

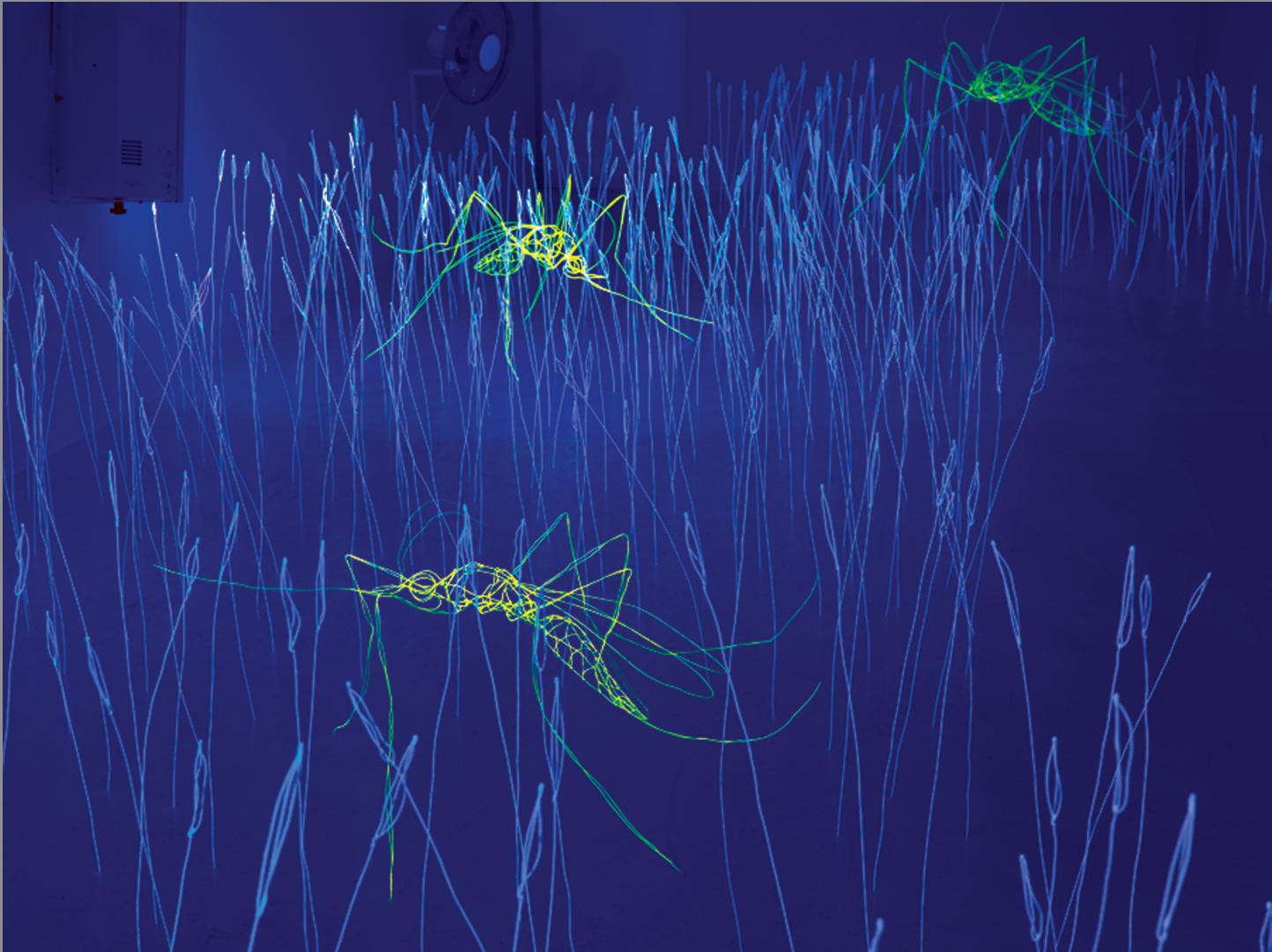
EMERGING INFECTIOUS DISEASES®

20
YEARS



Vectorborne Infections

May 2015



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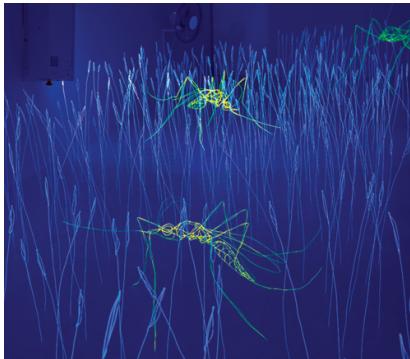
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On the Cover

Stefan Waibel (1970–)
Ideal Nature Machine, 2012

Installation (Metal wire, epoxy resin
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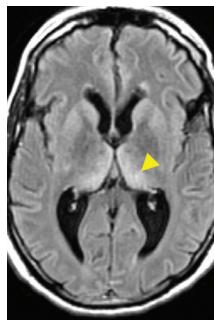
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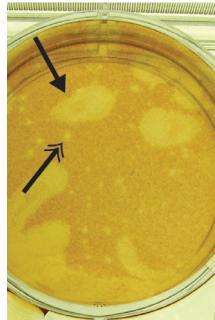
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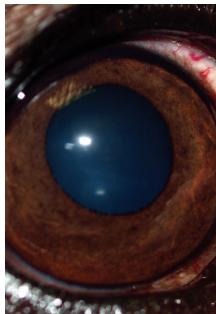
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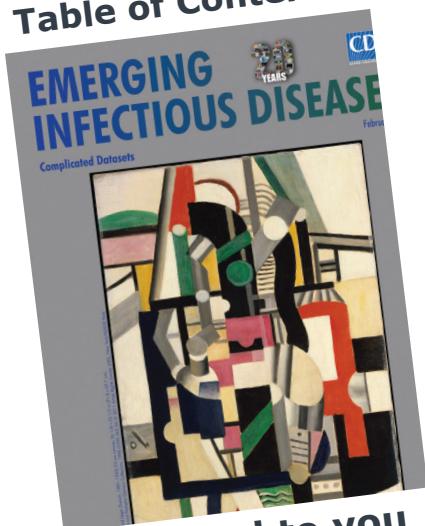
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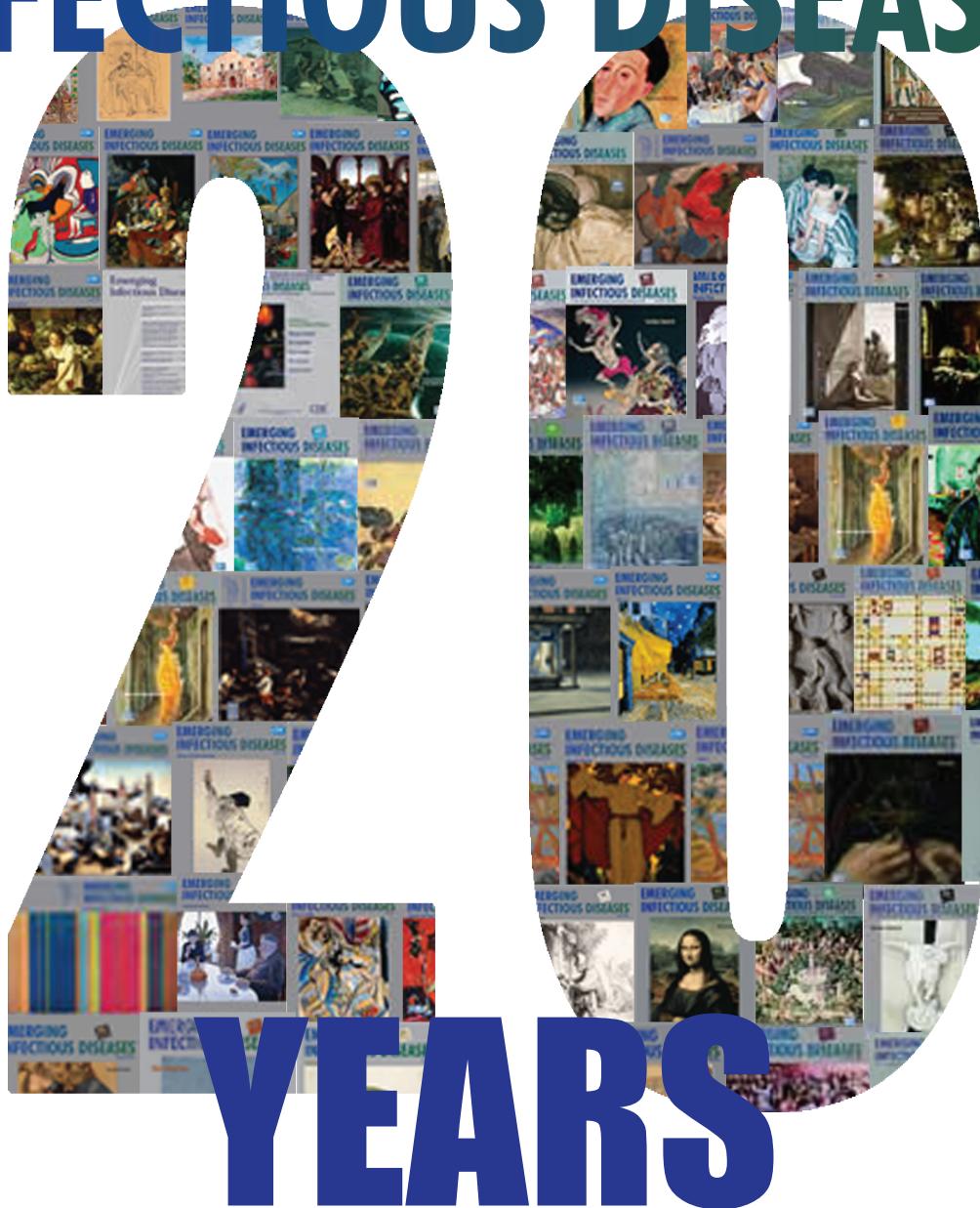
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Presenting the ongoing challenges
that emerging microbial threats
pose to global health



Detecting Spread of Avian Influenza A(H7N9) Virus Beyond China

Alexander J. Millman, Fiona Havers, A. Danielle Iuliano, C. Todd Davis, Borann Sar, Ly Sovann, Savuth Chin, Andrew L. Corwin, Phengta Vongphrachanh, Bounlom Douangneun, Kim A. Lindblade, Malinee Chittaganpitch, Viriya Kaewthong, James C. Kile, Hien T. Nguyen, Dong V. Pham, Ruben O. Donis, Marc-Alain Widdowson

During February 2013–March 2015, a total of 602 human cases of low pathogenic avian influenza A(H7N9) were reported; no autochthonous cases were reported outside mainland China. In contrast, since highly pathogenic avian influenza A(H5N1) reemerged during 2003 in China, 784 human cases in 16 countries and poultry outbreaks in 53 countries have been reported. Whether the absence of reported A(H7N9) outside mainland China represents lack of spread or lack of detection remains unclear. We compared epidemiologic and virologic features of A(H5N1) and A(H7N9) and used human and animal influenza surveillance data collected during April 2013–May 2014 from 4 Southeast Asia countries to assess the likelihood that A(H7N9) would have gone undetected during 2014. Surveillance in Vietnam and Cambodia detected human A(H5N1) cases; no A(H7N9) cases were detected in humans or poultry in Southeast Asia. Although we cannot rule out the possible spread of A(H7N9), substantial spread causing severe disease in humans is unlikely.

Novel low pathogenic avian influenza (LPAI) A(H7N9) virus emerged in February 2013 and, as of March 3, 2015, a total of 602 laboratory-confirmed human infections, including 227 deaths, had been reported (1–3). Most human cases have had live poultry or live-bird market (LBM)

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environmental exposure; person-to-person spread appears infrequent (4). However, as circulation of A(H7N9) virus becomes more widespread, the probability increases for mutations enabling efficient person-to-person transmission.

Similar fears accompanied the reemergence in 2003 of highly pathogenic avian influenza (HPAI) A(H5N1) virus, which caused ≈45 human cases in Vietnam and Thailand within 12 months. As of March 3, 2015, A(H5N1) virus had resulted in 784 human cases, including 429 deaths, in 16 countries and poultry outbreaks in 53 countries (3,5–7). In response to these outbreaks, avian influenza surveillance systems were created to monitor A(H5N1) activity and to detect other novel influenza viruses.

In contrast to the rapid international spread of A(H5N1) virus in poultry and humans within 12 months after its re-emergence, no autochthonous A(H7N9) cases in animals or humans have been reported outside mainland China (3), despite a higher incidence of reported A(H7N9) than A(H5N1) cases in humans; A(H7N9) detection in poultry and humans; and the presence of the virus in border provinces in southern, western, and northeastern China (2,8,9). Surveillance systems in Southeast Asia have detected new A(H5N1) cases in humans and poultry since the A(H7N9) virus was first identified (3). Whether the absence of reported A(H7N9) among humans or poultry outside mainland China represents a lack of spread or whether regional surveillance systems are insufficiently sensitive to detect A(H7N9) remains unclear. Because A(H5N1) virology and epidemiology differ from those of A(H7N9), assessing how A(H7N9) might spread and whether surveillance would detect it remains critical for countries to prepare control measures and to monitor virologic and epidemiologic changes.

We highlight differences and similarities between A(H5N1) and A(H7N9) viruses and hypothesize scenarios related to possible A(H7N9) virus spread. Then we describe human and animal influenza surveillance data from 4 Southeast Asian countries where A(H5N1) has been detected—Vietnam, Cambodia, Laos, and Thailand—to assess the likelihood that surveillance systems designed for A(H5N1)

would have detected human or animal A(H7N9) infections during the predominant months of A(H7N9) virus circulation during 2014.

Comparison of A(H5N1) and A(H7N9) Epidemiology and Virology

A(H5N1) and A(H7N9) have epidemiologic and virologic similarities and differences. These features have implications for detection by existing human and animal surveillance systems (Table 1).

Clinical Presentation and Demographics

Reported A(H5N1) and A(H7N9) infections in humans generally present as severe respiratory disease with fever, cough, and pneumonia, often leading to respiratory failure (1,4). Most A(H5N1) cases in Asia and A(H7N9) cases in China are detected in hospitals; therefore, existing hospital-based surveillance systems should detect severe infections of both viruses. However, hospital-based surveillance alone might be unlikely to detect mild cases or cases outside surveillance areas. Most A(H5N1) cases were reported in children and young adults (median age ≈17 years). In contrast, the median age of persons with A(H7N9) is 58 years; mild cases have been reported predominantly in children (4). Therefore, surveillance systems biased toward younger patients are less likely to detect A(H7N9) cases.

Seasonality

Most A(H5N1) infections in humans and poultry occur during November–May in China and Southeast Asia (3,14); similarly, most A(H7N9) cases occurred in February–May 2013 and December 2013–March 2014. Although A(H7N9) seasonality data are limited, A(H7N9) infections probably would increase during December–March; however, sporadic cases might be detected in other months.

Host Range and Pathogenicity in Animals

These viruses have some genetic similarities predicted to alter their adaptability to animal hosts. Many A(H5N1) and most A(H7N9) viruses have internal genes derived from LPAI A(H9N2) viruses, which might confer adaption to poultry (12). Additionally, internal genes shared by many of these viruses have several mutations demonstrated to enhance adaptation to mammalian hosts (PB2 E672K, PB1-F2, M1 N30D and T215A, and NS1 P42S). These and other shared mutations might affect viral adaptation to mammalian hosts and might explain similarly severe clinical infections in humans (12,15,16). These viruses currently have differences in host range and pathogenicity in animals that could affect surveillance. A(H5N1) circulates among domestic chickens, ducks, geese, and other poultry; sporadic outbreaks occur in wild migratory waterfowl species (5,17). A(H7N9) has been detected primarily in chickens

Table 1. Characteristics of influenza A(H5N1) and A(H7N9) infection and implications for surveillance system detection of A(H7N9) in humans and animals*

Characteristic	A(H5N1)	A(H7N9)	Reference	Surveillance system implications for A(H7N9) detection
Clinical signs and symptoms	Fever, cough, pneumonia, respiratory failure	Fever, cough, pneumonia, respiratory failure	(1,4)	SARI and ILI surveillance systems should detect with equal efficacy
Disease severity	Critical and fatal (60%)	Most are critical; mild infections reported in children	(4)	Hospital-based platforms would be most likely to detect cases
Patient age, y	<20	>60	(4)	Surveillance systems that do not cover older adults may not detect case
Seasonality	December–March (average)	Most cases in 2nd wave occurred December–March 2013–2014	(3)	Surveillance will be more likely to detect a case when the virus is circulating; however, additional data are needed to establish the seasonality of A(H7N9)
Geography	Primarily rural (farm)	Primarily urban (LBM)	(4)	Surveillance systems that do not cover visitors to LBMs may be unable to detect cases
Transmissibility from poultry or environment to humans	Appears low	Appears moderate	(10)	Surveillance systems should assess for poultry or environmental exposures, and known exposures should prompt testing in suspected cases of avian influenza
Person-to-person transmission	Appears uncommon	Appears uncommon	(1,11)	Surveillance systems will probably detect sporadic cases that have identifiable poultry exposures
History of poultry exposure	Common	Common	(4)	Animal surveillance is critical for detection in poultry and assisting with targeting control measures
Pathogenicity in chickens	High	Low	(5,11)	Infection with A(H7N9) does not appear to cause disease in poultry. Surveillance for detecting A(H7N9) in poultry requires targeted risk assessment and active testing.
Effects in wild bird species	Detected in wild bird species	Limited data	(11–13)	Poultry surveillance directed at either back-yard farms or commercial poultry farms (depending on prevalence) and LBMs should be sufficient to detect cases

*ILI, influenza-like illness; LBM, live-bird market; SARI, severe acute respiratory infection.

and infrequently in ducks, pigeons, and quail (10,18–20). A(H7N9) has not been detected among wild waterfowl. Experimentally, A(H7N9) viruses replicate well in poultry, quail, and Muscovy ducks but are less infectious and result in decreased shedding in other wild bird species, which may limit their ability to spread in these species (10). This feature may relate in part to a neuraminidase stalk deletion in A(H7N9), which is considered a marker of adaptation to poultry rather than to wild waterfowl (21); because similar neuraminidase stalk deletions have been documented in A(H5N1) viruses isolated from wild birds, the implications of this feature remain unclear (22).

