Program grant from Esso Highlands Limited, an ExxonMobil subsidiary.

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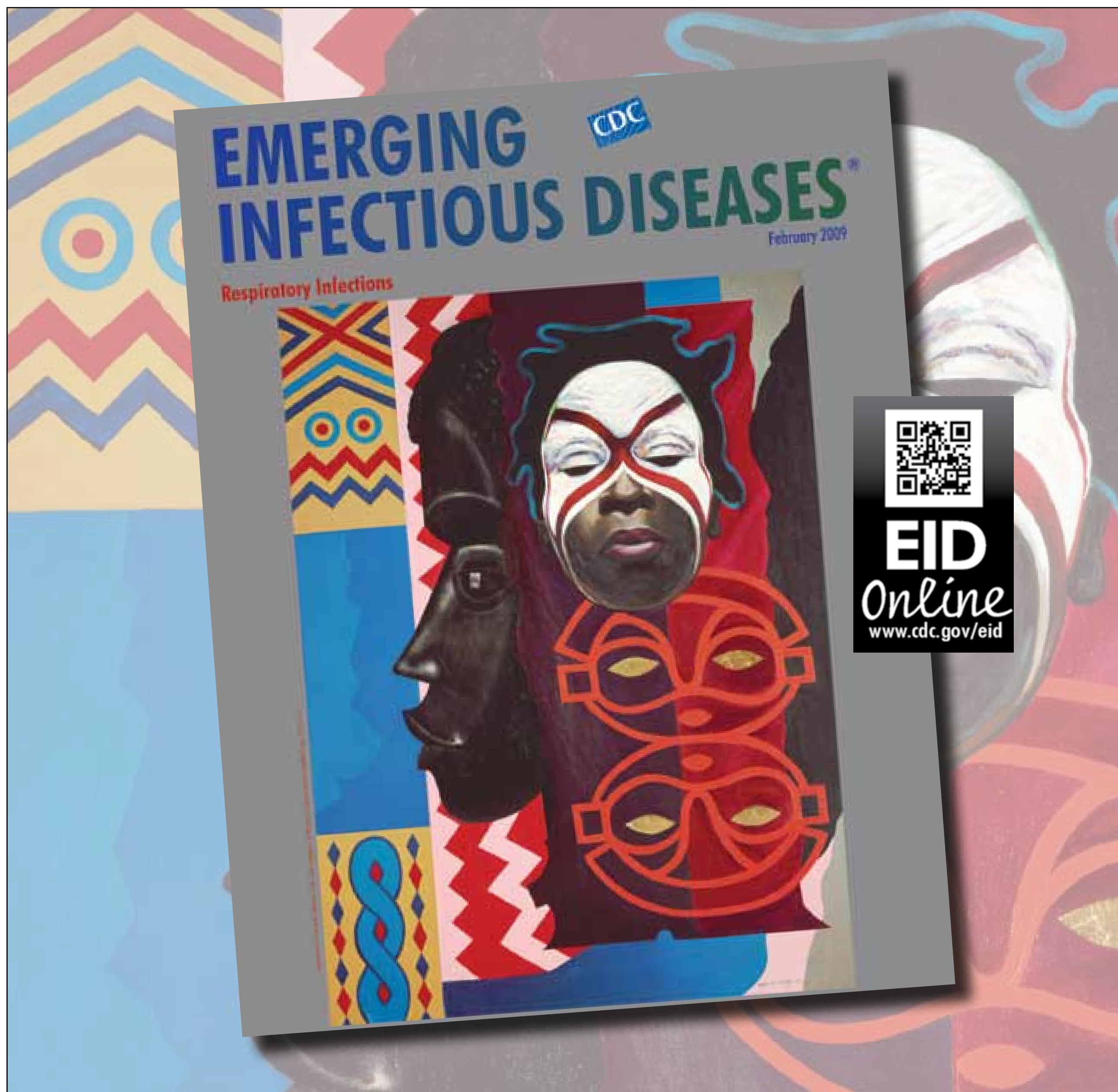

Clostridium difficile Infection Caused by Binary Toxin–Positive Strains

To the Editor: With interest, we read the article by Bacci et al. in which they conclude that *Clostridium difficile* strains containing the binary toxin gene were associated with a higher case-fatality rate after 30 days, even when the analysis was stratified for PCR ribotype (*1*). Although this was an appealing conclusion, in our opinion the study was severely limited by selection bias and confounding by underlying diseases. First, in Danish patients with a *C. difficile* infection (CDI), isolates were characterized only if they were isolated during outbreaks or from patients with severe disease or if isolates were found to be moxifloxacin resistant. Therefore, selection bias was likely to occur. Second, adjustment for concurrent conditions was not performed. This adjustment was especially warranted because outbreaks on specific hospital wards (e.g., intensive care units) could have influenced the all-cause mortality rate. Last, the selection of specific patients and strains questions the generalizability of the authors' conclusion.

In an approach to confirm the findings of Bacci et al. (*1*), we used data from a cohort study conducted during 2006–2009 in 13 Dutch hospitals (*2*). A total of 1,350 consecutive hospitalized patients with unformed feces and a positive *C. difficile* toxin test result were included in the study. We checked the 30-day survival for study patients in the Dutch Civil Registration System. For 626 (46%) of the patients, a *C. difficile* strain was available for PCR ribotyping and binary toxin gene characterization. Patient data (e.g., age, sex, hospitalization, and antimicrobial drug use in the 3 months before onset of diarrhea) were collected by review of electronic and paper patient charts and by contacting the treating physician. Underlying diseases present at hospital admission were classified into 7 disease categories (Table, footnote). In addition, during at least 6 months, the Charlson comorbidity index at admission was determined in 9 of the 13 hospitals (total of 357 CDI patients). Proportional hazards modeling was used for survival analysis. The Medical Review Ethics Committee of the Leiden University Medical Center approved this study

During the study period, CDI was endemic in all hospitals in the cohort study (13 cases/10,000 admissions). The all-cause risk for dying within 30 days was 22% (12/55) for persons infected with binary toxin–positive 027 strains, 15% (15/100) for those infected with binary toxin–positive non-027 strains, and 11% (50/471) for those infected with binary toxin–negative strains (Table). Selection bias (e.g., by primarily characterizing isolates of patients with severe disease) was unlikely because the number of deaths among CDI patients without strain characterization (100/724 [14%]) was similar to that among patients with a characterized strain (77/626 [12%]; $p = 0.41$). Thirty-day mortality rates were significantly higher among patients with CDI due to type 027 strains than among patients with binary toxin–negative strains (hazard ratio [HR] 2.2); additional adjustment for age and concurrent condition(s) resulted in a relatively constant HR of 2.0–2.4. Patients with CDI due to binary toxin–positive non-027 strains did not have a substantially higher 30-day mortality rate (HR 1.5); additional adjustment for age and concurrent condition(s) lowered the HR to 1.1–1.4, depending on the method of adjustment.

In accordance with findings in the Danish study, we observed a high 30-day mortality rate among persons infected with type 027 isolates. The 30-day mortality rate was lower among persons infected with non-027 binary toxin–positive isolates, especially after correction for concurrent condition(s); however, confidence intervals overlapped with those for type 027. Therefore, we cannot statistically contradict the conclusion of Bacci et al. (*1*). Nevertheless, because mortality rates in our study among patients with non-027 type CDI strongly resembled mortality rates among patients with CDI caused by binary toxin–negative isolates and because the Danish study was prone to bias and

Table. Thirty-day mortality rates, stratified by binary toxin status, for persons with *Clostridium difficile* infection, the Netherlands, 2006–2009*

Binary toxin status of infecting strain	Absolute 30-day mortality, No.	Absolute 30-day mortality, % (95% CI)	Relative 30-day mortality, HR (95% CI)†: No adjustment	Adjusted for age	Adjusted for age and concurrent condition: Method 1	Method 2	Method 3
Positive							
027, N = 55	12	22 (13–35)	2.2 (1.2–4.2)	2.0 (1.1–3.8)	2.4 (1.1–5.5)	2.0 (0.8–5.4)	2.0 (0.7–5.5)
Non-027, N = 100	15	15 (9–23)	1.5 (0.8–2.6)	1.4 (0.8–2.5)	1.3 (0.6–2.7)	1.1 (0.5–2.8)	1.1 (0.4–2.9)
Negative, N = 471‡	50	11 (8–14)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)	1.0 (ref)
Unknown, N = 724§	100	14 (11–17)	1.3 (0.9–1.9)	1.3 (0.9–1.9)	1.4 (0.9–2.2)	1.5 (0.9–2.3)	1.3 (0.7–2.4)

*HR, hazard ratio; ref, reference.
†In the model, age and Charlson index were added as continuous variables; all others were dichotomous. Method 1, adjusted for age and history of admissions and antimicrobial drug use in the prior 3 mos. Method 2, adjusted for age; diseases of the respiratory, digestive, circulatory, and genitourinary systems; endocrine diseases; neoplasms and other diseases; history of admissions and antimicrobial drug use in the prior 3 mos. Method 3, adjusted for age; history of admissions; antimicrobial drug use in the prior 3 mos.; and Charlson comorbidity index.
‡Binary toxin–positive non-027 strains belonged to 8 PCR ribotypes (76% type 078).
§Binary toxin–negative strains belonged to 64 different PCR ribotypes (23% type 014).

lacked adjustment for confounding, we think that the results of Bacci et al. (*1*) should be interpreted with caution. Furthermore, a large clinical study from 2008 concluded that *C. difficile* type 078, which is the most frequently found binary toxin positive non-027 strain, was not associated with a high all-cause mortality rate (*3*). A more recent publication confirmed this finding (*4*). Therefore, in our opinion, there is currently no convincing epidemiologic proof that binary toxin is a marker for infection with virulent *C. difficile.*

This work was supported by a grant from ZonMw (the Netherlands Organization for Health Research and Development; grant 4726).

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DOI: http://dx.doi.org/10.3201/eid1909.110814

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Spread of Kyasanur Forest Disease, Bandipur Tiger Reserve, India, 2012–2013

To the Editor: Kyasanur Forest disease virus (KFDV; family *Flaviviridae*, genus *Flavivirus*) was first recognized in 1956 in Shimoga District, Karnataka State, India (*1*). The natural cycle of KFDV involves 2 monkey species–black-faced langurs (*Semnopithecus entellus*) and red-faced bonnet monkeys (*Macaca radiata*)–and various tick species (genus *Haemaphysalis*). Monkeys become infected with KFDV through the bite of infected ticks; the virus is then transmitted to other ticks feeding on infected monkeys. KFDV infection causes severe febrile illness in some monkeys. When infected monkeys die, ticks drop from the body, thereby generating hot spots of infectious ticks that further spread the virus. In the enzootic state, KFDV circulates through small mammals (e.g., rodents, shrews, ground birds) and ticks (*2*).

Humans can also be infected with KFDV. In humans, the disease causes high fever, frontal headache, and severe myalgia, followed by bleeding from the nasal cavity, throat, gingivae, and, in some cases, gastrointestinal tract (*3*). In the natural KFDV cycle, humans are dead-end hosts.

KFD is unique to 5 districts (Shimoga, Chikkamagalore, Uttara Kannada, Dakshina Kannada, and Udupi) in the Malnad region of Karnataka State, India, where each year during January–May, 100–500 persons are affected by the disease (*2*,*4*). During December 2011–March 2012, a total of 215 suspected KFD case-patients were identified in 80 villages in Shimoga District; laboratory testing confirmed that 61 (28%) were infected with KFDV (*5*).

In November 2012, the deaths of 12 monkeys in Bandipur National Park, Chamarajanagara District, Karnataka State, were reported. At the same time, 6 humans from Mole Hole village and Madhur colony in the Bandipur Tiger Reserve who handled and incinerated the sick monkeys were reported to have clinical signs and symptoms typical of KFD (online Technical Appendix Figure 1, wwwnc.cdc.gov/EID/article/19/9/12-1884-Techapp1.pdf). The monkey handlers (20–55 years of age) were admitted to the local hospital in Gundlupet Taluk. Monkey autopsy specimens, serum samples from suspected human case-patients, and tick pools were collected by staff from the Virus Diagnostic Laboratory in Shimoga. The samples were sent to the National Institute of Virology in Pune for determination of the etiologic agent. Additional samples from humans with suspected KFDV infection, monkeys, and tick pools were received from Chamarajanagar District and adjoining border areas of Tamil Nadu State and Kerala State (Table).

Monkey brain and liver and tick pools were sonicated in 600 mL of Minimum Essential Media (GIBCO/BRL, Life Technologies, Grand Island, NY, USA), and 400 mL of media was added to the homogenate. TriPure Isolation Reagent (Roche Diagnostics, Indianapolis, IN, USA) was used to perform RNA extraction as described (*6*).

Samples were tested for KFDV by nested reverse transcription PCR (RT-PCR) and real-time RT-PCR as described (*6*); 12 of 21 human samples and 4 monkey samples were positive (Table). Two of 14 tick pools screened for KFDV by real-time RT-PCR were positive; however, 1 was weakly positive (Table). The PCR-amplified products were purified by using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany) and then sequenced. KFDV sequences from the samples showed 95.8%–98.1% similarity with prototype strain KFDV P9605. This finding supports the earlier conclusion that a high level of conservation exists for KFDV sequences (*7*). The phylogenetic tree formed 2 clades: the first

Table. Real-time reverse transcription PCR and nested reverse transcription PCR results for specimens screened for Kyasanur Forest disease virus, India, November 2012–May 2013*

Date of sample collection	Location of sample collection	No. samples positive/no. total		
		Human	Monkey	Tick pools
2012 Nov	Maddur Forest Range, Bandipur Tiger Reserve, Chamarajanagara District, Karnataka State	4/6	3/7	–
2013 Jan	Chamarajanagara District, Karnataka State	7/13	–	0/7
2013 Jan	Nilgiri, Tamil Nadu State	0/1	1/2	0/5
2013 Feb	Chamarajanagara District, Karnataka State	–	–	1/2
2013, May	Wayanad District, Kerala State	1/1	–	–
Total no. positive samples		12/21	4/9	1/14

*–, no samples from the area.

included mainly KFDV sequences from 1957–2006, the second included KFDV sequences (human and monkey) from Chamarajanagara District (online Technical Appendix Figure 2).

KFDV has not been detected previously in Chamarajanagara District, the location of Bandipur National Park. Affected areas in the district share a border with Mysore District (Karnataka State), Kerala State, and Tamil Nadu State. In addition,we subsequently found monkey samples from Nilgiri, Tamil Nadu, to be positive for KFDV.

The human case-patients from Chamarajanagara District were mainly forest workers involved in the incineration of the dead monkeys. Infection among these workers indicates that they did not follow appropriate biosafety procedures while handling the infected animals.

Our findings confirm that KFD has occurred outside the districts in Karnataka State where KFDV is known to be endemic. A hemagglutination inhibition antibody survey conducted during December 1988–January 1989 (*8*) indicated the possible existence of this disease in other regions of India. The presence of KFD becomes noticeable when enzootic infections occur and sentinel animals, like monkeys, start dying (*9*). Detection of KFDV in Chamarajanagara District, Tamil Nadu State (Nilgiri), and Kerala State indicates the presence of the virus in many evergreen and semi-evergreen forest areas of India. Infections in these areas may have been missed previously because of the lack of an organized surveillance system.

During the first week of December 2012, immediately after the KFD outbreak was confirmed, the Karnataka public health department vaccinated 322 persons, including villagers, forest officials, health workers, and members of local tribes in the Maddur Forest Range of Bandipur Tiger Reserve. Hot-spot areas caused by monkey deaths were dusted with malathion insecticide to kill ticks. In addition, to prevent additional human infections, epidemiologists recommended establishment of a health education campaign and the use of protective clothing and tick repellents, especially by persons frequently visiting forested areas.

Acknowledgments

We thank Anita Shete and Prasad Kokate for technical support and Sarah Cherian and Santosh Jadhav for help with construction of the phylogentic tree.

Financial support was provided by the National Institute of Virology, Pune, India.

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DOI: http://dx.doi.org/10.3201/eid1909.121884

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Chikungunya Virus Infection, Brazzaville, Republic of Congo, 2011

To the Editor: Chikungunya virus (CHIKV) is a positive single-stranded RNA virus within the genus *Alphavirus*, family *Togaviridae* (*1*–*3*). In humans, CHIKV infection has a rapid onset and is typically cleared in 5–7 days. Following transmission, CHIKV replicates in the skin and then disseminates to the liver and joints. The incubation period for CHIKV infection is 2–4 days. Signs and symptoms of infection include high fever, rigors, headache, photophobia, and a petechial or maculopapular rash, and most infected persons also report severe joint pain (*2*–*4*). CHIKV is transmitted through the bite of infected *Aedes aegypti* and *Ae. albopictus* mosquitoes. The current reemergence of CHIKV seems to be related to 1) CHIKV host switching from *Ae. aegypti* to *Ae. albopictus* mosquitoes, while retaining *Ae. aegypti* mosquitoes as a vector, and 2) introduction of *Ae. albopictus* mosquitoes, which were originally from Southeast Asia, into new areas of the world, including Africa. Severe epidemics of CHIKV infection have occurred in countries in the Indian Ocean region and Africa (*1*–*3*). We report an outbreak of CHIKV infection in Brazzaville, Republic of Congo, that was associated with the mosquito vectors *Ae. albopictus* and *Ae. aegypti*.