A(H5N1) viruses with the A/goose/Guangdong/1/1996-like hemagglutinin gene are classified as HPAI viruses because infections in chickens or other gallinaceous species cause high-level replication throughout many tissues, extensive shedding/environmental contamination, and generally high death rates (5,8,11). In contrast, A(H7N9) viruses are classified as LPAI because poultry infections cause low-level viral shedding and replication is limited to digestive and respiratory tracts; infected poultry typically remain asymptomatic. Nevertheless, some LPAI viruses, such as A(H9N2), have transmitted extensively in poultry in Asia (23), possibly because they have increased environmental stability relative to HPAI viruses (24). Poultry illnesses and deaths alert health authorities about possible A(H5N1) outbreaks and trigger enhanced surveillance in humans (5). Conversely, the low pathogenic nature of A(H7N9) means that passive surveillance cannot rely on the same triggers used for A(H5N1). To detect A(H7N9), systematic, risk-based surveillance and sampling of asymptomatic poultry is more appropriate.

Poultry Exposure and Transmissibility to Humans

Backyard poultry are a source for exposure to A(H5N1) virus, but this exposure has been reported less frequently among A(H7N9)-infected persons, whereas exposure to chickens (usually slower-growing yellow chickens or Silkie chickens) or environmental exposure in LBMs are the major risk factors for human A(H7N9) infection (4,14,25). Despite extensive testing reported by Chinese authorities, few A(H7N9)-positive samples have been detected in commercial farms (9). The sources of virus exposure to humans may change if A(H7N9) spreads further among backyard poultry and large commercial farms. Although poultry exposure is a risk factor for both viruses, A(H7N9) may be more transmissible from infected poultry or poultry environments to humans because it has a glutamine to leucine amino acid substitution at position 217 (position 226 in H3 numbering), whereas A(H5N1) virus maintains a more strictly conserved glutamine (avian consensus) at the equivalent H5 hemagglutinin position (10). This substitution confers a higher virus specificity to $\alpha 2,6$ sialic

acid receptors (which predominates on human respiratory epithelial cells), possibly explaining the high incidence of human A(H7N9) cases (10).

Potential Spread of A(H7N9)

Since A(H7N9) emerged, 2 complete waves of infections have occurred; the second wave is defined as cases occurring during October 1, 2013–September 30, 2014 and affecting mostly the southeastern provinces of China (Figure 1). A third (ongoing) wave is defined as cases since October 1, 2014. A(H7N9) virus does not transmit easily between humans, and person-to-person spread has been limited to 2 or possibly 3 generations of transmission (2,4). Assuming transmission remains unchanged, geographic spread probably will occur through travel of infected humans or infected poultry. Several persons exposed to A(H7N9) virus in China traveled to Hong Kong, Taiwan, Malaysia, and Canada; became ill; and were deemed to have imported infections (2). Additional sporadic A(H7N9) infections might occur in travelers, but appropriate isolation measures should prevent further spread (2).

LBMs create environments that can amplify avian influenza viruses and increase risk of human infection (5,19,26). Large informal poultry movements between China and Southeast Asian countries also pose a risk for spread (8,27). The first confirmed human A(H5N1) case outside China occurred in December 2003 in Vietnam and foreshadowed the virus' rapid regional spread in humans and poultry (Figure 2). Phylogeographic studies suggest that A(H5N1) virus was introduced to Vietnam from China through these poultry trade routes; A(H7N9) virus is similarly likely to be introduced in domestic poultry in Vietnam (27,28). However, because A(H7N9) preferentially infects different poultry species than A(H5N1), different poultry value chains might be implicated in this potential spread.

Wild migratory birds have contributed to spread of A(H5N1) virus along regional flyways (29,30). A(H7N9) virus was detected in a nonmigratory wild sparrow in China during spring 2013 but has not been identified in other wild bird species (13). This finding suggests that A(H7N9) infection is not widespread in wild birds, and the possible risk for regional spread by wild birds is currently low.

Surveillance for Avian Influenza in Southeast Asia

Vietnam, Thailand, Laos, and Cambodia each operate at least 2 national systems for influenza surveillance in humans: 1) sentinel inpatient-based severe acute respiratory infection (SARI) and 2) sentinel outpatient-based influenza-like illness (ILI). Additionally, most operate event-based or passive surveillance systems for pneumonia (Figure 3) with prespecified case definitions (online Technical Appendix Table, <http://wwwnc.cdc.gov/EID/article/21/5/>

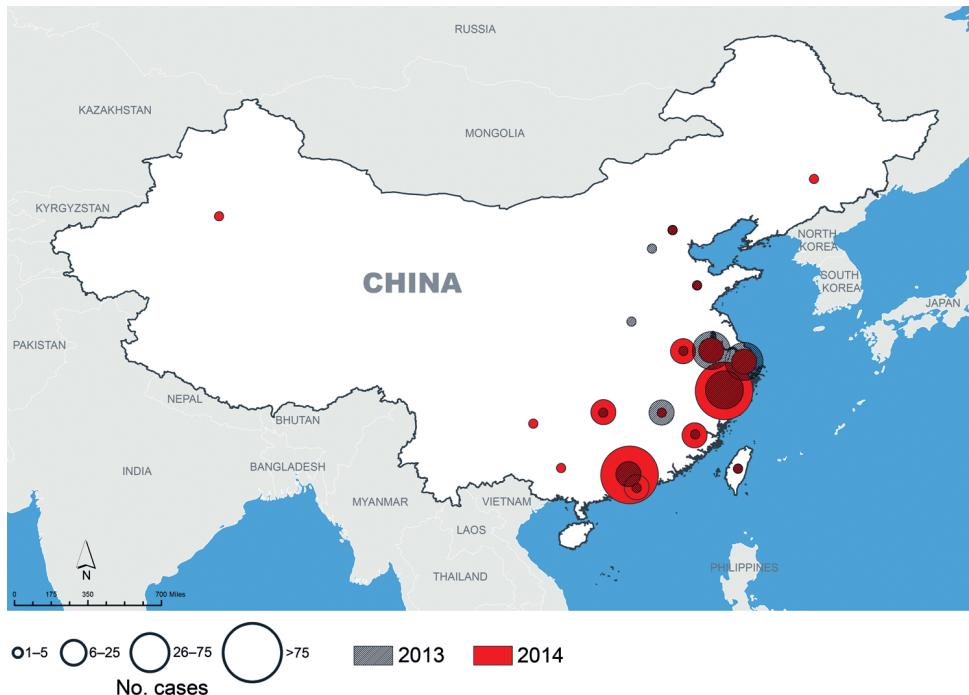


Figure 1. Avian influenza A(H7N9) in humans, China, 2013–2014. Data were obtained from the World Health Organization as reported from the National Health and Family Planning Commission (http://www.who.int/influenza/human_animal_interface/influenza_h7n9/en/).

14-1756-Techapp1.pdf). World Health Organization member states must report all cases of A(H5N1) and A(H7N9) in humans as required by the International Health Regulations (2005); the World Organization for Animal Health mandates reporting of outbreaks of HPAI in birds by the Terrestrial Animal Health Code (31,32).

Vietnam

Human Surveillance

Since May 2013, four regional public health institutes in Vietnam have had real-time reverse transcription PCR (rRT-PCR) A(H7N9) testing capacity, including the institutes housing the 2 National Influenza Centers (NICs)—the National Institute of Hygiene and Epidemiology in Hanoi and the Pasteur Institute in Ho Chi Minh City (33). Vietnam conducts sentinel SARI surveillance at 8 sites and ILI surveillance at 10 sites. Four SARI sites have operated continuously since April 2013. Another 4 were established in December 2013, including 3 in Lang Son and Quang Ninh provinces, which are entry points for Chinese poultry (34). Of $\approx 2,500$ SARI specimens tested for influenza by using Centers for Disease Control and Prevention (Atlanta, GA, USA) testing protocols during April 1, 2013–May 30, 2014, none were positive for A(H5N1) or A(H7N9) virus. During the same period, no ILI specimens tested positive for A(H7N9); 1 was A(H5N1) positive.

Since 2006, Vietnam has operated a nationwide passive surveillance system for pneumonia in all hospitals (35). This surveillance system identified 33 human A(H5N1)

virus infections, but no A(H7N9) virus infections have been detected since testing for this virus began in December 2013 (35). The median age of patients in this system was 43 years. Table 2 shows results from all 3 systems during April 1, 2013–May 30, 2014.

Animal Surveillance

The Vietnam Ministry of Agriculture and Rural Development, Department of Animal Health, National Center for Veterinary Diagnosis, obtained A(H7N9) laboratory testing capacity in June 2013. During December 5, 2013–March 6, 2014, the Department of Animal Health conducted active weekly surveillance for A(H7N9) and A(H5N1) using rRT-PCR in 13 traditional and nontraditional LBMs in Hanoi, Quang Ninh, and Lang Son provinces, areas historically known to contain markets selling smuggled poultry. No A(H7N9) virus was detected from 737 poultry oropharyngeal and cloacal specimens and 555 poultry cage fecal and water samples. Additionally, the Ministry of Agriculture and Rural Development conducted biweekly surveillance in 60 LBMs in 9 northern provinces bordering China (36). None of the 25,000 samples had tested positive for A(H7N9) as of August 2014 (36). Routine A(H7N9) surveillance is not performed on poultry farms.

Thailand

Human Surveillance

In April 2013, the Thai NIC in Nonthaburi acquired A(H7N9) testing capacity and has since trained 14 regional laboratories (33). SARI sentinel surveillance is

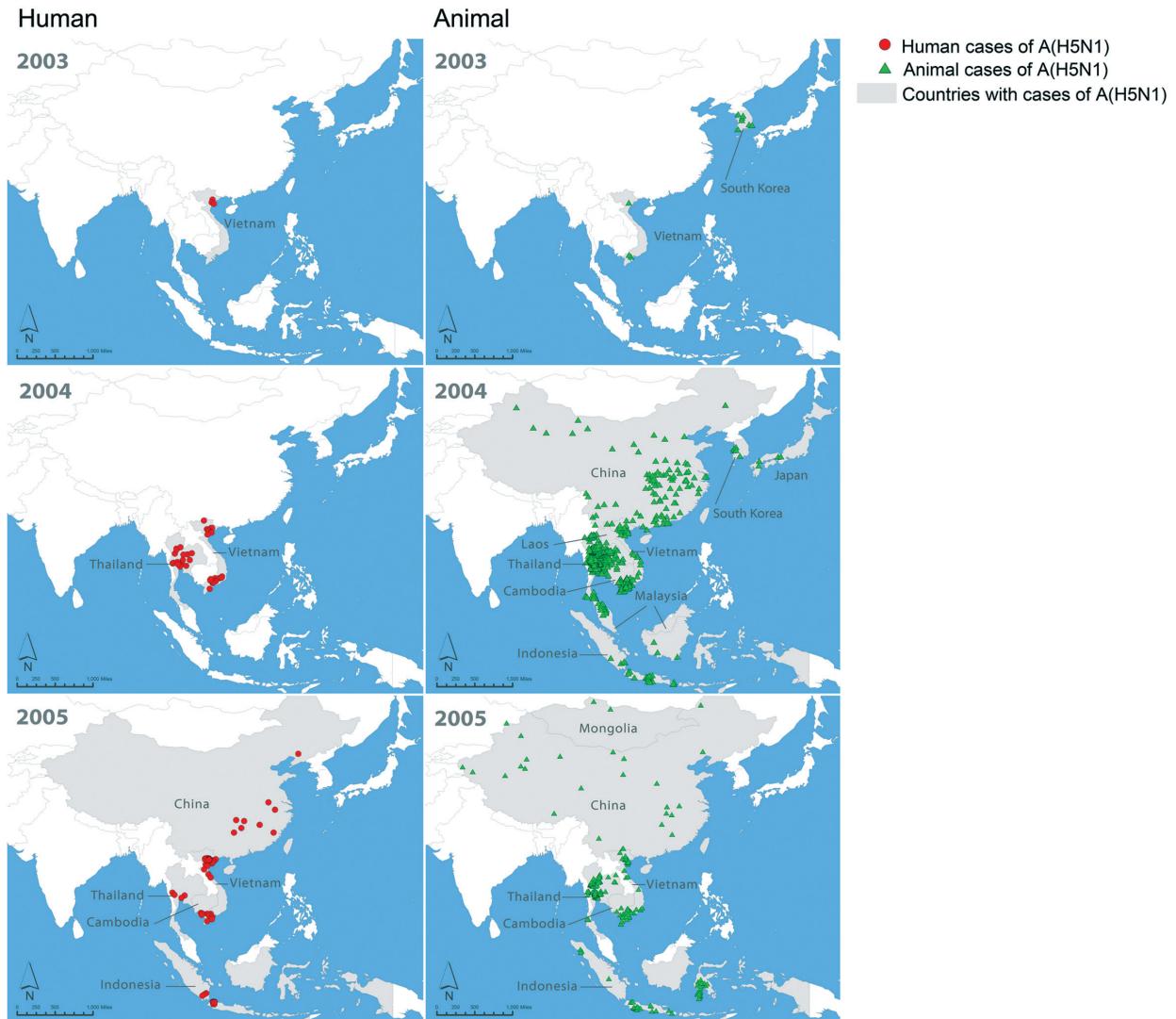


Figure 2. Initial 2-year spread of human cases and poultry outbreaks of influenza A(H5N1) in China and Southeast Asia, December 2003–2005. Data on A(H5N1) in humans were obtained from the World Health Organization (http://www.who.int/influenza/human_animal_interface/en/). Data on outbreaks of A(H5N1) in poultry were obtained from the World Organisation for Animal Health (outbreaks before 2005 from <http://www.oie.int/en/animal-health-in-the-world/the-world-animal-health-information-system/data-before-2005-handistatus/>; outbreaks after 2005 from http://www.oie.int/wahis_2/public/wahid.php/Wahidhome/Home).

conducted in hospitalized patients at 7 sites in 7 provinces; 2 were established after A(H7N9) emerged. ILI surveillance is conducted at 10 sites in 9 provinces, where 10 outpatients with ILI are sampled weekly for influenza testing.

Thailand operates 3 additional influenza surveillance systems: a national passive system for pneumonia in public outpatient and inpatient facilities; a national event-based system for reporting clusters of severe respiratory disease or respiratory illnesses associated with dead or dying poultry; and a severe and fatal pneumonia sentinel system in 30 hospitals nationwide. In the passive pneumonia system, physicians send samples at their discretion.

During April 1, 2013–May 30, 2014, no human cases of A(H7N9) or A(H5N1) were detected (Table 2). Among patients tested for A(H7N9) virus, the median ages were 29 years (pneumonia surveillance system) and 31 years (event-based system). Of 208 cases identified by the severe or fatal pneumonia system during July 2013–March 2014 (≈70% <5 years old), 14 were influenza A positive and none A(H7N9) positive.

Animal Surveillance

Poultry production in Thailand has strict biosecurity practices (5). Moreover, Thailand is a net poultry exporter and shares no borders with China. However, informal backyard

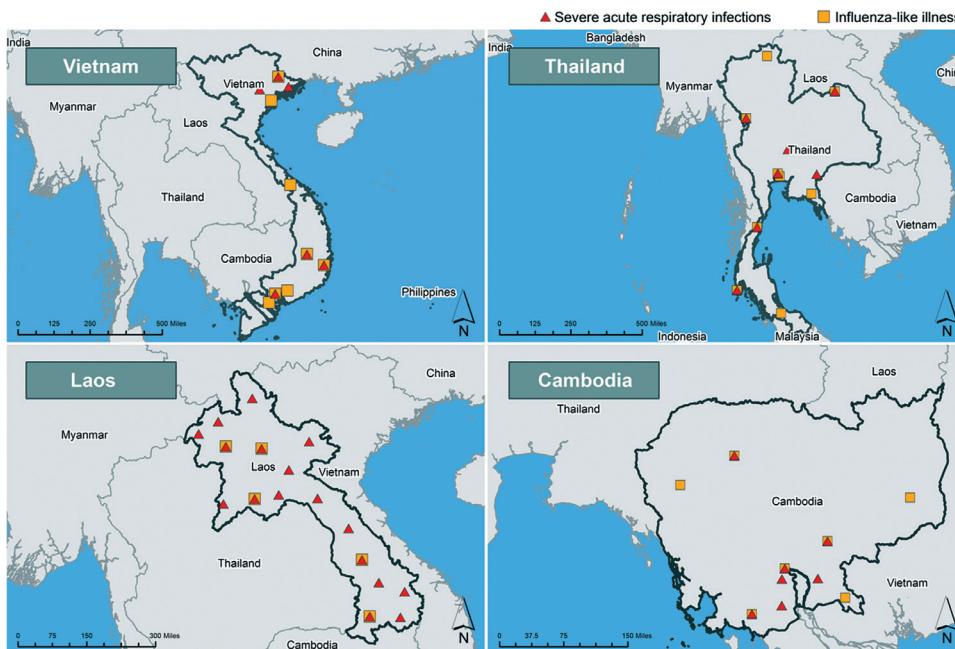


Figure 3. Severe acute respiratory infection (SARI) and influenza-like illness (ILI) sentinel sites in Vietnam, Thailand, Laos, and Cambodia. A given location might have >1 SARI or ILI sentinel site. SARI sites in Laos include planned SARI sites and 8 nonsentinel SARI sites. (The Lang Son and Quang Ninh province sites continue to operate. The Hanoi site operated until June 2014.)

poultry trade for LBMs at its Laos and Cambodia borders may present an indirect importation risk.