In June 2011, reports were received of an outbreak of a new drug-resistant form of malaria in southwest Brazzaville. Diagnostic capacity for detecting arboviruses, including CHIKV, by using molecular diagnostic assays was immediately implemented at the Laboratoire National de Santé Publique in Brazzaville. Blood samples were collected from 23 suspected case-patients, and serum samples were obtained and stored at 4°C. RNA from patients' serum samples was extracted by using the QIAamp Viral RNA Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. To determine if CHIKV, yellow fever virus, dengue virus, or malarial parasites were present in the serum samples, we performed quantitative reverse transcription PCR with Lightcycler 480 RNA Master Hydrolysis Probes (Roche Diagnostics, Mannheim, Germany) on the SmartCycler platform (Cepheid, Sunnyvale, CA, USA) (*5*–*7*). A cycle threshold (C_t) cutoff value of <40 was considered positive. Primary testing focused on CHIKV, and genomic RNA was detected in 21 of 23 patient serum samples (C_t 17.55–37.91) (Table). Neither yellow fever nor dengue virus genomic RNA was detected in any patient samples. However, malaria parasite genetic material was detected in the 2 samples that were negative for CHIKV (Table).

To investigate the involvement and distribution of potential vector mosquito species, we initiated an entomological study in the Makélékélé and Mfilou districts of Brazzaville, the 2 areas associated with the CHIKV

Table. Detection of virus genomic RNA in patient serum samples, Brazzaville, Republic of Congo, June 2011*

Patient sample ID	Virus, C_t			Malaria C_t
	Chikungunya	Yellow fever	Dengue	
1†	25.62	>40	>40	>40
2	>40	>40	>40	32.33
3	17.55	>40	>40	>40
4	37.91	>40	>40	>40
5	21.36	>40	>40	>40
6	21.00	>40	>40	>40
7	20.26	>40	>40	>40
8	19.78	>40	>40	>40
9	>40	>40	>40	33.71
10	25.77	>40	>40	>40
11‡	21.20	>40	>40	>40
12	22.98	>40	>40	>40
13	29.97	>40	>40	>40
14	23.50	>40	>40	>40
15§	31.97	>40	>40	>40
16	31.44	>40	>40	>40
17	33.54	>40	>40	>40
18	31.83	>40	>40	>40
19	34.63	>40	>40	>40
20	34.90	>40	>40	>40
21	33.46	>40	>40	>40
22	29.18	>40	>40	>40
23	33.16	>40	>40	>40

*Except as otherwise indicated, all samples were collected on June 27, 2011. ID, identification; A C_t cutoff value of <40 was considered positive. C_t, cycle threshold.
†Collected on June 23, 2011.
‡Collected on June 24, 2011.
§Collected on June 27, 2011.

outbreak. Mosquitoes were collected at various places in the 2 districts during different hours of the day (Technical Appendix Table, wwwnc.cdc.gov/EID/article/19/10/13-0451-Techapp1.pdf). Mosquitoes were separated by species, *Ae. aegypti* or *Ae. albopictus*, into 13 different pools. The pools were homogenized in lysis buffer (Buffer AVL; QIAGEN) by using sterile disposable tissue grinders, and RNA was extracted. We then analyzed the samples for the presence of CHIKV genomic RNA by following the procedures described above for the serum samples. All 13 mosquito pools were positive for CHIKV genomic RNA (C_t 23.5–34.63) (Technical Appendix Table).

Our study in Brazzaville identified CHIKV as the causative agent for the 2011 outbreak of febrile illness characterized by severe joint pain. In the affected Brazzaville communities, the illness had been named robot malaria because of its effects on posture and locomotion, effects that are typical of CHIKV infection. We also found that during the outbreak, CHIKV was present in *Ae. aegypti* and *Ae. albopictus* mosquitoes, suggesting that these mosquitoes played a role in dissemination and spread of the virus. These findings are in line with the increasing distribution of CHIKV and its vector, *Ae. albopictus* mosquitoes, in Africa.

Of note, the outbreak in the Republic of Congo seems to have been associated with *Ae. aegypti* and *Ae. albopictus* mosquitoes, whereas outbreaks of CHIKV infection in neighboring Gabon are predominantly associated with *Ae. albopictus* mosquitoes (*8*,*9*). Our study confirms the suspected presence of *Ae. albopictus* mosquitoes in the Republic of Congo. The identification of *Ae. albopictus* mosquitoes as a vector for CHIKV in remote areas in Gabon indicates that similar remote areas in the Republic of Congo could also be at risk during future outbreaks of CHIKV infection (*8*).

The availability of diagnostic capacity for arbovirus detection in sub-Saharan Africa is urgently needed for the deployment of potential intervention strategies. Improved surveillance and rapid identification of novel arbovirus–associated outbreaks would strengthen the potential for recognition of arbovirus-related diseases by health care providers and public health officials. In the absence of CHIKV-specific prophylactic or therapeutic control measures, the only current mitigation strategies are based on community outreach, which provides early alerts regarding outbreaks, and on control of the mosquito vectors (*10*).

This work was supported by the Division of Intramural Research of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, under the International Centers for Excellence in Research program.

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DOI: http://dx.doi.org/10.3201/eid1909.130451

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Chorea and Tick-borne Encephalitis, Poland

To the Editor: Tick-borne encephalitis (TBE) is caused by infection with vector-borne viruses classified in the family *Flaviviridae*. The disease is characterized by a biphasic course. In the first phase, fever, fatigue, malaise, headache, and arthralgia occur; in the second phase, neurologic signs develop, and the clinical spectrum ranges from mild meningitis to severe encephalitis, myelitis, and polyradiculitis, sometimes with a fatal outcome (*1*). The clinical signs and symptoms of TBE can be explained by affinity of TBEV to distinct regions of the CNS, such as multinodular to patchy encephalomyelitis accentuated in the spinal cord, brain stem, and cerebellum (*2*). According to the European Centre for Disease Prevention and Control, a notable rise in the incidence of TBE has been observed in Europe and Asia in recent years, and the disease is considered a growing public health concern in many parts of the world (*3*). In Poland, an average of 250 TBE cases are reported each year. Mean incidence is 0.75 cases/100,000 population, and incidence is highest in the northeastern part of the country, which is considered to be a TBE-endemic area (11.53 cases/100,000 inhabitants).

Chorea is defined as a state of excessive, spontaneous movements that are irregularly timed, nonrepetitive, randomly distributed, and abrupt in character (http://emedicine.medscape.com/article/1149854). This condition is a result of absent subthalamic nucleus inhibition, which increases motor activity through the motor thalamus. It is a dominant symptom of Huntington's disease, which is an inherited, progressive, neurodegenerative disorder. Nonhereditary causes of chorea, such as infectious chorea in the course of acute manifestations of bacterial or aseptic meningitis or encephalitis, have been described (*4*,*5*). We describe a case of chorea in the course of TBE in a man in Poland.

A 38-year-old man was admitted to the hospital in Białystok, Poland, because of fever (40°C), headache, vomiting, and drowsiness that had lasted for 1 week. The patient was a forest worker who reported frequent tick bites; for religious reasons, he had not been vaccinated against TBE. Physical examination revealed palsy in the seventh facial nerve, tremor in the facial muscles on the left side, muscle weakness in the left upper and lower limbs, and positive meningeal signs. Testing of cerebrospinal fluid (CSF) revealed inflammatory features (cytosis, 63 cells/µL, and protein concentration 68 mg/dL). Immunologic test results showed CSF IgM of 9.04 IU/mL and IgG of 55.1 IU/mL against TBE virus (cutoff 1.4 for IgM, 7.0 for IgG). Computed tomography scan and magnetic resonance imaging (MRI) of the brain revealed widening of ventricles. The patient was treated with mannitol, dexamethasone, third-generation cephalosporin, and diazepam; after signs and symptoms improved, the patient was discharged home on his request.

Eleven days after discharge, the patient was readmitted to the hospital because of deteriorating clinical signs (progressive muscle weakness and excessive tremor of the left-side limbs). Intensive propulsion, involuntary muscle movements (limbs and face), muscle weakness of left upper and lower limbs, and "walk dance" were observed (online Video, wwwnc.cdc.gov/EID/article/19/9/13-1804-V1.htm). Results of CSF analysis showed continued inflammatory features (pleocytosis, 64 cells/µL, and protein concentration 119.4 mg/dL). Results of testing for antibodies against TBE virus in CSF and serum were strongly positive (CSF IgM >14 U/mL, IgG 107 U/mL; serum IgM 7.92 U/mL, IgG 87.6 U/mL). Test results for *Borrelia burgdorferi* were negative 3 times. MRI of the brain showed lesions in the lenticular nucleus, caudate nucleus on the right side, and internal capsules of both sides (Figure).

After neurologic and psychiatric consultations, chorea in the course of TBE was diagnosed. The patient was treated with haloperidol, dexamethasone, pentoxifylline, nitrazepam, and midazolam, and his signs and symptoms improved.