The National Institute of Animal Health in Bangkok and Regional Veterinary Research and Development Centers perform diagnostic testing for avian influenza viruses, including A(H7N9). Active surveillance is conducted routinely in poultry farms in 77 provinces; viral isolation is performed on pooled samples of cloacal swabs (5 poultry per pool). During April 1, 2013–May 30, 2014, a total of 8,829 pooled samples from poultry farms were tested, and neither A(H5N1) nor A(H7N9) was detected.

Active poultry and environmental surveillance was conducted in 9 Bangkok LBMs during November 2013. A total of 1,853 samples consisting of 1,619 oropharyngeal or fecal swabs and poultry serum and 234 environmental water samples were tested for A(H7N9) by RT-PCR; none tested positive.

Cambodia

Human Surveillance

In Cambodia, testing capacity for A(H7N9) virus was available in September 2013 at the national laboratory network. The network comprises the Institute Pasteur; a World Health Organization NIC; and the National Institute Public Health Laboratory (33).

The Cambodia Ministry of Health operates 8 SARI sentinel sites in Phnom Penh and 6 provinces. Four have operated since 2009; 4 sites were added in provinces bordering Vietnam in 2013. Seven ILI sentinel sites operate in Phnom Penh and 6 additional provinces. During April 1, 2013–May 30, 2014, eight A(H5N1) cases and no A(H7N9) cases were detected in humans in Cambodia (Table 2).

The Ministry of Health also conducts event-based surveillance through CAM-EWARN (Cambodia Early Warning and Response Network) (37). CAM-EWARN operates in 1,199 sites ranging from specialty hospitals to health centers in all provinces and relies on voluntary reporting. Of 26 A(H5N1) cases in humans during 2013, 21 were reported through CAM-EWARN because these patients sought care at nonsentinel sites. Most cases reported through this system were in children <5 years of age. No human A(H7N9) cases have been reported through CAM-EWARN.

Animal Surveillance

The National Animal Research Institute and Institute Pasteur conducted poultry and environmental surveillance for A(H7N9) in 4 LBMs in 4 provinces during 2013. During this time, rRT-PCR was performed on 528 poultry throat and cloacal samples and 792 environmental samples; all were negative for A(H7N9). Routine A(H7N9) surveillance is not performed on poultry farms.

Laos

Human Surveillance

Since May 2013, the Laos NIC based in the National Center for Laboratory and Epidemiology has had testing capacity for A(H7N9) virus (33). The Hospital Sentinel Virological Surveillance Network comprises SARI surveillance in 7 sentinel sites in 5 provinces and ILI surveillance at 8 sites in 5 provinces. The SARI surveillance platform is expanding and will eventually comprise 26 sentinel sites in all provinces (Figure 3). During April 1, 2013–May 30, 2014, fifteen specimens from SARI patients were tested for

Table 2. Surveillance for SARI and ILI and passive surveillance for pneumonia for avian influenza in humans, 4 Southeast Asia countries, April 1, 2013–May 30, 2014*

Surveillance system	No. illnesses meeting case definition	Total no. samples tested	A(H5N1), no. positive/no. tested	A(H7N9), no. positive/no. tested
Vietnam				
SARI	11,558	2,485	0/798	0/798
ILI	29,027	3,770	0/0	0/0
Passive surveillance for pneumonia†	238	237	4/70	0/70
Thailand				
SARI	‡	1,025	0/106§	0/106§
ILI	‡	3,850	0/807	0/807
Passive surveillance for pneumonia	‡	157	0/157	0/43
Event-based surveillance	18 outbreaks	162	0/69§	0/69§
Surveillance for severe or fatal pneumonia	208	208	0/14§	0/14§
Cambodia				
SARI	2,282	2,282	7/219	0/0
ILI	1,567	1,567	1/10	0/0
Laos				
SARI	1,469	698	0/15	0/15
ILI	8,962	1,550	0/0	0/0

*ILI, influenza-like illness; SARI, severe acute respiratory infection.

†Severe viral pneumonia surveillance system.

‡Total number of illnesses meeting the case definition is unknown for ILI. For SARI, the number of illnesses meeting the case definition is unknown but believed to be close to 100% of persons sampled. Reporting to the passive pneumonia surveillance system is in accordance with physician discretion, and no clear case definition is applied.

§A(H5N1) and A(H7N9) testing is conducted only on specimens positive for influenza A virus.

A(H5N1) and A(H7N9), and none were positive; no specimens from ILI surveillance were tested for A(H5N1) or A(H7N9) virus (Table 2).

Animal Surveillance

The National Animal Health Laboratory (NAHL) acquired animal A(H7N9) testing capacity in April 2013. NAHL conducts routine surveillance in 4 LBMs in 3 provinces bordering China. A total of 892 poultry oropharyngeal specimens and 74 environmental specimens were negative for A(H7N9) virus by RT-PCR. An additional 892 poultry serum samples were negative for H7 antibodies. During April 1, 2013–May 30, 2014, NAHL collected 1,666 poultry swab samples and 137 environmental samples from poultry farms and villages in 3 provinces bordering China; all tested negative for A(H5N1) and A(H7N9).

Conclusions

A(H7N9) and A(H5N1) viruses can cause severe disease in humans, do not transmit easily from person to person, and are primarily linked to exposure to infected poultry or contaminated environments. However, several major differences—including older age of human A(H7N9) patients, greater risk for transmission from infected poultry to humans, and lower pathogenicity of A(H7N9) virus infection in poultry—have implications for A(H7N9) virus detection. A(H7N9) will circulate in poultry without the typical HPAI-associated morbidity and mortality and can spread undetected, making A(H7N9) passive surveillance and control in poultry species challenging. Systematic, risk-based poultry surveillance is appropriate but might still miss A(H7N9) cases. As probably occurred with A(H5N1), A(H7N9) may spread through informal poultry trade

between China and neighboring countries; however, the predilection of A(H7N9) for certain poultry species may favor different poultry value chains. This spread may have already occurred; although surveillance of poultry provides useful monitoring, it is unlikely to detect A(H7N9)-infected poultry until the virus becomes widespread because large sample sizes are needed to detect cases in a low-prevalence poultry population. Detection might not occur in poultry until a human case is identified, which would trigger additional poultry surveillance; notably, however, targeted poultry surveillance activities at known sites of exposure in China have had few detections despite extensive testing (20). The initial emergence of A(H5N1) in Hong Kong in 1997 was, like that of A(H7N9), associated with LBMs, but the virus has since become established in all poultry sectors, including endemicity in backyard poultry and outbreaks in wild birds (34). A(H7N9) virus might do the same, and its epidemiologic profile might change. However, the mean age of A(H5N1)-infected persons in 1997 was <10 years; no A(H5N1) cases have been reported in China in persons >65 years of age, which suggests that exposure alone cannot explain the age differences between persons with A(H5N1) and A(H7N9) infections.

Although we cannot rule out the possibility of human A(H7N9) disease in Southeast Asia, substantial A(H7N9) spread resulting in the widespread occurrence of severe infections in humans is unlikely to have occurred in Vietnam, Thailand, Cambodia, and Laos. All 4 countries have the laboratory capacity to detect A(H7N9) and well-developed hospital-based surveillance systems. Moreover, Vietnam, Thailand, and Cambodia operate passive pneumonia surveillance systems covering wide geographic areas and

include public and private health care facilities. Since 2006, when Vietnam established passive surveillance, the system has been critical to detecting A(H5N1) in humans. Similarly, of 26 human A(H5N1) cases detected by CAM-ERN in 2013, 18 sought care at private health care facilities and would not have been detected by sentinel platforms. Vietnam and Cambodia have detected A(H5N1) in humans since 2013, suggesting that severe A(H7N9) infections in humans have not occurred because either the virus has not spread to these countries or it has a lower incidence than A(H5N1). Sporadic traveler-associated A(H7N9) might be detected, but if person-to-person transmission remains limited, this mechanism should not contribute to continued spread of A(H7N9) in humans.

Although sentinel platforms in all countries may be adequate for detecting A(H7N9), their limited geographic coverage could miss cases. Efforts to expand these platforms may enhance case detection capabilities, especially in Laos, which relies solely on its sentinel surveillance systems. Therefore, robust passive reporting systems may be important to detect severe A(H7N9) cases early. Additionally, although these sentinel- and nonsentinel-based systems have performed well for A(H5N1), most detected cases occurred in children or young adults in Vietnam and Thailand or came from 1 pediatric hospital in Cambodia. Most surveillance systems cover all ages and should be able to detect illness meeting the case definition; gaps in age coverage (i.e., surveillance only at pediatric hospitals) would result in decreased sensitivity for detecting A(H7N9) because it is more likely to cause severe disease in persons >60 years of age.

Our analysis has limitations. We were unable to evaluate systems in other Asia countries; of particular concern is Myanmar, which has reported outbreaks of A(H5N1) and shares a long border with China across which it imports large quantities of poultry (3). Additionally, since July 2014, A(H7N9) has been detected in humans in Xinjiang Province in China (38), which borders 8 countries. A(H7N9) circulation in China's western region suggests that it is probably more geographically widespread than previously realized. Additionally, our analysis focused on surveillance programs operated by governments in Vietnam, Thailand, Cambodia, and Laos. Additional surveillance may exist for avian influenza, particularly through research studies and other entities our analysis does not cover.

Since 2013, we have observed the detection of multiple novel avian influenza viruses, including A(H7N9), A(H6N1), A(H5N6), and A(H10N8) (39,40). On the basis of our assessment, we believe substantial spread of A(H7N9) virus resulting in severe infections in humans is unlikely to have occurred in Vietnam, Thailand, Cambodia, or Laos. Given the virus' characteristics, there likely will be no obvious signal if it spreads to Southeast Asia. Well-designed, routine surveillance and astute clinicians are

essential for detecting the first case beyond China. The experience with A(H5N1) shows how countries in Southeast Asia designed systems capable of detecting and responding to avian influenza in poultry and humans, but vulnerabilities remain. Growing trade networks and economic integration mean weaknesses in individual surveillance systems can leave the entire region vulnerable. Governments must be vigilant against new and reemerging disease threats by rapidly responding to suspected outbreaks in animals and humans, educating health care professionals and the public, and working with partners to enhance animal health and public health systems.

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Recent US Case of Variant Creutzfeldt-Jakob Disease—Global Implications

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Learning Objectives

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

- Describe the clinical presentation of variant Creutzfeldt-Jakob disease, based on a case report and review
- Discuss diagnostic testing for variant Creutzfeldt-Jakob disease
- Determine the global implications of this report of a new US case of variant Creutzfeldt-Jakob disease

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Variant Creutzfeldt-Jakob disease (vCJD) is a rare, fatal prion disease resulting from transmission to humans of the infectious agent of bovine spongiform encephalopathy. We describe the clinical presentation of a recent case of vCJD in the United States and provide an update on diagnostic testing. The location of this patient's exposure is less clear than those in the 3 previously reported US cases, but strong evidence indicates that exposure to contaminated beef occurred outside the United States more than a decade before illness onset. This case exemplifies the persistent risk for vCJD acquired in unsuspected geographic locations and highlights the need for continued global surveillance and awareness to prevent further dissemination of vCJD.

P rion disorders are a unique class of diseases caused by pathologically misfolding proteins leading to neurodegeneration (1). Creutzfeldt-Jakob disease (CJD), one such prion disorder, is divided into 4 etiologic categories: sporadic (sCJD), familial, iatrogenic, and variant (vCJD). CJD is incurable and inevitably fatal.

The emergence of vCJD was linked to an earlier epidemic of bovine spongiform encephalopathy (BSE) in the United Kingdom through the consumption of contaminated beef (2). Since the first report in 1996, a total of 229 vCJD cases have been reported worldwide: 177 in the United Kingdom; 27 in France; and 25 distributed in 10 other countries, including the United States (3). Incidence of vCJD peaked in the United Kingdom and France in 1999 and 2004, respectively. Countries outside of the United Kingdom were apparently affected through importation of beef and live cattle from the United Kingdom (3,4). Only 5 vCJD cases have previously been reported in North America: 3 in the United States and 2 in Canada. All 5 patients had lived in either the United Kingdom (3 patients) or the Kingdom of Saudi Arabia (2 patients), prompting the conclusion that vCJD was acquired overseas rather than in the United States or Canada (5).

We report the fourth confirmed case of vCJD in the United States and note that, unlike for the previous 3 cases, the specific country in which this BSE/vCJD infection was acquired is less clear. We found no definite epidemiologic link to a country where other known vCJD patients probably had been infected. We also review the clinical features and diagnostic challenge of vCJD, with special consideration of the possible public health concerns and global risks inherent to this disease.

The Patient

The patient was a man in his forties who was born and raised in the Middle East. He lived in Russia for several years while completing a professional degree and briefly returned to the Middle East before taking up his final residence in the United States during the late 1990s.

Family members, friends, and co-workers were interviewed. The Texas Department of State Health Service's investigation ascertained and verified that he had resided in Lebanon, Kuwait, Russia, and the United States. The patient's family affirmed that, if the man had traveled to European countries, that travel was brief and infrequent. We found no evidence indicating that he had ever stayed in Great Britain, France, Ireland, or Saudi Arabia.

The patient's family and co-workers described him as healthy and high-functioning before illness onset. The condition first manifested in late 2012 as depression and anxiety. These symptoms were initially subtle and did not interfere with his daily activities. Delusions and hallucinations were observed shortly after the onset of illness and in subsequent months were associated with changes in behavior in the form of withdrawal, isolation, secrecy, aggression, poor judgment, and lack of insight (Figure 1).

Intermittent numbness and paresthesias of the left face and upper extremity began in the fifth month of illness. These symptoms were attributed to a motor vehicle collision within the previous weeks. A few months later, when the symptoms started to affect the contralateral side, brain and cervical spine magnetic resonance imaging (MRI) was performed and showed multilevel degenerative changes and spinal foraminal stenosis, leading to the conclusion that most of his signs and symptoms were caused by cervical disc disease. The etiology for his facial paresthesias remained unclear.

The patient's psychiatric condition gradually continued to worsen, and he was hospitalized multiple times in the 13th and 14th months after illness onset. Several diagnoses were entertained, including depression with psychotic features and bipolar disorder with psychosis. Despite treatment of symptoms, his condition continued to deteriorate.

Because of increasing agitation, the patient was once again brought to the emergency department in the 14th month after symptom onset, where the neurology service was consulted. He was restless, irritable, disinhibited, and impulsive. He exhibited choreiform movements, most pronounced in the left upper extremity, in addition to myoclonus and ataxia. Reflexes were reduced throughout the upper and lower extremities. MRI of the brain showed mild diffuse volume loss, T2 hyperintensity with subtle restricted diffusion in the pulvinar nuclei of bilateral thalami (pulvinar sign), and subtle restricted diffusion in the right frontal cortex (cortical ribbon sign) (arrows) (Figure 2). Analysis of cerebrospinal fluid (CSF) showed elevated protein at 120 mg/dL (reference range 15–60 mg/dL) and a normal cell count, prompting a course of intravenous steroids and plasmapheresis for suspected autoimmune encephalitis.

Extensive investigations for infectious and autoimmune encephalitides, malignancy, heavy metal intoxication, vitamin deficiency, and rheumatologic and endocrine disorders

were concomitantly pursued. CSF was sent to the National Prion Disease Pathology Surveillance Center (NPDPS; Cleveland, OH, USA) for evaluation of 14-3-3 and Tau protein levels, as well as real-time quaking-induced conversion (RT-QuIC) testing for prion detection (Table 1).

During the first several weeks after admission, the patient's behavior and chorea improved, which was interpreted as a possible response to immunomodulatory therapy. He therefore underwent further intravenous immunoglobulin treatment and received 2 doses of rituximab. After a few weeks of a relatively stationary course, his condition deteriorated. Dysarthria, truncal ataxia, and lower extremity weakness developed, with subsequent loss of his ability to ambulate.

After the laboratory tests for antibody-mediated autoimmune and paraneoplastic encephalidites yielded negative results, "probable vCJD" was diagnosed. The Texas Department of State Health Service and the Centers for Disease Control and Prevention (Atlanta, GA, USA) were notified of the suspected case. Samples of blood, urine, and CSF were sent to the NPDPS and the Medical Research Council Prion Unit (London, UK). Brain and tonsil biopsies were deferred because of concern that the patient would not tolerate either biopsy procedure.