One month later, follow-up testing and examination were performed. No abnormalities on physical examination or laboratory test results were observed except pleocytosis in CSF (35 cells/µL). The patient reported aphasia and deterioration of memory. Six months later, results of CSF testing were within normal limits, and repeat MRI showed resolution of inflammatory lesions.

In this case, chorea occurred in the course of TBE. The neurologic phase of TBE was not preceded by the expected first-phase signs and symptoms. The diagnosis was confirmed with serologic test results, which were strongly positive in serum and CSF, and virus neutralization testing

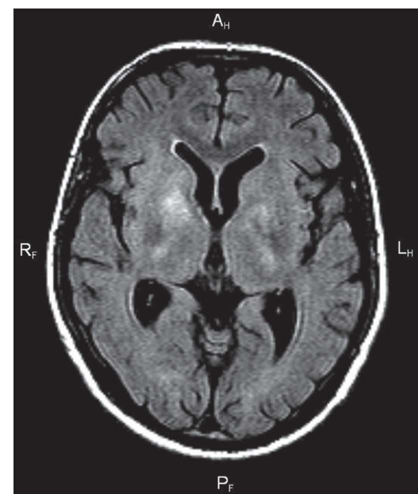

Figure. Fluid-attenuated inversion recovery magnetic resonance imaging of the brain of a 38-year-old man with tick-borne encephalitis and chorea. The image shows bilateral areas of hyperintensity in T2, affecting the nucleus caudate, internal capsule, and thalami.

performed as described in Stiasny et al. (*6*) showed a strongly positive titer of 1:320. We excluded hepatitis C virus; West Nile virus infection has not been reported in Poland. Because of his religious beliefs, the patient had not been vaccinated against TBE or yellow fever; he had not traveled abroad and had not been exposed to dengue virus. The differential diagnosis included Lyme disease, but the results of repeated serologic tests for *B. burgdorferi* were negative.

Chorea arises deep in the basal ganglia; high-definition MRI demonstrates caudate atrophy (*7*). In patients with TBE, MRI may reveal unilateral or bilateral thalamus lesions, as well as lesions in the cerebellum, brainstem, and nucleus caudalis (*8*). One case study presented a case of simultaneous lesions in the thalamus, stem, and spinal cord (*9*). Chorea developed in the patient reported here because of inflammatory lesions in the region of the thalamus. Supportive care is standard for patients with chorea (*10*), but after treatment for TBE, the lesions in this patient resolved, and the chorea abated.

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DOI: http://dx.doi.org/10.3201/eid1909.130804

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Streptococcus suis Meningitis and Bacteremia in Man, French Guiana

To the Editor: *Streptococcus suis* is a major swine pathogen and is increasingly recognized as the cause of an emerging zoonosis in humans. It is responsible for severe systemic infections, most commonly meningitis and sepsis, which lead to high rates of illness and death. Serotype 2 is considered to be the strain most pathogenic for humans and pigs (*1*,*2*).

The first reported infection in a human was in Denmark (*3*), but human infections have been now described worldwide, with a predominance in countries in Southeast Asia (*1*,*2*,*4*). However, only 2 cases of human infection have been described in South America, in Argentina in 2005 (*5*) and 2008 (*6*). One infection was related to a serotype 2 strain (*5*). We report a human case of *S. suis* infection with meningitis and bacteremia in an immunocompetent patient living in French Guiana.

In August 2011, a 42-year-old Haitian man was admitted to Cayenne Hospital (Cayenne, French Guiana) because of a 3-day history of fever, headache, vertigo, and vomiting. He was an unemployed illegal immigrant who had lived in French Guiana for 4 years. He had no particular medical history and no risk factors for immunodeficiency. At admission, he was lethargic, weak, and frail. He had a stiff neck, vestibular dysfunction, ataxia, and nystagmus. He did not have hearing loss or skin abnormalities. Cranial computed tomography with contrast and gadolinium-enhanced cranial magnetic resonance imaging did not show any abnormal findings.

Blood tests indicated thrombocytopenia (106×10^9 platelets/L), lymphopenia (0.57×10^9 lymphocytes/L), a high level of C-reactive protein (170 mg/L) and mild cytolysis

(3.8-fold increase in aspartate aminotransferase and 2.5-fold increase in alanine aminotransferase levels). There was no hyperleukocytosis (leukocyte count 7.7 × 10^9 cells/L). Cerebrospinal fluid (CSF) was slightly opalescent and had a protein level of 1.16 g/L, a glucose level of 2.2 mmol/L, and 270 leukocytes/mL (79% neutrophils and 21% lymphocytes). Gram staining of CSF showed numerous gram-positive cocci in pairs and short chains.

CSF and blood cultures after 12 and 48 hours, respectively, of incubation showed numerous α-hemolytic colonies on blood and chocolate agar. The bacteria were gram positive, optochin resistant, and catalase negative, and were reliably identified by using API 20 Strep (bioMérieux, Marcy l'Etoile, France) as *S. suis* (probability 99.7%).

Antimicrobial drug susceptibility tests were conducted by using Mueller-Hinton agar and the disk diffusion method. An E-test was performed for ampicillin (MIC<0.016 μg/mL). The isolate was resistant to gentamicin (500 μg/L), tetracycline, and norfloxacin, and sensitive to penicillin, ampicillin, amoxicillin, oxacillin, cefotaxim, pristinamycin, vancomycin, teicoplanin, trimethoprim/sulfamethoxazole, erythromycin, and lincomycin in comparison with published data (*7*). Slide agglutination with type-specific hyperimmune serum and specific multiplex PCR (*8*,*9*) identified the isolate as serotype 2. Multilocus sequence typing identified the isolate as a member of the sequence type 1 complex, which has spread throughout most countries in Europe. This complex has been strongly associated with isolates from patients with septicemia, meningitis, and arthritis (*10*).

After *S. suis* was identified, the patient reported contact with swine and that he had slaughtered 4 pigs 24 hours before onset of symptoms. During this activity, he injured his left thumb. The patient was empirically given a 2-day course of intravenous, high-dose ceftriaxone (3 g, 2×/d), which was replaced by intravenous high-dose amoxicillin (3g, 4×/d) for 14 days.

While the patient was receiving treatment for 2 days, moderate bilateral hypoacousia developed, which required adjunctive corticoid therapy. The hypoacousia developed into severe hearing loss in the left ear. Audiograms showed moderate sensorial hearing loss (50 dB) in the right ear on day 7 of treatment, which resolved 1.5 years later, and complete hearing loss in the left ear on day 7 of treatment, which that gradually decreased. However, the patient still has severe sensorinoral hearing loss (80 dB) in the left ear. The patient did not have signs or symptoms of endocarditis by cardiac ultrasonography.

Little data are available for circulation and epidemiology of *S. suis* in South America (*3*). We report a human case of *S. suis* infection with meningitis and bacteremia. Clinical and laboratory data, microbiological findings, and outcome for this case-patient were similar to those of the case-patients reported in Asia (*1*,*2*).

Our study shows that in nonendemic areas, infection with this pathogen, although not frequently reported, should be considered when diagnoses are made for patients who work in piggeries. Persons with occupational exposure to swine or pork products should be educated and made aware of this risk for infection.

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DOI: http://dx.doi.org/10.3201/eid1909.121872

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Alaria alata Infection in European Mink

To the Editor. Alariosis is a reemerging zoonotic disease caused by infection with larval stages of trematodes of the genus *Alaria*. The trematodes are found in wildlife that inhabit wetlands, and these animals may serve as possible reservoirs for these organisms that cause human infection (*1*). The main sources for human infection are suids and frogs (*1*). In humans, the clinical features of alariosis caused by infections with the North American species of *Alaria* vary from mild and asymptomatic to moderate with respiratory or cutaneous signs (*2*) or neuroretinitis (*3*), to severe-to-lethal anaphylactic shock caused by larva migrans (*4*,*5*). The genus *Alaria* has 7 species; only *A. alata* is found naturally in Europe (*6*), a species which has not thus far been shown to be responsible for human infections.

A. alata infection is common in its typical definitive host (red fox, *Vulpes vulpes*) and in certain paratenic hosts (wild boar, *Sus scrofa*) (*1*). However, the role of other paratenic hosts is poorly known. Among these, mustelids are reported to harbor mesocercariae of *A. alata* trematodes (*7*). The pathogenic effect of *A. alata* infection has been poorly studied, because most lesions described were in humans infected with other species of *Alaria*. Except for 2 experimental studies that described gross lesions produced by *A. alata* trematodes (*6*,*8*), to our knowledge, no data have been published concerning lesions produced by natural infection in nonhuman hosts. Our report provides a detailed description of the lesions, shown by microscopy, which suggests the pathogenic mechanisms.