By the 16th month of illness, left arm plegia and a severe bulbar palsy developed. Within 1 month, the patient became akinetic-mute and entirely bedridden. After the third episode of aspiration pneumonia, sepsis developed. After

discussion with the family, goals of care were restricted to comfort measures only. The patient died shortly thereafter, almost 18 months after initial onset of symptoms.

Results of Histopathologic Examination and Biochemical Tests

A brain-only autopsy was performed. Conventional histologic examination demonstrated numerous typical florid plaques that occasionally formed clusters and were often mixed with patches of spongiform change made of large vacuoles (Figure 3, panel A). This lesion pattern was present throughout the cerebral cortex, except for the hippocampal formation and lower temporal gyri. Basal ganglia and thalamus showed severe spongiform change with only scattered plaques. Prion plaques, but not well-formed florid plaques, were present in the granule cell layer of the cerebellum (Figure 3, panel B).

Prion protein immunostaining showed intense immunoreaction of the core of the florid plaques and patchy and granular deposits that were arranged in rounded clusters (Figure 3, panel C). In addition, the immunostaining highlighted cells with short processes stemming from the round perikaryon in a spoke wheel-like fashion (Figure 3, panel D). These cells were observed especially in the cerebral cortex and molecular layer of the cerebellum, where plaques and plaque-like deposits were also present. Clusters of intensely staining kuru plaques were seen in the granule cell layer of the cerebellum.

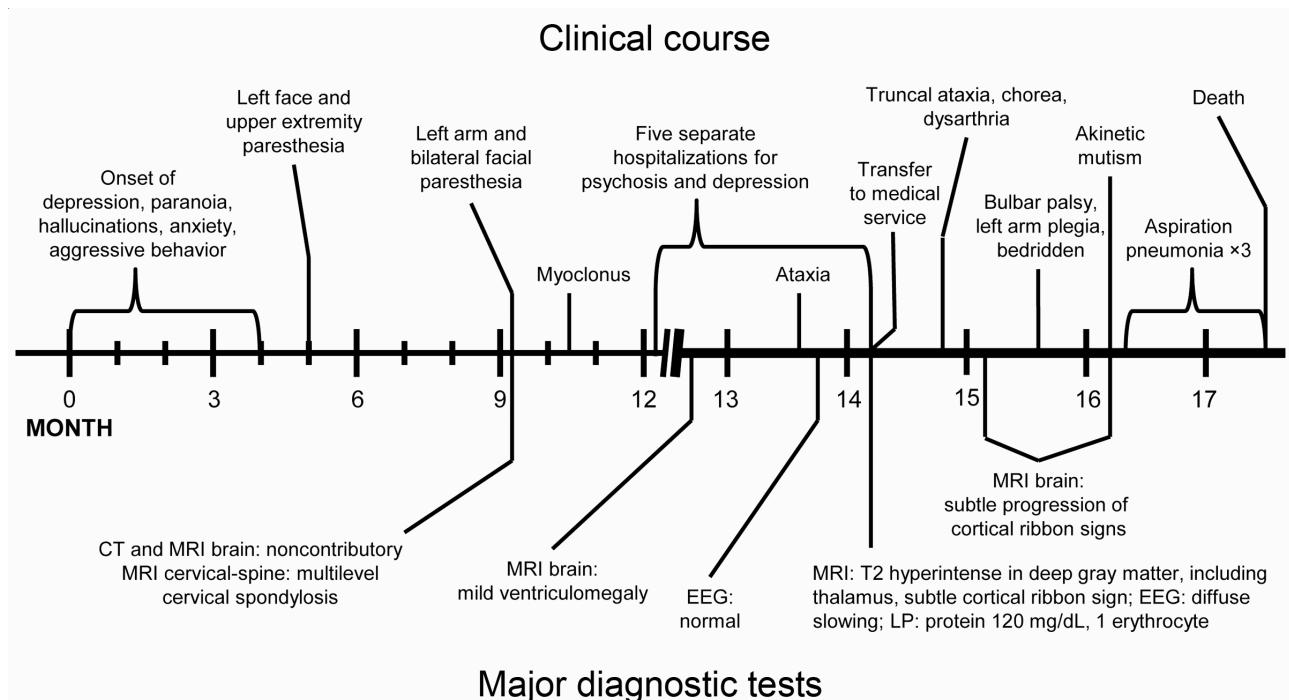


Figure 1. Timeline of course of illness and major diagnostic tests for US patient with variant Creutzfeldt-Jakob disease. CT, computed tomography; EEG, electroencephalography; MRI, magnetic resonance imaging, LP, lumbar puncture.

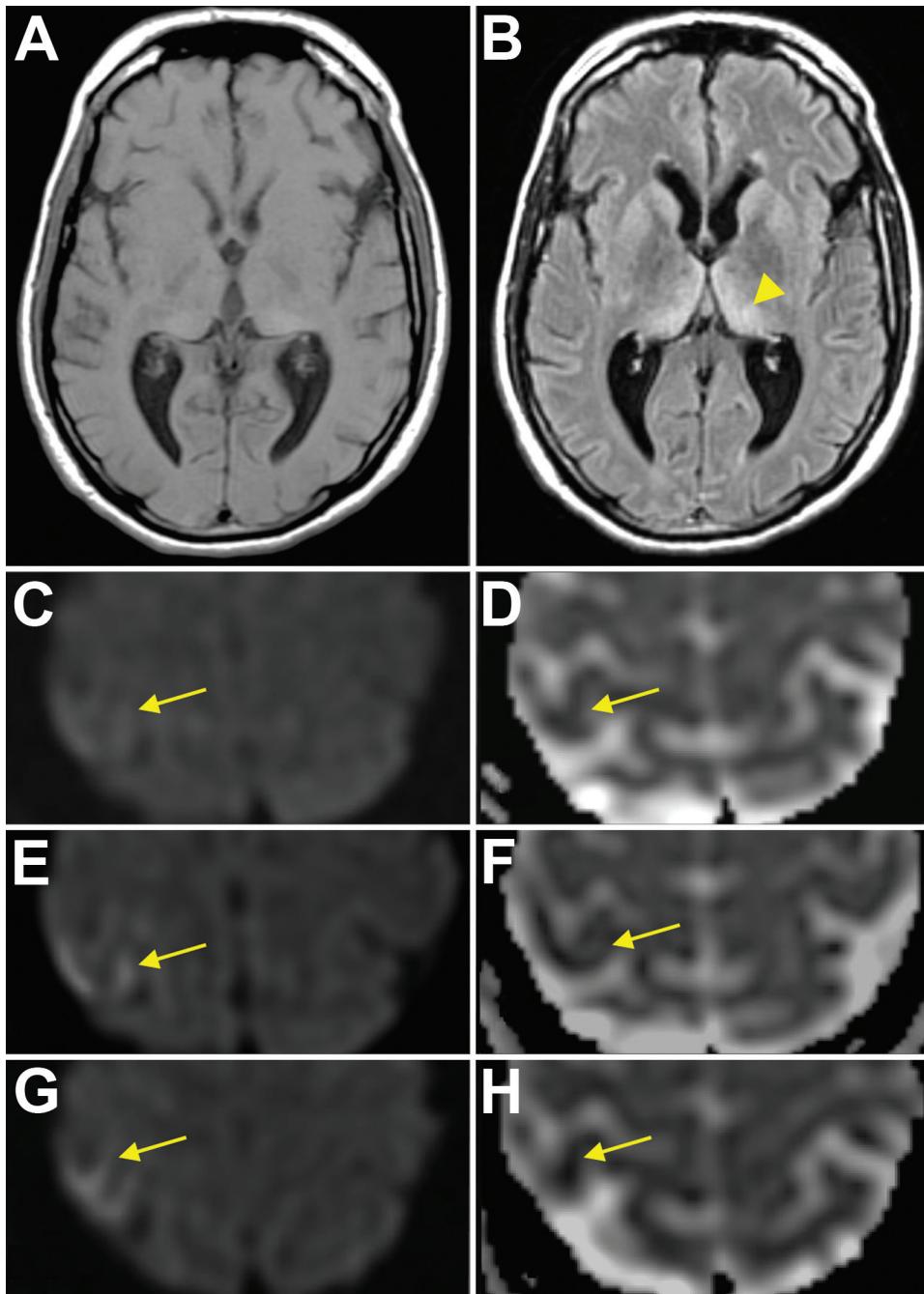


Figure 2. Magnetic resonance imaging (MRI) results for a US patient with variant Creutzfeldt-Jakob disease. T1 sequence (A) and T2 FLAIR sequence (B) show the “pulvinar” or “hockey stick” sign (arrowhead). Initial diffusion weighted imaging (DWI) (C) and apparent diffusion coefficient (ADC) (D) images show subtle restricted diffusion in the right primary motor cortex (arrows). Subsequent MRIs show increasing hyperintensity (arrows) on DWI (E, then G) and further attenuation (arrows) on ADC (F, then H), consistent with the “cortical ribbon” sign.

Western blot showed the typical electrophoretic pattern of the protease-treated scrapie prion protein (PrP^{Sc}) associated with vCJD. This pattern, commonly identified as 2B, is characterized by the 19 kDa molecular weight of the unglycosylated band of PrP^{Sc} after proteinase K digestion, unlike type 1, in which the size of the unglycosylated band is 21 kDa (Figure 4, panel A). In addition, the distribution of the PrP^{Sc} glycoforms differs; the diglycosylated form is the predominant form in vCJD, whereas the most represented band in sCJD is the monoglycosylated form.

Urine samples from this patient were collected before death and subjected to protein misfolding cyclic amplification (PMCA) analysis. Samples were tested twice, and both times the assay results were positive (Figure 4, panel B). Therefore, on the basis of histopathologic and biochemical analysis, the diagnosis of vCJD in the present case is definitive.

Discussion

When a rare disease manifests with a common symptom, that makes the diagnosis even more difficult. Unlike sCJD,

which often presents with the relatively rare symptom of rapidly progressing dementia, vCJD usually presents with psychiatric symptoms, which are relatively common. Because of these difficulties, diagnosis is usually delayed. Development of other neurologic manifestations (e.g., chorea,

ataxia) supports a diagnosis of sCJD or vCJD. Painful sensory complaints can be a particularly helpful clue pointing to vCJD because they are generally not reported with sCJD. In addition, vCJD onset occurs at a younger age than does sCJD (mean 26 vs. 65 years), and disease course of vCJD

Table 1. Relevant studies for the diagnosis of vCJD, United States*

Test†	Result (reference range)
Blood‡	
Albumin, g/dL	2.3–4.1 (3.4–5.0)
Ammonia, µmol/L	31 (11–32)
Anti-thyroglobulin antibody, IU/mL	<1.0 (0.0–0.9)
Anti-thyroid peroxidase antibodies, IU/mL	5 (0–34)
Ceruloplasmin, mg/dL	32.5 (20.0–60.0)
Creatine kinase, U/L	39–257 (0.6–1.3)
C-reactive protein, mg/dL	0.098 (0.0–0.3)
Erythrocyte sedimentation rate, mm/h	7 (0–15)
Ethanol level, g/dL	Undetectable (0.0–0.08)
Hemoglobin A1c, %	5.0 (4.3–6.1)
Protein, g/dL	5.1–7.8 (6.4–8.2)
Rapid plasma reagin	Nonreactive
Toxoplasma IgG, IU/mL; IgM, AU/mL	<3.0, <3.0 (<5.9, <7.9)
Thyroid stimulating hormone, U/mL	1.78 (0.36–3.74)
Vitamin B1, nmol/L	254.5 (66.5–200.0)
Vitamin B12, pg/mL	597–926 (211–911)
Cerebrospinal fluid§	
Electrophoresis	No oligoclonal bands
Glucose, mg/dL	46–53 (50–80)
Protein, mg/dL	90.0–204.2 (15–45)
Erythrocytes, cells/µL	1–2 (0)
Leukocytes, cells/µL	1–3 (0–5)
Angiotensin converting enzyme, U/L	1.5 (0.0–2.5)
Epstein-Barr virus PCR	Not detected
Herpes simplex viruses 1 and 2	Not detected
VDRL	Nonreactive
Stain	No organisms found
Culture	No growth
Other diagnostic tests	
Electroencephalography x3	Mild to moderate diffuse slowing; no epileptiform activity
MRI brain	14 mo after initial symptoms: bilateral T2 hyperintensities in the thalamic pulvinar nuclei and, to a lesser extent, in the caudate and lentiform nuclei. Subtle cortical ribbon sign over the right motor cortex At 15 mo: persistent pulvinar and cortical ribbon sign; resolution of caudate and lentiform nuclei T2 hyperintensities At 16 mo: persistent pulvinar and cortical ribbon sign, with interval development of subtle T1 hyperintensities
MRI, C/T/L-spine	Cervical and lumbar spondylosis; no cord compression
CT angiogram head and neck	No intracranial vascular abnormalities; mild large vessel atherosclerotic disease without significant stenosis
CT chest/abdomen/ pelvis; scrotal ultrasound:	No malignancy detected
CJD-specific laboratory tests	
Blood	
PRNP genotype	Codon 129 methionine homozygous; otherwise no mutations
Direct detection assay	Negative
Urine PMCA	Positive for scrapie prion protein
Cerebrospinal fluid	
14-3-3 protein	Negative
Tau protein, pg/mL	358, negative
RT-QuIC	Negative

*Includes tests that rule out vCJD mimics. Routine serum electrolytes and cell counts were otherwise normal. CJD, Creutzfeldt-Jakob disease; CT, computed tomography; MRI, magnetic resonance imaging; PMCA, protein misfolding cyclic amplification; RT-QuIC, real-time quaking-induced conversion; vCJD, variant CJD; VDRL, Venereal Disease Research Laboratory.

†Urinalyses were negative for heavy metals, drug toxicity, and copper.

‡Blood tests for antinuclear antibody, antineuronal nuclear antibody (ANNA1, Anti-Hu antibody), anti-Purkinje cell antibody (anti-Yo antibody), anti-smooth muscle/ribonucleoproteins; anti-Sjögren's-syndrome-related antigen A, and anti-Sjögren's-syndrome-related antigen B, *Aspergillus* antibody, *Blastomyces* antibody, *Coccidioides* antibody, hepatitis panel, HIV-1/HIV-2, and *Borrelia burgdorferi* PCR were all negative.

§Negative for cryptococcal antigen; IgM against West Nile virus, St. Louis encephalitis virus, California encephalitis virus, eastern equine encephalitis, western equine encephalitis virus, and West Nile virus; fluorescent treponemal antibody; and autoimmune/paraneoplastic panel (Dalmau).

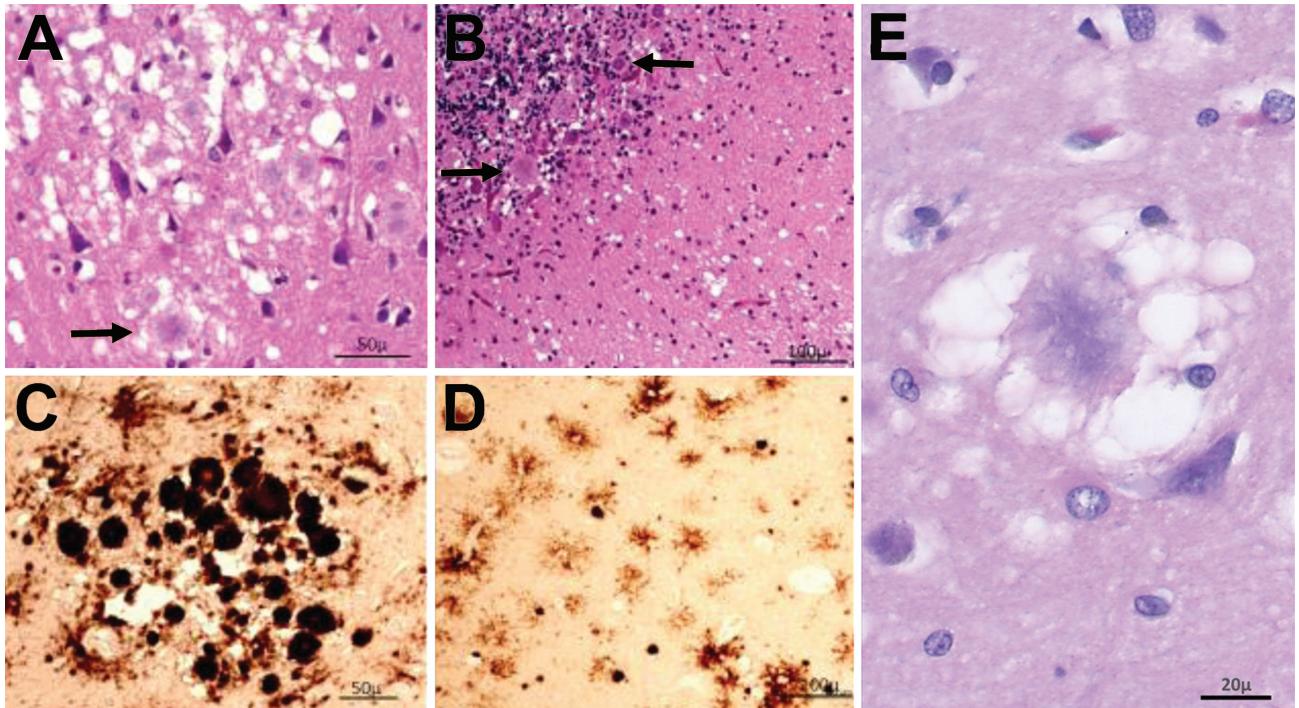


Figure 3. Results of histopathologic and immunohistochemical analyses for a US patient with variant Creutzfeldt-Jakob disease. A) Hematoxylin and eosin staining shows many typical florid plaques (arrow) occasionally forming clusters; large vacuole spongiform change is also present (original magnification $\times 10$). B) Plaques often not of the florid type along with spongiform change are present in cerebellum (arrows; original magnification $\times 20$). C, D) Prion protein immunostaining confirms the presence of PrP in the plaques, which intensely immunostained (C; original magnification $\times 10$), and highlights small round cells surrounded by short processes (D, antibody 3F4; original magnification $\times 40$). E) Under high magnification, a prion plaque with spongiform change is seen in the left frontal cortex (original magnification $\times 400$).

is longer than that of sCJD (mean 14 months vs. 4 months) (7,8). Despite the statistical differences in onset and duration, the distinction between sCJD and vCJD is not always clear (9). In 1 study, 13 sCJD patients with psychiatric presentation had clinical characteristics more similar to vCJD than sCJD (younger age at onset and longer survival), and depression was the most common presenting symptom (10). Regardless, in the patient reported here, clinical onset and duration of illness were typical of vCJD. The clinical differentiation between sCJD and vCJD is reflected by the World Health Organization diagnostic criteria (7,8,11,12) (Table 2).