One adult female European mink (*Mustela lutreola*) was found dead during standard surveillance operations in which box traps were used; this trapping was part of biodiversity and ecology studies in the central part of the Danube delta in Romania (45°08′ N, 29°19′E) in March 2010. The corpse was deep-frozen and analyzed after 3 months in the laboratory. During necropsy, multiple, well-defined, whitish

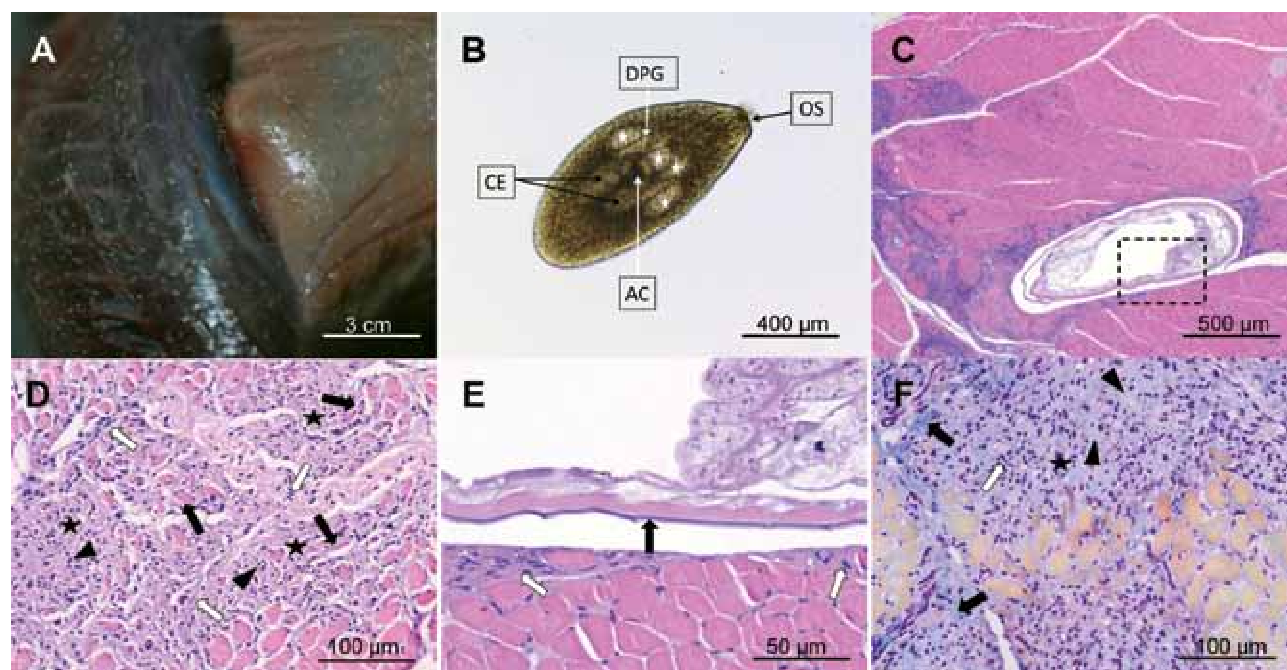

Figure. Lesions produced by mesocercariae of *Alaria alata* in European mink. A) Mesocercariae in the muscle and subcutaneous tissue produce whitish, round or slightly oval, well-defined nodules. B) Free mesocercarium after artificial digestion, showing the characteristics of *A. alata* mesocercarium: piriform body with anterior oral sucker (OS), acetabulum (AC) positioned in the center of the parasite, 2 pairs of large, finely granulated penetration glands (white stars), limiting the anterior part of the acetabulum, linear ducts of penetration glands (DPG) converging to the oral opening of the oral sucker and large double ceca placed posterior to the acetabulum. C) Histologic section showing an encysted mesocercarium in the muscle (parasite is surrounded by a capsule and pericystic inflammation, which extends to the surrounding muscular tissue). D) Mononuclear leucocytes (arrowheads) scattered between the fibroblastic proliferations (white arrows) and collagen deposits (black stars). Muscle fibers are atrophic due to compression (black arrows). E) Microscopic detail of the inset from panel C. Some mesocercariae (indicated by black arrow) are enclosed in a thin, pale staining capsule (white arrows). Note the lack of leukocyte response. F) Migration route of the parasite (route with center marked by the black star), followed by invasion of nonnecrotic muscle fibers by mononucleate inflammatory cells (white arrow) located mainly in the center of the migration tract and fibrous connective tissue with collagen fibers densely packed at the periphery (black arrows) and more loosely in the center (arrowheads). Hematoxylin–eosin stain (panels C, D, E); Masson trichrome (panel F); original magnifications x40 (panels B and C), x200 (panels D and F), and x400 (panel E). A color version of this figure is available online (wwwnc.cdc.gov/EID/article/19/09/13-0081-F1.htm).

nodules were observed in most muscular and subcutaneous tissues (Figure, panel A), with no evident preferential localization. We collected samples from these tissues for artificial digestion (*9*,*10*) and histologic examination, using the routine paraffin-embedding protocol and the following staining methods: hematoxylin-eosin, Masson trichrome, and Gordon and Sweet.

Artificial digestion released parasites (6 larvae/5 gm tissue) with typical larval trematode structures (Figure, panel B). By microscopy, we observed that morphologic features of these larvae were consistent with *A. alata* mesocercariae (*6*). Histopathologic examination confirmed the presence of parasitic forms in muscle sections (Figure, panel C). The mesocercariae were located in the connective fibrous tissue of the perimysium or between the muscle fibers. The typical structure of muscle fibers was altered around the larvae, with inflammatory cell reactions, represented mainly by lymphocytes, macrophages, and plasma cells (Figure, panel D). In other areas, the inflammatory reaction around the parasite was minimal or absent (Figure, panel E). In certain histologic sections, the damaged muscular tissue was replaced by granulation tissue in various stages of development (Figure, panel F). The maturity of the granulation tissue differed substantially, depending on the muscular areas examined. Some lesions were found in adult connective tissue, formed by mature collagen scar fibers (type I collagen) and few inflammatory cells, whereas other lesions had reticulin fibers (type III collagen) with numerous inflammatory cells. The lesions of the subcutaneous connective tissue consisted of an inflammatory reaction (panniculitis). The inflammation was characterized by a low number of mononuclear leukocytes and fibrinous exudate and fibroplasia.

The polyphasic nature of muscle and subcutaneous lesions produced by *A. alata* infection in its paratenic host appears to be caused by mesocercarial migration. This view is sustained by the presence of mononuclear cells that it infiltrates and by the appearance of the granulomatous tissue in various stages of maturation, which leads to muscle and subcutaneous fibroplasia. The reparatory nature of the lesions suggests that the inflammation is probably the result of direct tissue damage rather than an immune reaction targeted toward the parasitic antigens. This assumption could explain the local absence of inflammatory reaction around the parasites. The lack of inflammation was previously observed also with *A. americana* infection of humans (*4*). The structure of all mesocercariae observed by microscopy suggested that they were alive and active before the mink carcass was frozen. Because no mesocercariae were surrounded by adult connective tissue or by granulomatous inflammation, together with the multiple presences of migratory routes, the continuous mobility of the parasites through the host's tissues was strongly suggested.

Although data on the pathologic changes caused by *Alaria* spp. in general, and *A. alata* parasites in particular, are scarce, the migration pattern and the lesions seem to be dependent on the particular parasite and host species. The reparatory nature of the lesions suggests that the inflammation is the result of direct tissue damage rather than an immune reaction targeted toward the parasitic antigens.

The research was conducted with the support of Unitatea Executivă pentru Finantarea Învătământului Superior, a Cercetării, Dezvoltării si Inovării (UEFISCDI) grant PCE236/2011.

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DOI: http://dx.doi.org/10.3201/eid1909.130081

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Serologic Survey of Plague in Animals, Western Iran

To the Editor: Plague has been one of the most devastating infectious diseases in human history. The etiologic agent, *Yersinia pestis*, primarily affects rodents and is usually transmitted to humans through infective flea bites. Endemic plague foci result from circulation of the plague bacillus in its rodent reservoir, the source of human plague cases (*1*). Carnivores such as dogs and foxes, which prey on rodents and eat their fresh carcasses, are valuable sentinel animals for plague serosurveillance in disease-endemic foci, although their infections are usually asymptomatic (*2*,*3*).

Plague epidemics have caused loss of human life in various parts of Iran. During 1947–1966 in western Iran, 9 human epidemics occurred and caused 156 deaths. The last case of human plague was reported in 1966 (*4*). Field investigations identified 4 *Meriones* rodent species as *Y pestis* reservoirs; 2 were resistant (*M. persicus* and *M. libycus*), and the other 2 (*M. tristrami* and *M. vinogradovi*) were susceptible to death from infection (*4*,*5*). The epidemiologic investigations demonstrated a 3–4 year plague epizootic cycle in Iran (*5*). The last official report of plague in rodents in Iran dates back to 1978, in Sarab County in the East Azarbaijan Province (*6*). Plague surveillance was ignored for more than 3 decades and then restarted in 2011 in Iran.