Three well-known diagnostic tests can point the clinician to sCJD: MRI (diffusion restriction and T2 hyperintensity of the cortex and basal ganglia), electroencephalography (periodic sharp wave complexes), and CSF analysis (elevated 14-3-3/Tau levels) (13). In contrast, for vCJD, electroencephalography and CSF analysis do not show any specific changes. The only positive finding of diagnostic value in vCJD from these tests is the pulvinar sign on MRI, which is not entirely specific for vCJD and can be absent in up to 9% of cases, even after multiple MRIs (14). According to World Health Organization criteria, the only way to

diagnose “definite vCJD” while the patient is living is a brain biopsy (15). In contrast to sCJD, lymphoid tissue in vCJD has a high detection rate of PrP^{Sc} (16), so tonsil biopsy has become an alternative to brain biopsy, with recent investigations showing high sensitivity and specificity (17).

The panel of diagnostic tests for CJD used for many years at the NPDPSA includes level determinations of 14-3-3 and Tau proteins in the CSF. These tests are highly sensitive for sCJD, the most common form of human prion disease, but not for vCJD. A negative 14-3-3 has a negative predictive value of 63%, and a negative Tau has a negative predictive value of 81% (18). If both tests are negative, the negative predictive value for vCJD rises to 84% (18), which leaves ≈ 3 of 20 patients with a false-negative result, as in the patient reported here.

RT-QuIC, a relatively new test available at the NPDPSA, can detect minute amounts of prions in CSF by amplifying PrP^{Sc} using recombinant PrP as substrate. If positive, RT-QuIC is very specific for diagnosing sCJD, but it has been reported to be negative in vCJD (3), as in the CSF sample of the patient reported here.

Analysis of the PrP gene coding regions demonstrating the presence of methionine homozygosity at codon 129 has

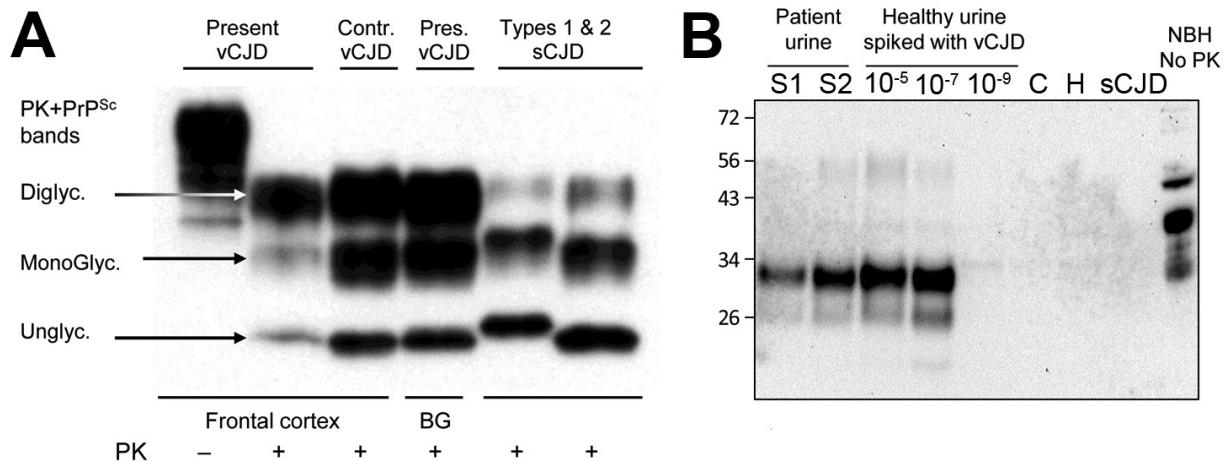


Figure 4. Results of biochemical testing of a US patient with variant Creutzfeldt-Jakob disease (vCJD). **A**) Immunoblot of vCJD patient and controls. All the PK-treated preparations show similar electrophoretic profiles characterized by 3 bands displaying different mobilities according to number of the linked sugar moieties. The samples from this case and another vCJD case used as positive control (third lane) show the overrepresentation of the diglycosylated band, whereas in sCJD cases, the monoglycosylated band is the most prominent. In both vCJD cases, the unglycosylated band co-migrates with type 2 (sixth lane) as indicated by the type 1 and 2 controls. The PK-untreated preparation (first lane) is used as control of PK digestion. Total brain homogenate, antibody 3F4. **B**) PrP^{Sc} detection in urine by protein misfolding cyclic amplification (PMCA). A urine sample from the patient was processed as previously described (6), and the supernatant fraction was run in duplicate (S1, S2). Samples were subjected to 96 PMCA cycles in the presence of 10% TgHuM brain homogenate, used as the substrate for PMCA. PrP^{Sc} signal was assessed by Western blot after PK digestion. As a positive control, vCJD brain homogenate was spiked at 10⁻⁵, 10⁻⁷, and 10⁻⁹ dilutions into urine from a healthy person and processed at the same time and in the same manner for PMCA. Lane C, PMCA-negative control (no sample); lane H, urine from a healthy person; sCJD, urine from an sCJD patient. CJD, Creutzfeldt-Jakob disease; Contr, control; MonoGlyc, monoglycosylated; NBH, normal brain homogenate without PK treatment used as an electrophoretic migration marker; Pres, present; PK, proteinase K; PrP^{Sc}, scrapie prion protein; sCJD, sporadic CJD; Unglyc, unglycosylated.

been found in all cases of proven primary vCJD examined (3,19), including in the case reported here. However, because >40% of the population can be methionine homozygous, sequencing of PrP gene coding regions is helpful only in providing supportive evidence for increased susceptibility to prion disease (20).

One experimental test for vCJD is a blood-based direct detection assay that takes advantage of the high affinity of PrP^{Sc} for metal surfaces to capture and immunodetect the minute amounts of PrP^{Sc} expected to be present in the blood of vCJD patients (21). Application of this test on specimens collected from >5,000 persons did not indicate false-positive findings; however, the sensitivity reached only 71% (22) and was negative in the patient in our report.

Another new experimental test is based on PrP^{Sc} detection in urine. This test uses PMCA technology to amplify and then visualize traces of PrP^{Sc} that might be present in the patient's urine and has nearly 93% sensitivity and 100% specificity (6). Although the high accuracy of the assay might decrease when larger cohorts are examined, this entirely noninvasive test is likely to be useful in screening for vCJD. This test was the only one conducted on a sample obtained before the death of the patient reported here that yielded a positive result. PrP^{Sc} was detected in this patient after only 1 round of 96 PMCA cycles, indicating that

the amount of PrP^{Sc} in urine was higher than that in most of the other vCJD patients analyzed (6).

The neuropathology of vCJD is distinct from that of sCJD, and the classification system for neuropathologic subtypes of sCJD has been described in detail elsewhere (23–25). The diagnosis of vCJD was definitively confirmed in this case by the postmortem histologic and PrP^{Sc} examinations, which demonstrated the widespread presence of florid plaques, the typical electrophoretic profile of the PrP^{Sc} (3), and the PrP immunostaining demonstrating rounded cells surrounded by short delicate processes resulting in a feathery appearance (26). The identity of these cells remains to be determined.

This case highlights several diagnostic challenges presented by vCJD. Studies on a vCJD cohort from the United Kingdom have shown a mean interval of 2.5 months between clinical onset and the first medical examination. The overall average delay between onset and suspicion of vCJD is 8.9 months (7). This case met similar delays, despite the typical clinical presentation (3).

The significantly elevated protein concentration in the CSF in this patient, up to 204 mg/dL, was a confounder. CSF protein in vCJD is usually normal; the highest concentration previously reported was 90 mg/dL (19). We remain unsure about the source of this finding. The cortical ribbon

Table 2. World Health Organization diagnostic criteria for vCJD and sCJD*

Diagnosis	vCJD	sCJD
Possible	Progressive psychiatric disorder lasting >6 mo with no alternate explanation; at least 4 of the following: early psychiatric symptoms, persistent pain and/or dysesthesia, ataxia, chorea/ dystonia/myoclonus, and dementia; EEG without periodic sharp wave complexes typical for sCJD	Progressive dementia <2 y duration (typically <6 mo); at least 2 of the following: myoclonus, visual or cerebellar disturbance, pyramidal or extrapyramidal dysfunction, akinetic mutism; EEG atypical (not showing periodic sharp wave complexes) or not done
Probable	Meets criteria for possible vCJD plus: EEG not consistent with sCJD; and bilateral pulvinar high signal on MRI of brain OR progressive psychiatric disorder lasting >6 mo with no alternate explanation; and positive tonsil biopsy	Meets criteria for possible sCJD plus: Typical EEG findings (generalized periodic sharp wave complexes at ≈ 1 Hz); and/or positive 14-3-3 assay in CSF and clinical duration leading to death in <2 y
Definite	Neuropathologic confirmation of vCJD	Neuropathologic confirmation of sCJD

*See (11,13). CJD, Creutzfeldt-Jakob disease; CSF, cerebrospinal fluid; EEG, electroencephalography; MRI, magnetic resonance imaging; sCJD, sporadic CJD; vCJD, variant CJD.

sign on MRI, although a common finding in sCJD, was also not previously reported in vCJD (14).

Another major difficulty in this case was proving the diagnosis with a high level of certainty before death so the goals of care could be adjusted accordingly. Despite our clinical suspicion, results of all the initial laboratory tests were negative. The pulvinar sign, which is relatively sensitive and specific for vCJD (14), did not exclude other disorders that rarely have similar features, including Wernicke's encephalopathy and inflammatory limbic encephalitis (27–29). By the time brain or tonsil biopsy was considered, the patient was considered unable to tolerate the procedure. The only positive test specific for vCJD in this patient was PrP^{Sc} detection in urine using the PMCA test. Unfortunately, the result became available only after his death.

Perhaps the most challenging aspect of this case is identifying the geographic location of the patient's exposure to prions. The patient, despite being a US citizen, was born and raised outside of the Americas in 3 countries that were importing UK beef at a time (during 1980–1996) when it was at increased risk for BSE contamination. According to the 2010 US Census, US citizens born outside the Americas constituted <5% of the US population, and not all of this small subgroup would have resided in the United Kingdom or in countries that had imported UK beef during the increased risk period, a residency history common to the 5 previously identified North American vCJD patients (30).

Exportation of BSE-contaminating beef from the United Kingdom during 1980–1996 and/or BSE-infected live cattle during 1980–1990 most likely is the main cause of BSE exposure outside the United Kingdom (4). During 1980–1996, the patient in our report spent >6 years in 2 countries—Kuwait and Russia—to which UK beef was exported. During that time, he also lived <1 year in Lebanon. Although the size of the Kuwaiti population was a few magnitudes smaller, based on UK export data for 1980–1996, the same order of magnitude ($\approx 2.5 \times 10^3$ metric tons) of beef was exported to Kuwait and to the Soviet Union/Russia

when this patient resided in those 2 countries (4; Her Majesty's Revenue and Customs, Overseas Trade Statistics, Crops & Trade Branch, Analysis and Evidence Team, unpub. data). These data suggest that the patient's risk of eating possibly BSE-contaminated UK beef would have been substantially greater during his stay in Kuwait than during his stay in Russia. The risk that he ate such meat was lower in Lebanon than in Russia, primarily because of his relatively short stay in Lebanon during 1980–1996 and the fact that >85% of the 1980–1996 UK beef was imported to Lebanon after he moved to Russia.

This patient lived in the United States for 14 years before vCJD developed. A >14-year incubation period can be consistent with the mean incubation period for vCJD: 11.6–16.7 years, estimated by published models of the vCJD epidemic (31–33). Each of these models predicts a skewed curve toward much longer incubation periods than the mean as the vCJD epidemic wanes over time, similar to other human prion disease outbreaks (31,34,35). In addition, each of these models reflects a strikingly younger distribution of vCJD patients in the United Kingdom and supports the published concept that susceptibility to the prion infection that will cause vCJD peaks among adolescents and declines rapidly with age thereafter (36). Given that this patient did not come to the United States until his late 20s, this age-dependent susceptibility factor favors a conclusion that he was infected before he moved to the United States.

We deemed surgical or transfusion routes of vCJD transmission to or from the patient to be unlikely because the only known surgical procedure he had undergone was a circumcision performed in Kuwait when he was ≈ 8 –10 years of age, and he had no history of receiving or donating blood. A review of records at the Gulf Coast Regional Blood Center and the American Red Cross provided further evidence that he had not been a US blood donor.

Given the markedly declining incidence of vCJD globally, this patient is only the fourth patient worldwide confirmed to have this disease since the beginning of 2012; the other 3 were from the United Kingdom and France (3). This

case underscores that the diagnosis of vCJD should not be dismissed if the patient has not resided in a country with a known endemic case of vCJD. Given the several decades' long potential incubation periods estimated from epidemiologic modeling, the international occurrence of additional vCJD cases can be reasonably anticipated (34,35).

Furthermore, UK surveys of archived appendix tissues indicate an approximate prevalence of asymptomatic vCJD infection of 1 in 2,000 persons born during 1941–1985 (37). Depending on genetic subtype, one may harbor the pathogenic prion and never develop symptoms (3). However, because the agent is transmissible through blood transfusions, organ transplants, and surgical instrumentation, iatrogenic propagation of the disease remains a real possibility (3).

The detection of a definitive case of vCJD in the United States highlights the importance of continuing enhanced national human prion disease surveillance. According to the World Organisation for Animal Health, the BSE status of the 3 countries where the patient reported here resided during the critical exposure period was “undetermined,” suggesting the lack of a systematic BSE surveillance system.

The potential difficulty in making the clinical diagnosis in many patients with vCJD and the delay with which the disease is first suspected raises the concern that vCJD can be missed. The need for neuropathology expertise and advanced neuropathologic techniques is probably an important limiting factor in some parts of the world. The MRI examination, along with the newly developed blood and urine tests, are among the most helpful premortem tests to diagnose vCJD. A postmortem brain and lymphoreticular tissue autopsy examination remains critical to confirm the diagnosis.

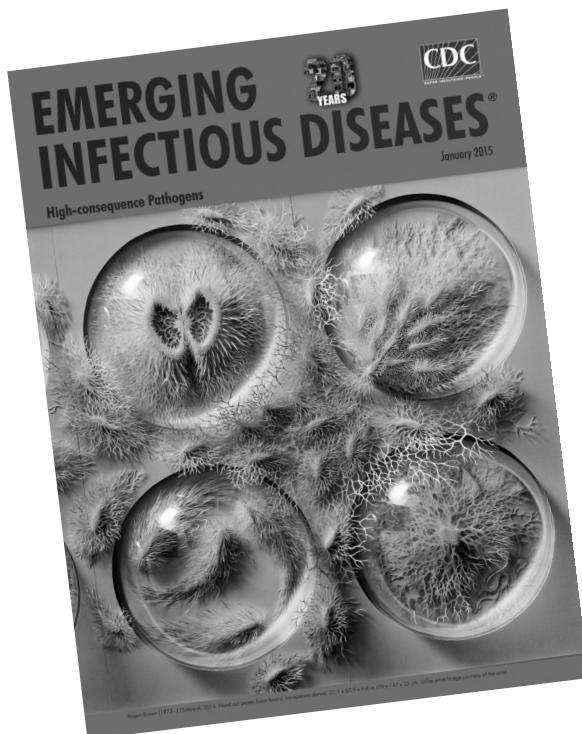
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Novel Thogotovirus Associated with Febrile Illness and Death, United States, 2014

Olga I. Kosoy, Amy J. Lambert, Dana J. Hawkinson, Daniel M. Pastula, Cynthia S. Goldsmith, D. Charles Hunt, J. Erin Staples

A previously healthy man from eastern Kansas, USA, sought medical care in late spring because of a history of tick bite, fever, and fatigue. The patient had thrombocytopenia and leukopenia and was given doxycycline for a presumed tickborne illness. His condition did not improve. Multiorgan failure developed, and he died 11 days after illness onset from cardiopulmonary arrest. Molecular and serologic testing results for known tickborne pathogens were negative. However, testing of a specimen for antibodies against Heartland virus by using plaque reduction neutralization indicated the presence of another virus. Next-generation sequencing and phylogenetic analysis identified the virus as a novel member of the genus *Thogotovirus*.