This study was designed to investigate plague among resident animals in western Iran, specificallyregion localities along the border between the Kurdistan and Hamadan Provinces, where plague in wildlife has been repeatedly reported (enclosed by 47.900° and 48.284° north latitude and 35.4616° and 35.7829° east longitude). The epidemiologic team was based at the Akanlu Research Center of the Pasteur Institute of Iran, in a village ≈100 km from Kabudar Ahang, Hamadan Province, at an altitude of ≈1,600 m. The study was conducted during June–September in 2011 and 2012. In 2011, a large area (2,000 km^2) was selected and, because only 1 *Y. pestis*–positive dog sample was found, in 2012, , the study area was reduced to 1,200 km^2 and confined to localities in which the *Y. pestis*–positive dog sample was identified the previous year; 3 additional *Y. pestis*–positive dogs and 1 *Y. pestis*–positive rodent were found in 2012.

The average number of traps used per night per locality was 13. A total of 46 rodents were entrapped from 26 localities in 998 traps (4.61% success) during the first year, and 52 rodents were captured in 30 localities in 1,164 traps (4.46% success) during the second year. They were mostly members of the *Meriones* genus, although a few *Microtus socialis irani* and 1 *Ellobius lutescens* rodents were also caught (Table). A total of 281 fleas were collected on 70.41% of trapped rodents (Table), corresponding to an average flea index of 4.10 for infested rodents. All fleas were *Xenopsylla* spp. ELISA was performed as described (*7*) to detect antibodies against *Y. pestis* F1 capsular antigen. Samples positive by ELISA were confirmed by using the inhibition ELISA method (*8*). Of 98 trapped rodents, 1 (1.02%) had IgG against F1 (Table), an *M. persicus* jird caught in 2012.

Sheepdogs that lived in the study areas were also used as sentinel animals. Blood samples were collected from 58 sheepdogs in 15 villages in 2011 and from 59 sheepdogs in 8 villages in 2012. Of 117 dog serum samples analyzed, 4 (3.42%) had IgG titers against F1, 1 in 2011 and the other 3 in 2012 (Table). Finally, wild animals such as jackals, foxes, rabbits, and hedgehogs were hunted in the study areas, and blood samples were taken immediately. None of the serum samples obtained from 3 foxes, 2 jackals, 8 rabbits, and 1 hedgehog had IgG against F1 (Table).

Because a well-established plague focus existed in Iranian Kurdistan, with animal cases occurring until 1978 (*9*), complete extinction of this focus is most unlikely. Our study demonstrates that animal reservoirs (*Meriones* rodents) and flea vectors (*Xenopsylla* spp.) shown to be central to the plague ecologic cycle in Iran still are found in high numbers in a previously active focus. The fact that 70% of trapped rodents were infested with fleas, with an average *Xenopsylla* spp. index of 4.10, may be considered as circumstances most favorable for the onset of plague epizootics. Furthermore, the detection of *Y. pestis*–specific IgG in 1.02% of trapped rodents and 3.42% of sentinel dogs is highly suggestive of active circulation of *Y. pestis* in its natural animal reservoir. Because *Y. pestis* antibodies last only for ≈6 months in dogs (*2*), seropositivity of these dogs indicates newly acquired infections.

This fact that *Y. pestis*-–positive animals were found over the 2-year surveillance period suggests that this area could be an active plague focus. Therefore, although no official reports of human plague in Iran have been made since 1966, this study indicates that the epidemiologic conditions needed to trigger an outbreak have been met. It is thus of utmost importance to maintain and strengthen the health system with plague surveillance in western Iran.

Table. Number of fleas and serum samples with *Yersinia pestis* F1 antibody collected from various animals during field investigations, Iran, 2011–2012*

Animal/species	No. serum samples	No. serum samples with antibodies against F1	No. fleas collected
Rodents			
2011			
Meriones persicus	39	0	33
Meriones vinogradovi	3	0	0
Microtus socialis irani	4	0	1
2012			
Meriones persicus	24	1	146
Meriones libycus	26	0	101
Microtus socialis irani	1	0	0
Ellobius lutescens	1	0	0
Total	98	1	281
Sheepdogs			
2011			
ND	58	1	ND
2012			
ND	59	3	ND
Total	117	4	
Miscellaneous			
Jackals			
2011			
Canis aureus	2	0	ND
2012			
C. aureus	0	0	ND
Foxes			
2011			
Vulpes vulpes	1	0	ND
2012			
V. vulpes	1	0	ND
Rabbits			
2011			
Lepus capensis	1	0	ND
2012			
L. capensis	7	0	ND
Hedgehogs			
2011	0	0	
ND			ND
2012	1	1	
ND			ND
Total	14	0	

*ND, not determined.

Acknowledgments

We appreciate the financial support of the Pasteur Institute of Iran and Center for Disease Control of the Iranian Ministry of Health and Medical Education (grant no. 582). We also express our gratitude to Mohammad Mehdi Goya and Mahmoud Soroosh for their scientific and logistic support; to Behzad Esfandiari, Hamed Hanifi, and Mohammad Hanifi, who helped us with sampling; and to Manijeh Yousefi-Behzadi, who assisted us with laboratory tasks.

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DOI: http://dx/doi.org/10.3201/eid1909.121829

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Livestock Density as Risk Factor for Livestock-associated MRSA, the Netherlands

To the Editor: We challenge the conclusions of Feingold et al. that "regional density of livestock is a notable risk factor for nasal carriage of LA-MRSA for persons with and without direct contact with livestock" (*1*). They did not study nasal carriage of methicillin-resistant *Staphylococcus aureus* (MRSA), but they retrospectively analyzed 87 culture-confirmed MRSA cases reported to a reference laboratory. These were a mixture of clinical disease isolates and screening (nose, throat, and perineum) isolates that were unevenly distributed between the groups (*2*). Because their analysis aimed to assess exposure risk by residential location, they should have excluded the 5 persons who acquired MRSA outside the Netherlands.

Table. Pig density in the Netherlands, United States (excluding Alaska), and major pig-producing states

Location	No. pigs	Area, km^2	Pig density, pigs/km^2	Relative pig density*
The Netherlands	12,100,000†	41,518	291.4	1
United States	67,500,000†	8,108,782‡	8.3	35.0
Iowa	19,700,000	145,744	135.2	2.2
North Carolina	8,600,000	139,393	61.7	4.7
Minnesota	7,600,000	225,174	33.8	8.6

*Pig density in the Netherlands divided by pig density in other locations.
†US data were obtained from a quarterly US Department of Agriculture report (http://usda01.library.cornell.edu/usda/nass/HogsPigs//2010s/2012/HogsPigs-09-28-2012.pdf).
‡Alaska was excluded because of minimal swine industry.

Retrospective case–control studies preclude direct estimation of incidence, prevalence, or risk. However, because of the symmetric property of odds ratios, disease odds ratios can be inferred indirectly from the estimated exposure odds ratios in case–control studies (*3*). However, this case–case study design has no true controls, precluding valid inferences of absolute or relative risks. The higher ratio of livestock-associated (LA)–MRSA to a typeable strain of MRSA (T-MRSA) in rural cases could be attributable to higher risk for LA-MRSA in rural areas, lower risk for T-MRSA in rural areas, or both.

To illustrate this point, suppose urban dwellers had equal prevalence rates of LA-MRSA and T-MRSA of 5%, and rural dwellers had prevalence rates of 2% for LA-MRSA and 1% for T-MRSA. The ratio approach used would indicate that rural dwellers had twice the risk for LA-MRSA than urban dwellers, when the absolute risk is 2.5 times higher in the urban group. At best, their conclusion could be viewed as a hypothesis that should be tested.

Three large community-based studies with better methods collectively refute this hypothesis. Across these studies, LA-MRSA prevalence (44%) was >180 times higher in 352 occupationally exposed persons than in 2,094 rural residents without farm exposure (0.24%) (*4*–*6*). Prevalence in family members of livestock workers was intermediate (5.2%). These consistent observations indicate that exposure to LA-MRSA in livestock-dense regions is a common occupational risk for livestock workers, a lesser indirect risk to their family members, and a negligible risk to persons without livestock or farm contact.

Finally, the contention of Feingold et al. that pig production in the Netherlands is "greatly overshadowed by the density of pig-farming operations in the United States" is mistaken (*1*) (Table). Pig density in the Netherlands is 35 times higher than in the United States, and more than twice that in Iowa.

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DOI: http://dx.doi.org/10.3201/eid1909.121577

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In Response: We thank Davies et al. for their letter (*1*) responding to our report (*2*). We appreciate the opportunity to address their comments, some of which raise appropriate concerns.

Davies et al. correctly note that the original methicillin-resistant *S. aureus* (MRSA) registry data were derived from swab specimens and clinical isolates; the distribution of anatomic sites differed between the cases and controls in our study. However, all study participants were proven MRSA carriers. As per their comment in their letter (*1*), we reran multivariate models excluding 5 persons who acquired MRSA outside the Netherlands. We found odds ratios (and $p<0.05$) similar to those originally reported for covariates of municipality-level livestock densities.