The genus *Thogotovirus* (family *Orthomyxoviridae*) contains ≥ 6 distinct viruses, including Araguari, Aransas Bay, Dhori, Jos, Thogoto, and Upolu viruses (1–3). These viruses have been primarily associated with either hard or soft ticks and have a wide geographic distribution (1–8). The only virus in this genus known to occur in the United States is Aransas Bay virus, which was isolated from soft ticks (*Ornithodoros* spp.) collected from a seabird nest off the coast of Texas (3).

Two viruses in the genus *Thogotovirus* (Thogoto and Dhori viruses) are currently known to cause human infection and disease. Antibodies against Thogoto virus have been identified in humans living in parts of Europe, Asia, and Africa (1,4,6,8). Two persons from Nigeria infected with this virus were identified in 1966. The first patient was a man with a febrile illness in whom neuromyelitis optica later developed. The second patient was a 14-year-old boy in whom meningitis developed and who died 6 days later because of complications of sickle cell disease (9).

Antibodies against Dhori virus in humans have been reported in a similar distribution as those against Thogoto virus (1,6,8,10). Five patients with disease have been described after accidental laboratory exposure to Dhori virus; encephalitis developed in 2 of these patients (11). We report a novel Thogotovirus associated with a febrile illness and death that occurred in a man in the United States in 2014.

The Case-Patient

The patient was a previously healthy man >50 years of age from Bourbon County, Kansas, USA. While working outdoors on his property in late spring 2014, the patient had several tick bites and found an engorged tick on his shoulder several days before he became ill with nausea, weakness, and diarrhea. The following day, a fever, anorexia, chills, headache, myalgia, and arthralgia developed. On the third day of illness, the patient went to his primary care physician, who empirically prescribed doxycycline for a presumed tickborne illness because of his history of tick bites, symptoms, and no reported travel outside the immediate area. The following morning, the patient's wife found him obtunded (experiencing reduced consciousness) but arousable. He was taken by ambulance to a local hospital.

At the hospital, he had a temperature of 37.3°C, a pulse rate of 84 beats/min, and an increased blood pressure of 151/65 mm Hg. The patient had a papular rash on his trunk, but otherwise results of his physical examination were unremarkable. Initial laboratory findings showed leukopenia (2,200 cells/ μ L), lymphopenia (absolute lymphocyte count 550 cells/ μ L), thrombocytopenia (72,000 cells/ μ L), mild hyponatremia (sodium 133 mmol/L), hypokalemia (potassium 3.0 mmol/L), a creatinine level (0.8 mg/dL) within the reference range (0.6 mg/dL–1.2 mg/dL), a slightly increased level of blood urea nitrogen (25 μ g/dL), and increased levels of aspartate aminotransferase (138 U/L) and alanine aminotransferase (86 U/L). He was admitted because of the principal problems of dehydration, syncope, and possible tickborne illness. He was given an intravenous (IV) fluid bolus, then maintenance fluids, and doxycycline (200 mg IV every 12 h for the first 24 h, then 100 mg IV every 12 h).

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Despite doxycycline therapy, the patient continued to report malaise and anorexia, and periodic fevers (maximum temperature 38.8°C) developed. At day 8 postillness onset, the patient was transferred to a tertiary care center for further evaluation and management. Patient samples collected before transfer showed no serologic evidence of Rocky Mountain spotted fever, Lyme disease, or ehrlichiosis.

At initial assessment at the tertiary care center, the patient was febrile (temperature 39.4°C) and had a nontender left axillary lymphadenopathy; a diffuse maculopapular rash on his chest, abdomen, and back; petechiae on his soft palate and lower extremities; and bibasilar crackles in the lung fields. Laboratory testing continued to show mild leukopenia (3,600 cells/ μ L) but also showed worsening thrombocytopenia (34,000 cells/ μ L). His renal function was normal, but his aspartate aminotransferase level had increased to 119 U/L. Doxycycline treatment (100 mg IV every 12 h) was continued, and the patient was evaluated further for a potential etiology of his illness.

Hematologic results suggested that his persistent thrombocytopenia and leukopenia were secondary to acute bone marrow suppression. A chest, abdomen, and pelvis computed tomography scan with contrast showed trace pleural effusions, bibasilar atelectasis, and multiple prominent abdominal lymph nodes. At day 9 postillness onset, he remained lucid and interactive, but he continued to have episodic high fever (temperature >39°C) and progressive dyspnea developed, which resulted in a need for supplemental oxygen. A chest radiograph showed new findings of pulmonary venous congestion and interstitial edema, suggestive of progressive heart failure or fluid overload, and an echocardiogram showed global hypokinesis.

Because of increasing supplemental oxygen needs and progressive lactic acidosis, he was transferred to the intensive care unit and given broad-spectrum antimicrobial drugs on day 10 of his illness. His renal function began to deteriorate and his aminotransferase levels continued to increase. The patient was intubated because of acute respiratory distress syndrome and was given 3 vasopressor medications because of shock. The patient subsequently had sustained ventricular tachycardia with persistent hypotension and eventual pulseless electrical activity with refractory shock. After multiple resuscitations, the decision was made to withdraw further care, and he died shortly after being extubated, 11 days after first becoming ill. An autopsy was not performed.

Results of comprehensive evaluations for tickborne diseases, including serologic testing for Rocky Mountain spotted fever, tularemia, brucella, babesiosis, and Q fever; molecular testing for *Ehrlichia* spp. and *Anaplasma phagocytophilum*; and blood thin smears for *Babesia* spp. were negative. Results of evaluations for fungal pathogens (*Aspergillus* spp. galactomannan, antibodies against

Histoplasma spp., and *Histoplasma* spp. antigen in serum and urine) were negative. Evaluations for cytomegalovirus, Epstein-Barr virus, and parvovirus showed past infection. Test results for hepatitis B and C viruses, West Nile virus, and HIV were also negative. Blood, sputum, and urine bacterial cultures were negative. A whole blood specimen collected 9 days after illness onset was sent to the Centers for Disease Control and Prevention (CDC) (Fort Collins, CO, USA) for Heartland virus testing as part of an active institutional review board-approved protocol.

Materials and Methods

Clinical Specimen Handling and Evaluation

At CDC, EDTA-treated blood, along with serum separated from that blood, were tested for Heartland viral RNA and neutralizing antibodies by real-time reverse transcription PCR (RT-PCR) and plaque reduction neutralization test (PRNT) with 6-well plates with confluent Vero E6 monolayers, according to protocols described elsewhere (12,13). Standard virus isolation methods were also used. In brief, 200 μ L of undiluted and 1:10 dilutions of blood or serum specimens were inoculated onto confluent Vero cells in T25 flasks. Inoculated flasks were then incubated at 37°C and reviewed for cytopathic effect daily.

Viral Genome Sequencing, RT-PCR, and Phylogenetic Analyses

Supernatants collected from standard virus isolation cell cultures were subjected to next-generation sequencing (NGS) methods by using the Ion Torrent PGM sequencer (Life Technologies, Grand Island, NY, USA) and methods as described (14). After novel viral sequences were identified by NGS, a real-time RT-PCR was designed to target the newly derived sequences and applied to blood and serum samples by using methods described (15). Phylogenetic analyses were conducted on deduced amino acid sequences from multiple genomic segments of selected viruses of the same viral family by using MEGA 5.05 software (<http://www.megasoftware.net/>) as described (16).

Results

Isolation and Identification of Virus in Blood and Serum

Blood and serum showed negative results for Heartland viral RNA and antibodies against this virus. However, heterologous viral (non-Heartland viral) plaques were noted in PRNT cell culture wells, which indicated the presence of another virus (Figure 1). Standard virus isolation methods showed a substantial cytopathic effect at day 3 postinoculation in cells that were inoculated with blood or serum specimens. These findings were confirmed by repeated isolation attempts.

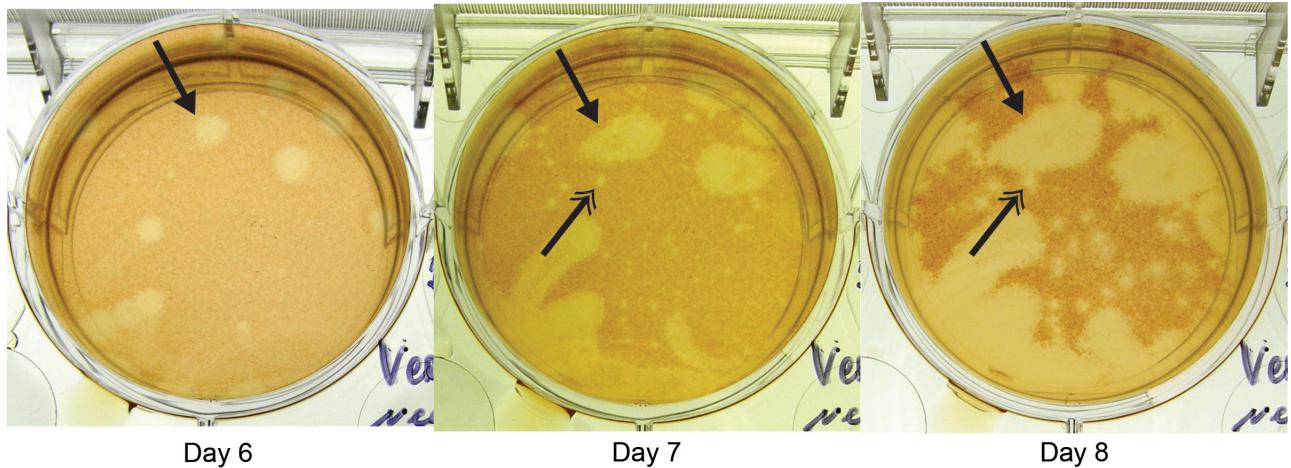


Figure 1. Plaque reduction neutralization test of patient sample for Heartland virus, showing images of the same well obtained days 6, 7, and 8 postinoculation at a dilution of 1:20. Arrows with single heads indicate appearance of a novel virus plaque beginning at day 6. Arrows with double heads indicate development of a typical Heartland virus plaque, apparent on day 7 and more evident on day 8, generated from a control strain added to each well in defined quantities to identify Heartland virus-specific antibodies in the patient sample.

Negative stain and thin-section electron microscopy showed pleomorphic viral particles consistent with viruses in the family *Orthomyxoviridae* (Figure 2). NGS methods applied to cell culture supernatants from multiple

isolations showed the presence of novel orthomyxoviral RNA. We observed $\approx 70\%$ overall average nucleotide sequence percentage identity with Dhori virus in multiple genomic segments. Blood and serum samples were verified as

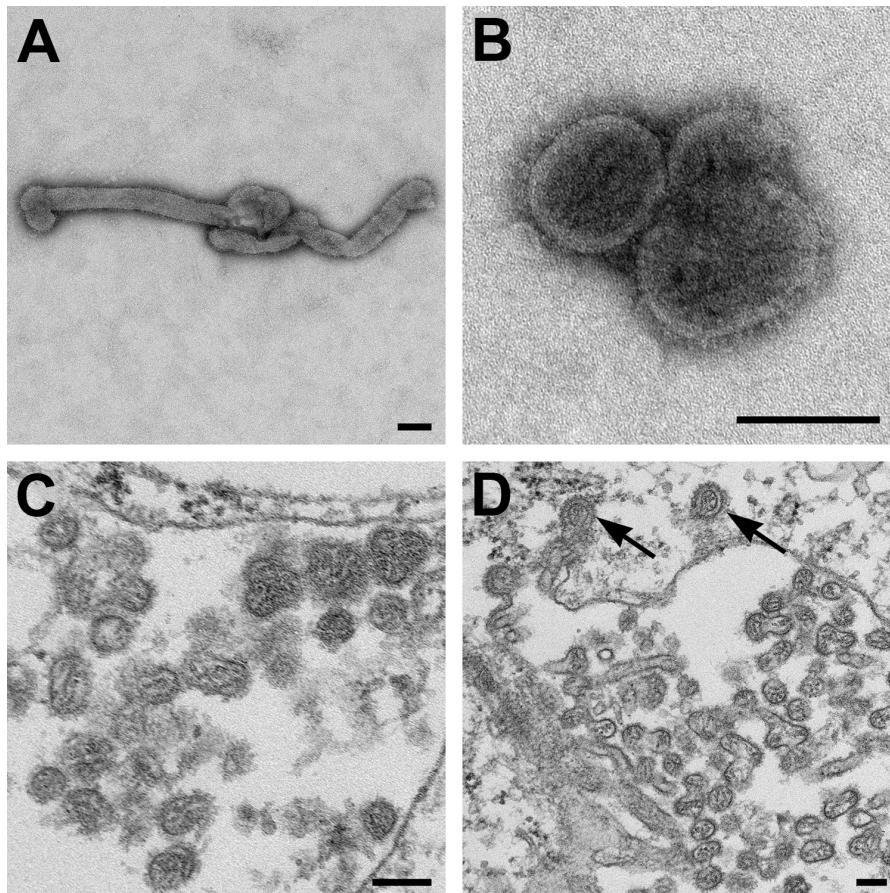


Figure 2. Electron microscopic images of novel Thogotovirus isolate. Filamentous (A) and spherical (B) virus particles with distinct surface projection are visible in culture supernatant that was fixed in 2.5% paraformaldehyde. Thin-section specimens (C and D), fixed in 2.5% glutaraldehyde, show numerous extracellular virions with slices through strands of viral nucleocapsids. Arrows indicate virus particles that have been endocytosed. Scale bars indicate 100 nm.

the source of the novel virus by real-time RT-PCR–based detection of viral RNA in these samples.

Phylogenetic Analyses

Three phylogenies, each generated by a neighbor-joining method applied with 2,000 bootstrap replicates for grouping analysis, were chosen as representative of overall genetic relationships of selected viruses (Figure 3). The novel virus was found to group with strong support along with Dhori virus, and the closely related Batken virus, in all trees.

Discussion

Using traditional techniques (i.e., PRNT and culturing on animal cells) in combination with NGS, we isolated a novel virus from a blood sample collected 9 days after illness onset from a previously healthy man. It is likely that this novel Thogotovirus, which we are proposing to call Bourbon virus after the county of residence of the patient, was the cause of his illness. Although it is unclear what role the virus played in the death of the patient, the high level of viremia, as shown by multiple isolations from the blood of the patient 2 days before his death, suggests that this might have contributed to the death of the patient.

The patient had a history of tick exposure, as well as symptoms and laboratory findings (i.e., leukopenia and thrombocytopenia) consistent with a tickborne illness. Several tickborne pathogens, such as *Ehrlichia chaffeensis*, *Rickettsia*, and Heartland virus, are present in eastern Kansas and adjacent areas (17–19). However, the patient did not respond to doxycycline therapy initiated 3 days after illness onset and had negative results for these and other tickborne pathogens.

Of the 7 symptomatic human infections that have been associated with viruses in the genus *Thogotovirus*, most case-patients have had neurologic findings (e.g., meningitis, encephalitis) without any described abnormalities in blood counts (9,11). Although cerebrospinal fluid was not tested for the patient reported, his clinical signs and symptoms were not suggestive of neurologic infection. Furthermore, the patient did not have any respiratory symptoms that would be expected with other viruses that are known human pathogens in the large family of *Orthomyxoviridae*, such as influenza virus (1).