Davies et al. apparently pooled data from 3 studies and stated that there is negligible risk for livestock-associated–MRSA among persons who do not have livestock or farm contact. Each of these studies was designed differently and had different comparison groups, and each report conceded that factors such as indirect human or environmental transmission could have exposed study participants who lacked known farm risk factors.

In addition, Davies et al. state that our observed association of increased odds of livestock-associated–MRSA compared with a typeable strain of MRSA in regions with higher livestock densities should be limited to hypothesis generation. We agree, as stated in the final sentence of our report (*2*). However, a similar association was confirmed in a recent study in the Netherlands that included MRSA-negative controls (*3*).

To clarify our statement comparing pig densities in the United States and the Netherlands (*2*), we referred to density in terms of animals per operation, a relevant parameter for other zoonotic diseases, including swine and avian influenzas. To state this information in a different manner, in 2007, a total of 60% of the 67.7 million pigs raised in the United States were raised on farms with >5,000 pigs, but only 22% of the 11.66 million pigs raised in the Netherlands were raised on farms with >5,000 pigs (*4*,*5*).

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DOI: http://dx.doi.org/10.3201/eid1909.130876

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Neuroinfections (What Do I Do Now?)

Don Gilden

Oxford University Press, New York, New York, USA, 2013

ISBN-10: 0199926638

ISBN-13: 978-0199926633

Pages: 204; Price: US $34.99

This edition in the series of What Do I Do Now? books provides a nicely organized, concise, and informative guide for the clinician on the approach to the clinical features, differential diagnosis, and management of several neurologic infectious diseases, as well as postinfectious neurologic syndromes such as acute disseminated encephalomyelitis. The illnesses and pathogens covered in this book include those that the average clinician is likely to encounter in routine clinical practice, as well as some uncommon conditions rarely encountered outside the arena of clinical neurovirology.

Each section starts with a brief clinical vignette, describing the history, clinical findings, and other relevant medical data of a particular patient. At the end of the short vignette, readers are instructed to ask themselves what they would do in this particular clinical situation. The following section then goes into greater detail about the differential diagnosis, testing, and management and treatment approaches associated with the particular pathogen. In the process, the reader learns not just about the particular pathogen in question, but about other possible etiologies that may produce a similar clinical situation. Each chapter finishes with key, take-home items regarding the illness, as well as select references relevant to the pathogen/syndrome. The reader is provided with insight into the diagnostic thought process that the practicing neurologist goes through in thinking through issues around neurologic infections.

This book would be most useful to medical students, residents, and other general practitioners who wish to explore further approaches to the patient with an apparent neurologic infection because of its practical, clinical-based approach. Although perhaps less useful as a reference text, it may also serve as a useful addition to the clinician's library in those instances in which one would like a quick, concise synopsis of particular infections or etiologies that are being suspected in a particular patient. In summary, Neuroinfections (What Do I Do Now?) serves as a helpful and informative summary of major etiologies of neurologic infections, in an enjoyable, easy-to-read format.

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etymologia

Staphylococcus [staff″ə-lo kok′əs]

From the Greek *staphyle* (bunch of grapes) and *kokkos* (berry), *Staphylococcus* is a genus of gram-positive spherical bacteria that commonly cause surgical and skin infections, respiratory disease, and food poisoning. In 1880, Scottish surgeon Sir Alexander Ogston first described staphylococci in pus from a surgical abscess in a knee joint: "the masses looked like bunches of grapes." In 1884, German physician Friedrich Julius Rosenbach differentiated the bacteria by the color of their colonies: *S. aureus* (from the Latin *aurum*, gold) and *S. albus* (Latin for white). *S. albus* was later renamed *S. epidermidis* because of its ubiquity on human skin.

–Giancarlo Licitra

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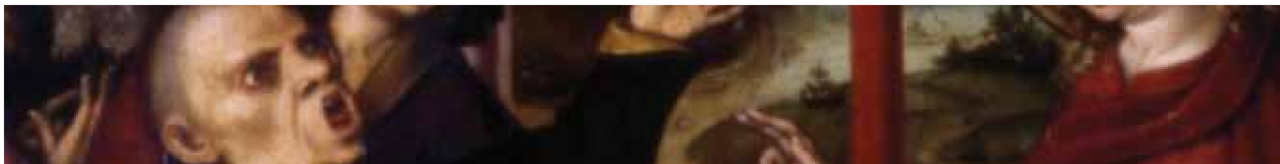
DOI: http://dx.doi.org/10.3201/eid1909.ET1909



When at night I go to sleep / Fourteen angels watch do keep[1]

Polyxeni Potter

Every year, on Whit Tuesday, pilgrims crowd the small town of Echternach, Luxembourg, to join a Dancing Procession, a tradition, legend has it, rooted in miraculous healings as far back as the 8th century at the site of Saint Willibrord's sarcophagus. The procession, first mentioned in the city archives in 1497, moves rhythmically, three steps forward, two steps back, and slowly–five steps needed to advance one–the folk dancers, four or five abreast, holding on to each other.

Willibrord, known as Saint Witt (Vitus) in Germany and as Saint Guy in France, was an English missionary to Denmark and the Netherlands and the first Bishop of Utrecht, who founded the Benedictine Abbey of Echternach. Word of healings at his graveside spread far and wide: a woman brought there unable to walk left "using her own legs." A man with fainting spells and tremor in the limbs was cured.

Willibrord became the patron saint of patients with neurologic diseases: paresis, epilepsy, and what was then known as choreomania or the "dancing disease," an epidemic of strange behavior of crowds. They would suddenly form circles and dance to exhaustion, "their limbs jerked and they collapsed snorting, unconscious and frothing." This epidemic of uncontrolled dancing spread throughout Germany and the Netherlands. Outbreaks also occurred in France and Britain. "Peasants left their ploughs, merchants their workshops, and housewives their domestic duties to join the wild revels."

Paracelsus (1493–1541) described the dancing mania as chorea (Gr. *χορεία* = dance) Sancti Viti and raised it from superstition to a disorder manifested in three types, one of them, chorea naturalis (arising from physical causes). The dancers were not uniformly hysterical. Some, who exhibited neurologic symptoms of unknown origin, were removed from the procession by the authorities. Others were the relatives and friends of the sick, who danced for the healing of their beloved.

Felix Platter, in his Praxeos Medicae (1602), referred to Saint Vitus' dance as "that frightening and remarkable though rare disease," an epidemic in which not just women but men too were afflicted with dancing that went on for weeks. He noted a possible parallel with a peculiar "jumping condition of the limbs," known to the Arabs and concluded that if this disease is not from the devil, it must come from God himself as punishment.

The term Saint Vitus' dance became popular after early epidemiologist Thomas Sydenham (1624–1689) used it in his classic description of acute chorea, "St Vitus dance is a sort of convulsion which attacks boys and girls from the tenth year until they have done growing. At first it shows itself by a halting, or rather an unsteady movement of one of the legs, which the patient drags. Then it is seen in the hand of the same side. The patient cannot keep it a moment in its place, whether he lay it upon his breast or any other part of his body. Do what he may, it will be jerked elsewhere convulsively."

The Miracle of Saint Vitus, on this month's cover, is an account of chorea-like illness. As the clinical features were heterogeneous, Saint Vitus' dance became an umbrella term for an assortment of kinetic disorders. In this scene, the unknown painter captures for posterity the frightful plight of a person with neurologic illness. Helpless and misunderstood, he awaits a miracle that will loosen his muscles, unlock his mouth, and steady his limbs. At the same time, the painting sheds light on how a saint and his name might have become intricately connected with the activities in Echternach and elsewhere.

The martyr Saint Vitus lived in Sicily and was mentioned in historical documents as early as the 5th century. He was one of 14 Holy Helpers, grouped together in the Middle Ages, when the Black Death was spreading throughout Europe decimating the population. These saints were pressed to action because of their healing powers against a variety of disorders, from headache and abdominal pain to bubonic plague. Saint Vitus' feast day was celebrated by dancing before his statue because he was the patron saint of dancers and entertainers. But he was also the protector of those sick with "unsteady step, trembling limbs, limping

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DOI: http://dx.doi.org/10.3201/eid1909.AC1909

[1]"Fourteen Angels" of the lost children's prayer from the 19th-century German composer Engelbert Humperdinck's fairy opera Hansel and Gretel: www.fourteenangels.org/the-prayer.html.

knees, bent fingers and hands, paralysed hands, lameness, crookedness, and withering body." The signs and symptoms mimicked the movements of dance.

This Saint Vitus' early life is clouded in legend. Hagiographic texts describe him as a spiritual and holy child, possessing extraordinary powers, much to the chagrin of his family. During his brief life, he was hounded for his rejection of idolatry and traveled around to escape persecution, all the while performing miracles. He is known for healing Roman Emperor Diocletian's son, who was thought to be possessed by demons because he twitched uncontrollably. In a singular gesture of ungratefulness the emperor pronounced his son's cure unholy magic and had Vitus thrown into a cauldron of boiling oil.