Phylogenetic analyses indicated that Bourbon virus is most closely related to Dhori and Batken viruses. However, the branch lengths suggest a relatively distant evolutionary distinction of Bourbon virus from Dhori and Batken viruses, which have only been described in the Eastern Hemisphere. Dhori, Batken, and Thogoto viruses have been identified in various hard tick species (1). However, Batken virus also has been identified in mosquitoes (1). It is currently unknown how Bourbon virus is transmitted to humans. However, illness onset of the patient in late spring

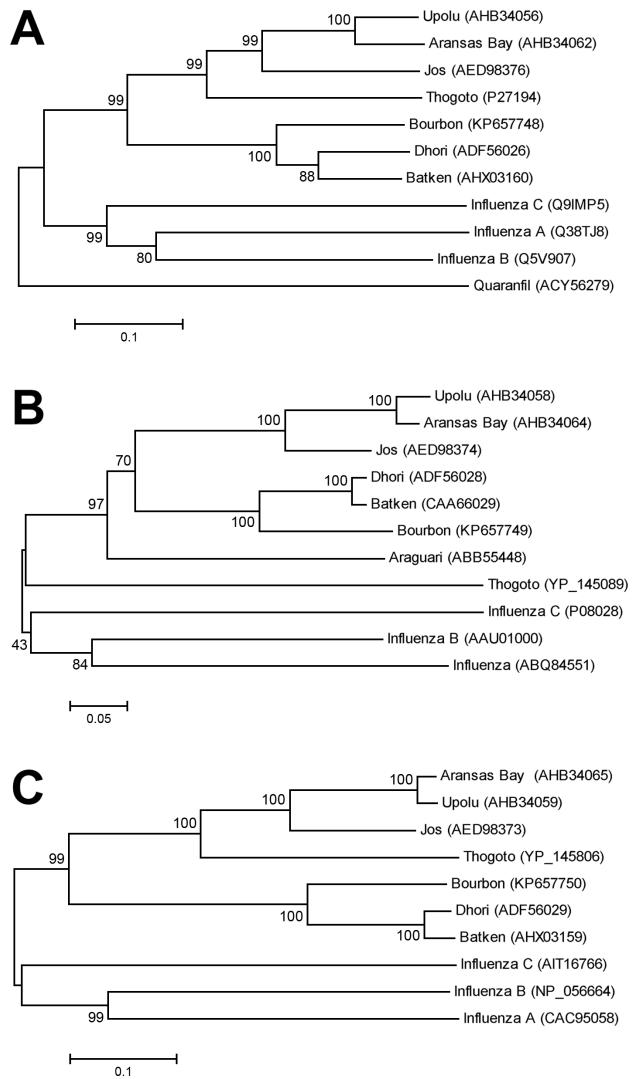


Figure 3. Phylogenies of deduced amino acid sequences of representative genes of Bourbon virus in comparison to homologous sequences of selected orthomyxoviruses. A neighbor-joining method was used for inference of each phylogeny with 2,000 replicates for bootstrap testing. Values at nodes are bootstrap values. A) PA polymerase subunit (segment 3). B) Nucleocapsid protein (segment 5). C) Membrane protein (segment 6). GenBank accession numbers appear next to taxon names. Scale bars indicate number of amino acid substitutions per site.

and a history of finding an embedded tick before becoming ill support the notion that Bourbon virus might be transmitted by ticks. Therefore, to potentially prevent Bourbon virus disease, as well as other tickborne diseases, persons should be advised to avoid tick bites by using an insect repellent registered with the US Environmental Protection Agency to be effective against ticks, wearing long sleeves and pants, avoiding bushy and wooded areas, and performing tick checks after spending time outdoors.

The discovery of Bourbon virus, in addition to recent discoveries of tick-associated Heartland and severe fever with thrombocytopenia syndrome viruses (19,20), suggests that the public health burden of these pathogens has been underestimated. As nonselective molecular methods of pathogen identification (i.e., NGS sequencing) become more widely used, ideally in combination with classical microbiologic techniques, it is anticipated that similar discoveries will be made in the future.

It is currently not known how many human infections and disease cases might be attributable to this novel pathogen. On the basis of limited information for our case-patient, health care providers might consider Bourbon virus as a potential infectious etiology in patients in whom fever, leukopenia, and thrombocytopenia develop without a more likely explanation and who have shown negative results for other tickborne diseases (e.g., ehrlichiosis, anaplasmosis, or Heartland virus disease) or have not responded to doxycycline therapy. Work is planned to identify additional human infections with this novel virus, as well as to explore its potential geographic distribution. Also, more comprehensive virologic characterizations and field work are ongoing to better understand the biology of, and to identify potential vectors and reservoirs for, Bourbon virus. These data will be critical to further characterize the epidemiology and illness caused by Bourbon virus and to implement potential prevention and control measures.

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Transmission of Hepatitis C Virus among Prisoners, Australia, 2005–2012

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Hepatitis C virus (HCV) is predominantly transmitted between persons who inject drugs. For this population, global prevalence of HCV infection is high and incarceration is common and an independent risk factor for HCV acquisition. To explore HCV transmission dynamics in incarcerated populations, we integrated virus sequences with risk behavior and spatiotemporal data and analyzed transmission clusters among prisoners in Australia. We detected 3 clusters of recent HCV transmission consisting of 4 likely in-custody transmission events involving source/recipient pairs located in the same prison at the same time. Of these 4 events, 3 were associated with drug injecting and equipment sharing. Despite a large population of prisoners with chronic HCV, recent transmission events were identified in the prison setting. This ongoing HCV transmission among high-risk prisoners argues for expansion of prevention programs to reduce HCV transmission in prisons.

Hepatitis C virus (HCV) is a blood-borne virus that infects 3–4 million persons each year (1). In industrialized countries, transmission of HCV is largely attributed to injection drug use (2). The association between injection drug use, HCV infection, and imprisonment is very close (3). People who inject drugs (PWID) account for a large proportion of the incarcerated population in the United States, Canada, Europe, and Australia (4–7), and injection drug use is prevalent during incarceration (8,9). Globally, the prevalence of HCV infection among prisoners is ≈30% (10,11). A meta-analysis of 30 studies conducted in different countries revealed a clear association between the prevalence of HCV infection among prisoners and a history of injection drug use (6).

A recent meta-analysis of HCV incidence studies among prisoners revealed a mean incidence of 16.4 (95% CI 0.8–32.1) cases per 100 person-years (11). We recently

documented incidence of 14.1 (95% CI 10.0–19.3) cases per 100 person-years in 37 prisons in New South Wales (NSW), Australia, and identified recent injection drug use and Aboriginal and Torre Strait Islander descent as independent risk factors for HCV seroconversion (12). This analysis also identified high prevalence of injection drug use and sharing of injecting equipment in prisons (12). Furthermore, 13 incident cases were identified in a subcohort of 114 prisoners continuously imprisoned (i.e., without release to the community) during the study period (incidence 10.3 cases/100 person-years).

Prisons can be regarded as an enclosed network of facilities within which prisoners are frequently moved. In NSW, prisoners are often transferred between prisons (e.g., because of changes in prisoner security classifications) and temporarily moved for brief periods (e.g., to go to court or obtain medical treatment). In addition, prison sentences in Australia are typically short (average 7–9 months), but reincarceration rates are high (13).

The HCV genome evolves rapidly by mutations caused by highly error-prone replication mechanisms, which generate a swarm of constantly evolving variants (quasispecies) during every infection (14). HCV is classified into 7 genotypes and 67 subtypes (15). At the nucleotide level, each virus subtype differs by up to 25% and genotypes differ by up to 33% (16). The hypervariable region (HVR) of the HCV genome is the most variable; hence, this region is commonly used in molecular epidemiologic studies to detect clusters of persons infected via recent transmission events (17). We used sequences covering envelope (E) 1 and partial E2 (HVR1).

Acute HCV infection is largely asymptomatic; hence, the precise timing and source of transmission are usually unknown. Accordingly, virus sequencing and phylogenetic analysis have been used to reconstruct probable transmission chains from prevalent cases (18–20). Although broad linkages between HCV-infected persons have been demonstrated, previous efforts to identify probable transmission pairs among infected persons by using a

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combination of social network information and phylogenetic analysis techniques suggested that social and genetic distances were only weakly associated (21). By contrast, a recent report from a study that used this same approach among both prevalent and incident (newly infected) case-patients, identified probable clusters evidenced by proximity of social network and clustering analysis of core HCV sequences in a community-based cohort of PWID (22).

Our study used an integrated analysis of molecular, epidemiologic, and spatiotemporal data from a well-characterized cohort of longitudinally followed PWID. We used incident case detection in prisons to identify clusters of recent HCV transmission.

Methods

Hepatitis C Incidence and Transmission Study

The Hepatitis C Incidence and Transmission Study in Prisons (HITS-p) is a prospective study of a cohort of 498 prisoners with a history of injection drug use recruited from 37 prisons in NSW during 2005–2012 (12,23,24). At the time of preenrollment screening, all HITS-p participants were not infected with HCV; 181 subsequently became infected (12,23,24).

Study Cohort

For our study, we considered a HITS-p subset of 79 prisoners infected with HCV genotype 1 or genotype 3 for which HCV E1-HVR1 sequences were available. At ≈6-month intervals during participants' incarceration, we collected demographic information, lifetime and follow-up risk behavior data, and blood samples for HCV serologic and virologic testing (12,23,24). These data were collected by a trained research nurse whose employment was independent of the prison system (12).

HCV Testing and Estimated Date of Infection

Blood samples were tested for presence of HCV RNA and antibodies as described elsewhere (12,23,24). For participants who had seroconverted at the incident time point (the time of sampling when a person is found to have already seroconverted), the date of infection was estimated as the midpoint between the first HCV antibody-positive and the last HCV antibody-negative test result. For participants who were HCV RNA positive but HCV antibody negative at the incident time point, the date of infection was estimated to be 51 days before the date of sampling (25).

Statistical Analyses

We used *t*-tests (for continuous variables) and χ^2 tests (for categorical variables) to compare the demographic

characteristics and risk behavior of newly infected participants with those of noninfected participants (significance level = 0.05). We used the Wilcoxon rank-sum test to assess differences in number of movements.

Sequencing of the E1-HVR1

The region encoding the last 171 bp of core, E1, and HVR1 (882 bp [nt 723–1604]) was compared with HCV strain H77 (GenBank accession no. AF009606). These sequences were then amplified by nested reverse transcription PCR as described elsewhere (26).

Phylogenetic Analysis

ClustalW (implemented in MEGA 5.2.1 [27]) was used for alignment of genotypes 1 and 3 E1-HVR1 sequences. Alignments were visually inspected and manually edited. The HKY model with gamma distribution and a proportion of invariable sites was selected as the best-fit evolutionary model by using JModelTest (28). Separate phylogenetic trees for the genotype 1 and genotype 3 alignments with a maximum-likelihood approach were generated by using PhyML (29). To check for the robustness of the trees, we performed a 1,000-bootstrap test.

Clustering Analyses

Clusters of recent HCV transmission were detected by using PhyloPart (30), a software program that identifies genetically related sequences from a given tree by use of a statistical algorithm based on analysis of pairwise patristic distances (the amount of change between any 2 sequences as depicted by the branch lengths in a phylogenetic tree). PhyloPart considers any subtree as a cluster if the median pairwise patristic distance among its members is below a set percentile threshold of the distribution of all pairwise patristic distances in the given tree (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-1832-Techapp1.pdf>).

Validation Analyses of Clusters of Recent HCV Transmission

Records for each participant (consisting of time, date, and location of entry and exit from each prison) during 2005–2012 were obtained from the NSW Department of Corrective Services. Recent HCV transmission events were validated by integrating the estimated date of infection, incarceration time and location, and the reported risk behavior of participants during follow-up in each of the phylogenetically designated clusters.

For each cluster of cases indicating recent transmission, potential transmission pairs (source and recipient) are identified as any 2 participants co-located in the same prison for at least 24 hours. The source was identified as the participant with an estimated date of infection earlier

than the time of co-location with the other participant. The recipient was identified as the participant who was HCV antibody negative before co-location and who became HCV antibody positive within 12 months after co-location with the source participant. Clusters of >2 participants were considered valid with the identification of at least 1 transmission pair.

Risk behaviors (assessed prospectively during interviews at 6-month intervals) were available for the HITS-p cohort and included injection drug use and other blood-to-blood contact but excluded risks associated with sexual behavior (12). Information about drug injection and sharing of injecting equipment were obtained “since coming into prison” or “since the last interview” in association with “injected drugs,” “frequency of injecting drugs,” “use of injecting equipment after someone else,” and “frequency of use of injecting equipment.”

Results

Participants

From 181 newly infected participants (incident case-participants) in the HIT-P cohort, 102 were excluded from the study because they were infected with an HCV genotype other than 1 or 3. The study cohort thus comprised 79 viremic incident case-participants. Most (49 [62%]) participants were male, mean \pm SD age was 28 ± 7.2 years, 18 (23%) were of Aboriginal and Torres Strait Islander descent, and 61 (77%) had completed ≤ 10 years of formal education. The study cohort included 69 (87%) participants who had been previously imprisoned, and most had lifetime risk factors for blood-borne virus acquisition at baseline (Table 1). No significant differences in demographics and lifetime risk behaviors were found between the 79 study cohort participants and the 317 non-infected HITS-p cohort participants, other than previous

imprisonment and having ever injected drugs while in prison (Table 1). There were no significant differences between the 79 study cohort participants and the 102 excluded infected participants (Table 1).

Phylogenetics

A total of 129 sequences of E1-HVR1 were obtained from the 79 participants; 26 participants were infected with HCV genotype 1a, 5 with genotype 1b, 44 with HCV genotype 3a, and 4 with HCV genotypes 1a and 3a at different times. These reinfection cases were included in both the genotype 1 and genotype 3 analyses with the corresponding genotype-specific sequences. For participants infected with genotype 1, sequences were available from 1 viremic time point for 19 participants, from 2 time points for 10, and from 3 time points for 6. For participants infected with genotype 3, sequences were available from 1 viremic time point for 28 participants, from 2 time points for 15, and from 3 time points for 5. Phylogenetic trees were constructed for the genotype 1 and genotype 3 E1-HVR1 sequences (Figure 1).

Clustering

The optimal cutoff patristic distance designating recent transmission clusters was determined first by investigation of a range of percentile thresholds from the distribution of pairwise patristic distances (online Technical Appendix Methods). As expected at the minimum percentile value, only within-participant clusters were detected, while at the maximum, all sequences for each genotype were included in a single between-participant cluster (Figure 2). On this basis, the chosen cutoff patristic distance for designation of between-participant clusters was 0.099 for genotype 1 and 0.095 for genotype 3 (corresponding to 0.034 and 0.022 nt substitutions/site in the E1-HVR1 region, respectively).

Table 1. Demographic characteristics and lifetime risk behavior of prisoners in New South Wales, Australia, 2005–2012*

Characteristic	Infected prisoners/ study cohort, n = 79†	Noninfected prisoners, n = 317	p value‡	Infected prisoners excluded, n = 102§	p value¶
Mean (\pm SD) age, y	28 (7.2)	28 (7.0)	0.71	26 (6.5)	0.13
Median (\pm SD) time since initiation of injecting, y	6.5 (6.3)	7 (6.3)	0.81	7 (6.1)	0.60
Male sex	49 (62)	216 (68)	0.41	60 (59)	0.78
Aboriginal and/or Torres Strait Islander	18 (23)	58 (18)	0.44	37 (36)	0.07
≥ 10 y of education	61 (77)	238 (75)	0.73	84 (82)	0.50
Previously imprisoned	69 (87)	215 (68)	0.001	77 (75)	0.07
Ever had a tattoo	58 (73)	228 (72)	0.84	74 (73)	1
Ever injected drugs in prison	26 (33)	67 (21)	0.04	42 (41)	0.33
Ever shared injecting equipment in prison	23 (29)	61 (19)	0.06	37 (36)	0.43

*Data are expressed as no. (%) unless otherwise indicated. HITS-p, Hepatitis C Incidence and Transmission Study in Prisons.

†Study cohort = viremic participants from the HITS-p cohort.

‡2-sided comparison of participants from the study cohort and noninfected participants from the HITS-p cohort.

§102 prisoners were excluded because they were infected with an HCV genotype other than 1 or 3.

¶2-sided comparison of participants from the study cohort and infected participants excluded from the study.

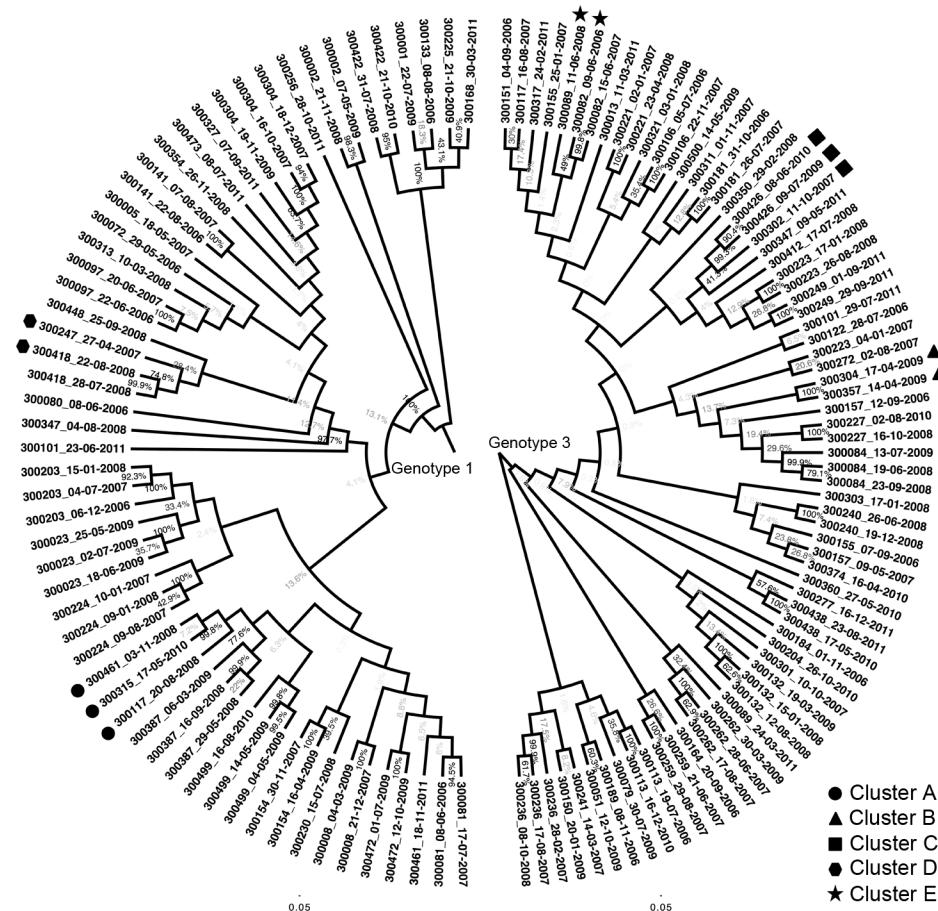


Figure 1. Phylogenetic trees composed of 129 sequences from 79 participants infected with hepatitis C virus genotypes (gt) 1a, 1b, or 3a, New South Wales, Australia, 2005–2012. Names on the tips of the tree represent participant identification numbers and are followed by the sample collection date. Each phylogenetic tree was generated separately from a maximum-likelihood model by using an HKY substitution model with gamma distribution. Bootstrap values are >80% for all branches of identified transmission clusters. Bootstrap values between branches representing sequences from the same host were lower than those between host branches. Identified transmission clusters are labeled with symbols. Scale bars indicate nucleotide substitutions per site.