The Miracle of Saint Vitus was a product of the Nuremberg School, a tradition named after a major artistic and commercial center of the times. The Franconian city, which in its early days had no well-known painter at the helm, at its peak boasted none other than the great Albrecht Dürer. Long and enduring, this artistic tradition encompassed the late Gothic and early Renaissance art of Northern Europe, which was rich and diverse, embracing eastern and western elements and culminating in a Gothic style softened with features from Italian art and the advent of oil painting.

"Realism of particulars," along with symbolism, characteristic elements of Franconian painting, is at work in this theatrical scene. The architectural details place the viewer in a palatial context with stately columns and marble floor. On the right stands the emperor in regal attire—crown, scepter, and robe richly trimmed at the sleeve and hemline; on the left, his stricken son, head shaved and arms flailing. The onlookers' faces reflect ignorance, curiosity, suspicion, revulsion, fear, mockery.

The attendants trying to control and stabilize the emperor's son recall the "fourteen angels" of the lost children's prayer and symbolize the Fourteen Holy Helpers providing assistance to the patient, who falls back as his legs give out under him: "Two my head are guarding, / Two my feet are guiding." Unable to control his body, he is at their mercy: "Two upon my right hand, / Two upon my left hand." His face distorted, dystonic mouth frozen, eyes transfixed, he is finished. "Two to whom 'tis given / To guide my steps to heaven."

At the center of the painting, the young saint outshines the emperor, who seems diminished, secondary, frightened, and helpless, even as he extends the index finger of authority. In this boldly colored room, against the atmospheric perspective beyond the window, the saint is king. He is attentive and focused, one hand touching the patient's cloth, the other raised in a symbolic gesture.

The modern version of Saint Vitus' dance, Sydenham chorea, the prototype for an autoimmune-mediated chorea following infectious illness, is still characterized by involuntary movements, which "flit and flow unpredictably from one body part to another." They can be caused by several infections, among them HIV and tuberculosis.

The epidemiologic features of neurologic disorders are confusing and rival in complexity the cryptic descriptions of the lives of saints. Diseases of the same cause are expressed differently and those with different cause sometimes look the same, a difficulty also encountered often in the study of emerging infections. While in times past those affected sought the answer in theological miracles, now we sometimes look for scientific miracles, or at least statistical ones.

Bizarre behavior, whether frantic or somnolent, is caused by a growing procession of ailments, among them rabies, nodding syndrome, unexplained epidemic epilepsy associated with onchocerciasis, diphtheric polyneuropathy, acute flaccid paralysis, brucellosis, encephalitis, or various fevers of unknown origin. Bizarre behavior still relies for relief on miracles, which today's science measures as probability (p values). Epidemiologists at a slow but steady pace, generate surveillance data around the globe, three steps forward, two steps back. The hope is to enable patients with neurologic disorders to gain control over their bodies and only dance when they choose to.

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Article Title

Mumps Postexposure Prophylaxis with a Third Dose of Measles-Mumps-Rubella Vaccine, Orange County, New York, USA

CME Questions

1. There are reports of an outbreak of mumps in your community. As you prepare to see patients who may be infected, what should you consider regarding mumps?

A. Two doses of the measles-mumps-rubella (MMR) vaccine provides over 99% protection against mumps
B. Mumps is only communicable after the onset of parotitis
C. Up to one-quarter of individuals infected with mumps virus may be asymptomatic
D. Public health policies in schools effectively eliminate the risk of the spread of mumps

2. You begin to receive phone calls from patients interested in a third dose of MMR as postexposure prophylaxis. What is the policy of the Advisory Committee on Immunization Practices (ACIP) regarding postexposure prophylaxis with MMR?

A. It should be administered to household contacts only
B. It should be administered to children between the ages of 5 and 12 years only
C. It should be applied broadly in affected communities
D. It should not be used at all

3. You see a 4-year-old boy in your clinic that day who appears to have mumps. Which of the following statements regarding the presentation of cases of mumps in the current study is most accurate?

A. The median age of index case-patients was 9 years
B. Most cases had not received 2 doses of MMR
C. Nearly all secondary case-patients who did not receive postexposure prophylaxis were young children
D. Secondary cases were far more common in households in which the index case-patient had received fewer than 2 doses of MMR

4. What was the main result of using MMR as postexposure prophylaxis against mumps in the current study?

A. MMR was not associated with a numerical or statistical benefit
B. MMR was associated with a lower rate of secondary cases of mumps, but the result was not statistically significant
C. MMR significantly reduced the risk of secondary cases of mumps
D. MMR caused more cases of mumps than it prevented

Activity Evaluation

1. The activity supported the learning objectives.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
2. The material was organized clearly for learning to occur.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
3. The content learned from this activity will impact my practice.				
Strongly Disagree				Strongly Agree
1	2	3	4	5
4. The activity was presented objectively and free of commercial bias.				
Strongly Disagree				Strongly Agree
1	2	3	4	5

EMERGING INFECTIOUS DISEASES

Upcoming Issue

Chagas Disease and Breastfeeding

Transmission of Immunogenic *Mycobacterium africanum* Strains, The Gambia

Plasmodium vivax Malaria during Pregnancy, Bolivia

Genetic Recombination and *Cryptosporidium hominis* Virulent Subtype IbA10G2

Increased Incidence of Invasive Fusariosis with Cutaneous Portal of Entry, Brazil

Coccidioidomycosis-associated Hospitalizations, California, 2000–2011

Emergence of Vaccine-Derived Polioviruses, Democratic Republic of Congo, 2004–2011

Clonal Distribution and Virulence of *Campylobacter jejuni* Blood Isolates, Finland, 1998–2007

Melioidosis in Traveler from Africa, Spain

Rickettsia slovaca Infection in Humans, Portugal

Reassortant Avian Influenza (H5N1) Viruses with H9N2-PB1 Gene in Poultry, Bangladesh

Evolution of Influenza A Virus H7 and N9 Subtypes, Eastern Asia

Hepatitis E Virus among Persons Who Inject Drugs, San Diego, California, USA, 2010

Human Infections with New Subspecies of *Campylobacter fetus*

Transition of Febrile Illness Etiology from Malaria to Dengue Fever, Northwestern Ecuador

Subclinical Avian Influenza A(H5N1) Infection in Human, Vietnam

Chikungunya Fever Outbreak, Bhutan, 2012

Primary Multidrug-Resistant *Mycobacterium tuberculosis* in 2 Regions, Eastern Siberia

Melanesian Variant of HTLV-1 and Adult T-cell Leukemia in Indigenous Australians

Declining Influenza Vaccination Coverage among Health Care Workers, Hong Kong

Complete list of articles in the October issue at http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

September 5–10, 2013
Options for the Control of Influenza VIII
Cape Town, South Africa
http://www.isirv.org

September 10–13, 2013
ICAAC 2013
(Interscience Conference on Antimicrobial Agents and Chemotherapy)
Denver, Colorado, USA
http://www.icaac.org

October 2–6, 2013
2nd annual IDWeek
San Francisco, CA, USA
A combined meeting of the Infectious Diseases Society of America (IDSA), the Society for Healthcare Epidemiology of America (SHEA), the HIV Medicine Association (HIVMA) and the Pediatric Infectious Diseases Society (PIDS)
http://idweek.org

November 2–6, 2013
APHA
American Public Health Association's 141st Annual Meeting and Exposition
Boston, MA, USA
http://www.apha.org

November 4–7, 2013
3rd ASM-ESCMID Conference on Methicillin-resistant Staphylococci in Animals:
Veterinary and Public Health Implications
Copenhagen, Denmark
http://www.asm.org/conferences

November 5–7, 2013
ESCAIDE 2013
European Scientific Conference on Applied Infectious Disease Epidemiology
Stockholm, Sweden
http://www.escaide.eu

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EMERGING INFECTIOUS DISEASES®

JOURNAL BACKGROUND AND GOALS

What are "emerging" infectious diseases?

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

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

Why an "Emerging" Infectious Diseases journal?

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

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

What are the goals of Emerging Infectious Diseases?

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

   - ★ Investigates factors known to influence emergence: microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.
   - ★ Reports laboratory and epidemiologic findings within a broader public health perspective.
   - ★ Provides swift updates of infectious disease trends and research: new methods of detecting, characterizing, or subtyping pathogens; developments in antimicrobial drugs, vaccines, and prevention or elimination programs; case reports.

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

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

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Emerging Infectious Diseases is published in English. To expedite publication, we post some articles online ahead of print. Partial translations of the journal are available in Japanese (print only), Chinese, French, and Spanish (http://wwwnc.cdc.gov/eid/pages/translations.htm).

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Research. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

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Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

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Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

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