To assess the effect of the time interval between sampling points on the distribution of pairwise patristic distances, and hence the designated thresholds, we studied the relationship between the time of collection and the pairwise patristic distance between all the sequences available for the study cohort (longitudinally within-participant and between-participant). The pairwise patristic distances between hosts was independent of the time interval (Figure 3). The degree of viral divergence reflected by patristic distances among sequences from within the same participant increased with the time interval between the collection time points. Within the time window analyzed (up to 4 years), within-participant genetic distances remained smaller than those from between-participant pairs. Only a small proportion of the between-participant genetic distances were within the range of within-participant pairs.

Further validation analyses including sequences from a single-source HCV outbreak (online Technical Appendix Results 1) showed that within-participant evolution could generate patristic distances greater than those observed between the sequence of the source and infected recipients when collected up to 23 years after transmission. However, the median distribution of these distances revealed that

between-participant distances were significantly higher than within-participant differences.

Last, to assess the potential effect of virus diversity within the quasispecies of a single-source host and the potential transmission of a minor variant to a new recipient, the distribution of pairwise patristic distances between all E1-HVR1 variants within the quasispecies from 2 time points collected over 1 year from 2 participants followed from primary HCV infection was analyzed to a sensitivity of variants representing 1% of the quasispecies (online Technical Appendix Results 2). Again, the maximum within-participant genetic distance within the quasispecies did not exceed the genetic distances between consensus sequences identified in between-participant analyses.

Clusters of Recent Transmission and Spatiotemporal Validation

One cluster of recent transmission was detected among 57 genotype 1 sequences (Figure 1, cluster A). This cluster consisted of 3 participants (nos. 117, 461, and 315); median pairwise patristic distance was 0.058. Two clusters were detected among genotype 3 sequences. The first (Figure 1, cluster B) consisted of 2 participants (nos. 304

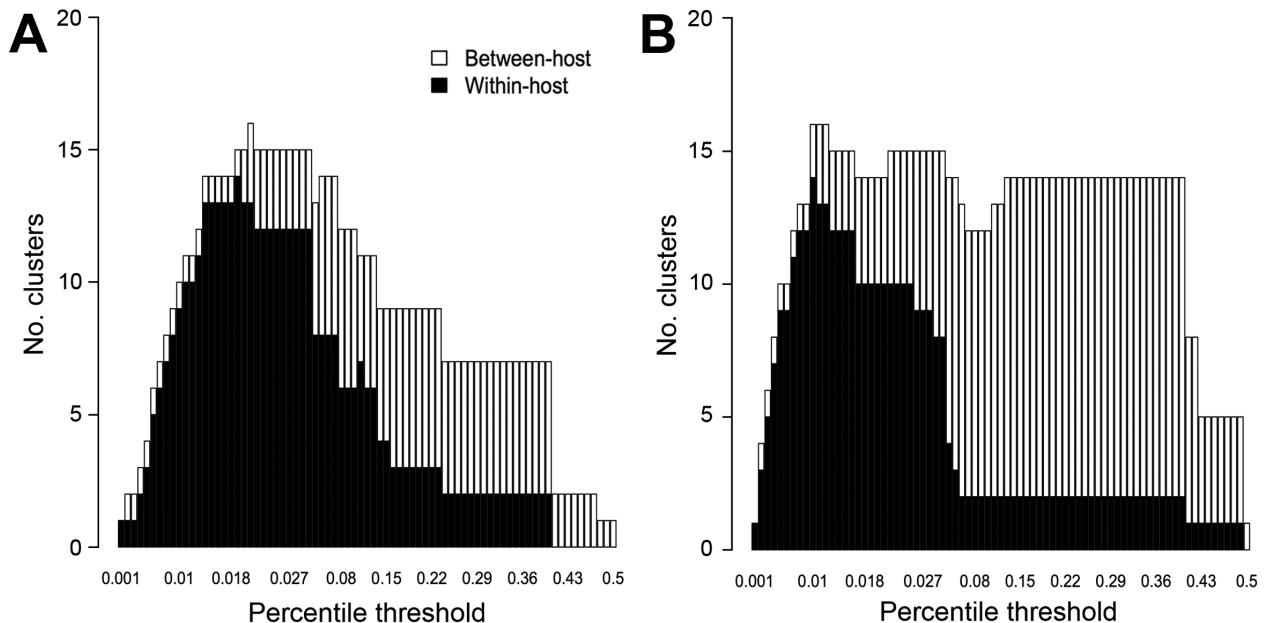


Figure 2. Analysis of hepatitis C virus transmission clusters identified across a range of percentile thresholds among prisoners in New South Wales, Australia, 2005–2012. Analysis shows the relationship between the number of clusters detected and the percentile thresholds from the distribution of genetic distances generated by using genotype 1 (A) and genotype 3 (B) sequences. At the lowest percentile threshold, only clusters containing sequences from the same participant are detected (black bars). When this threshold is increased, clusters of sequences from distinct participants arise (white bars).

and 357); median pairwise patristic distance was 0.011. The second cluster (Figure 1, cluster C) consisted of 2 participants (nos. 426 and 302); median pairwise patristic distance was 0.090. Two more clusters were detected just above the designated patristic distance cutoff (online Technical Appendix Results 3). The estimated date of infection, incarceration time and location, and reported risk behavior for each cluster member were analyzed to provide convergent evidence for likely transmission events (Table 2).

These dynamic participant movements were reconstructed for each transmission cluster. In cluster A, HCV was likely to have been transmitted from participant 315 to participants 117 and 461 (Figure 4). The estimated date of infection with genotype 1a for participant 315 was October 30, 2007; this participant had been in the same prison as participant 117 for 22 days (December 31, 2007–January 22, 2008). Both participants reported injecting drugs and sharing injecting equipment during the period of co-location. Participant 117 was then found to be viremic with genotype 1a in a sample obtained on August 20, 2008, giving an estimated date of infection of February 27, 2008. In another likely transmission event, participant 315 had been in the same prison with participant 461 on 2 occasions: for 13 days (June 29–July 11, 2008) and for 9 days (September 24–October 1, 2008). Both participants reported injecting drugs and sharing injecting equipment

during the period of co-location. Participant 461 was then found to be viremic with genotype 1a according to a sample dated November 3, 2008; estimated date of infection was October 6, 2008 (Video 1, <http://wwwnc.cdc.gov/EID/article/21/5/14-1832-F1.htm>). In transmission cluster B, HCV was likely to have been transmitted from participant 304 to 357. Estimated date of infection with genotype 3 for participant 304 was March 17, 2007; this participant had been in the same prison with participant 357 for 28 days, October 26–November 23, 2007. Both participants reported injecting drugs (although participant 304 did not report sharing injecting equipment) during the period of co-location. Participant 357 was then found to be viremic with genotype 3 according to a sample dated April 17, 2009; estimated date of infection was September 11, 2008 (Video 2, <http://wwwnc.cdc.gov/EID/article/21/5/14-1832-F2.htm>). In transmission cluster C, HCV genotype 3 was likely to have been transmitted from participant 302 to participant 426. Estimated date of infection for participant 302 was May 22, 2005; this participant had been in the same prison with participant 426 for 9 days, December 9–18, 2008. Both participants reported injecting drugs and sharing injecting equipment during this period of co-location. Participant 426 was then found to be viremic according to a sample obtained on July 9, 2009; estimated date of infection was December 21, 2008 (Video 3, <http://wwwnc.cdc.gov/EID/>

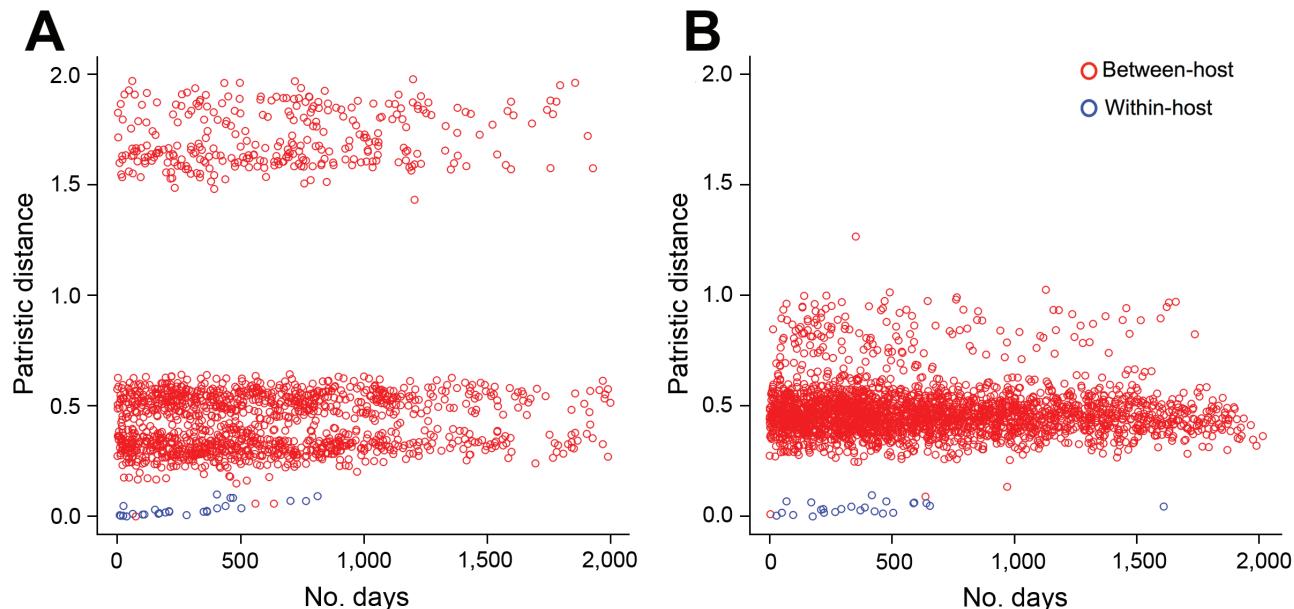


Figure 3. Analysis of pairwise patristic distances between hepatitis C virus sequences from the same participant (within-participant) sampled over time, and from between participants also sampled over time, among prisoners in New South Wales, Australia, 2005–2012. Analysis shows pairwise patristic distances as a function of the time interval between 2 sampling time points: within-participants (blue circles) and between-participants (red circles) for genotypes 1 (A) and 3 (B). A) Blue circles represent data from 35 participants, for a total of 57 sequences; B) blue circles represent data from 49 participants, for a total of 73 sequences.

article/21/5/14-1832-F3.htm). Of note, participant 302 is female, and participant 426 is male. Despite the short period of co-location, it is unlikely that prisoners of different sex could interact directly in the prisons, although shared use of a single injection device may have been possible.

Relationship between Phylogenetic Clustering and Movement Dynamics

In NSW, a high number of prisoner movements are common; prisoners are often transferred between correctional centers or released to the outside community. During the study period (2005–2012), participants from the HITS-p cohort were moved to a different location (a prison or the outside community) a mean of 17 times (online Technical Appendix Table 2), and the 79 participants in the study cohort moved a mean (\pm SD) of 22 ± 13.55 times, with a mean of 4 ± 2.83 release events. The 7 participants from the 3 clusters of recent HCV transmission moved to a different location a mean of 28 ± 15.75 times, a significantly greater number of times than for the HITS-p cohort as a whole ($p = 0.002$) and for the subcohort of uninfected participants ($p < 0.001$). These differences remained significant when movements from one prison to another and release to outside community were tested separately ($p < 0.05$ for all).

Discussion

Our molecular epidemiology analysis combined with detailed spatiotemporal and behavioral risk data identified

several clusters of recent transmission of HCV infection within NSW prisons. This study shows direct evidence of ongoing HCV transmission among PWID in a prison setting.

Previous phylogenetic studies have examined associations between HCV infection and risk and demographic characteristics, including injection drug use (17,21,22,31,32). Moreover, those studies have defined transmission clusters with a threshold value fixed a priori, such as a maximum genetic distance of 2%–5% (17), or with a bootstrap cutoff value (22). Here, an empirically optimized threshold, which can also be larger than the typical threshold fixed in previous studies, was used to search for clusters of recent transmission exclusively among incident case-participants.

Despite a high prevalence of chronic HCV infection in prison populations, 3 clusters of transmission were identified in phylogenetic analysis of only 79 participants with recent HCV infection identified during 2005–2012. During this period, $\approx 20,000$ persons were imprisoned annually in NSW; HCV antibody prevalence was $\approx 30\%$ (33,34), which equates to $\approx 4,500$ persons with chronic HCV infection (assuming 25% of those cleared infection) who were imprisoned annually. When discounted for 40% recidivism (13), this calculation yields $\approx 19,000$ infected prisoners who may have acted as sources for HCV transmission over the study period. In our analysis, the numbers of movements were higher among newly infected

Table 2. Probable HCV transmission events identified by using phylogenetic analysis, spatiotemporal information, and risk behavior information, New South Wales, Australia, 2005–2012*

Cluster	Transmission, participant ID no.	Period of co-location	Prison ID†	Patient ID	Estimated date of infection	HCV genotype	ATSI	Continuously in prison‡	Equipment sharing§	OST§	Heroin use§
A	315 → 117	2007 Dec 31–2008 Jan 22	AT	315	2007 Oct 30	1a	No	Yes	Yes	No	No
				117	2008 Feb 27	1a	No	No	Yes	No	Yes
	315 → 461	2008 Jun 29–Jul 11 2008 Sep 24–Oct 1	AE	3 15	2007 Oct 30	1a	No	Yes	Yes	No	No
				461	2008 Oct 6	1a	No	No	Yes	No	Yes
B	304 → 357	2007 Oct 26–Nov 23	AB	304	2007 Apr 17	3a	No	No	No	No	No
				357	2008 Nov 11	3a	No	Yes	Yes	No	Yes
C	302 → 426	2008 Dec 9–18	AP	302§	2005 May 22	3a	Yes	No	Yes	No	No
				426	2008 Dec 21	3a	Yes	No	Yes	Yes	No

*All prisoners were injection drug users during the period of co-location. ATSI, Aboriginal and/or Torres Strait Islander descent; HCV, hepatitis C virus; ID, identification; OST, opioid substitution therapy.

†Prisons are identified by codes for de-identification purposes.

‡Continuously in prison 6 mo before estimated date of infection.

§Female patient. All others were male.

participants than among noninfected participants, suggesting that transmission is associated with frequent movements between prisons and from prison to the outside community. Such frequent movements could increase the chance of contact with infected persons or could be otherwise associated with behavior that puts a person at increased risk for HCV transmission.

It is possible that recently infected participants are more likely than chronically infected participants to transmit infection (35). This possibility could result from higher infectivity of the transmitted founder viruses, which are intrinsically adapted for successful transmission and dominate the acute phase of infection (14). In contrast, a high circulating viral load is associated with an increased probability of vertical HCV transmission (36,37). However, in our study of PWID, the viral loads (recorded in the blood samples close to the time of transmission) in the source case-participants in the clusters were only low to moderate (data not shown). An alternative explanation is the possibility that these clusters are part of an existing network of high-risk PWID across prisons.

The genetic diversity between variants within the quasispecies during a single infection can become substantial because of the high mutation rate of the virus and the selection pressures of the host immune response. This diversity could influence transmission events because a minor variant in the source can be preferentially transmitted and then dominate the virus population in the recipient host. Therefore, consensus sequencing might not be sufficient for detection of clusters in which transmission is driven by rare variants. Despite the fact that

the maximum genetic distances observed within the quasispecies in the selected samples studied here did not exceed the mean genetic distance between hosts, it remains possible that additional transmission clusters may have become evident had this approach been used for all samples.

Our study has several limitations. First, the virus populations involved in transmission events occurring several months after infection might differ from those involved in the acute phase of infection because of the rapid diversification of the virus genome. Therefore, these findings may underestimate ongoing transmission in prisons. Second, although the viruses infecting persons in the clusters were closely related, there is a possibility that unknown participants outside the cohort were also part of the transmission chains; hence, the identified recipient could have been infected by an intermediary source. This possibility may be relevant to probable indirect transmission of HCV from a female participant to a male participant in cluster C because male and female prisoners are segregated in prisons in Australia. Third, because the proposed method uses information collected only during incarceration, data on injecting and sharing behavior in the outside community were not available. Indeed, only 20 (25%) prisoners in the study cohort were continuously imprisoned in the 6 months before the estimated date of infection. Finally, risk behavior could have been underestimated because of the underreporting of sensitive and socially stigmatized behavior during interviews.

From a global perspective, public health control programs have had relatively limited effects on mitigating

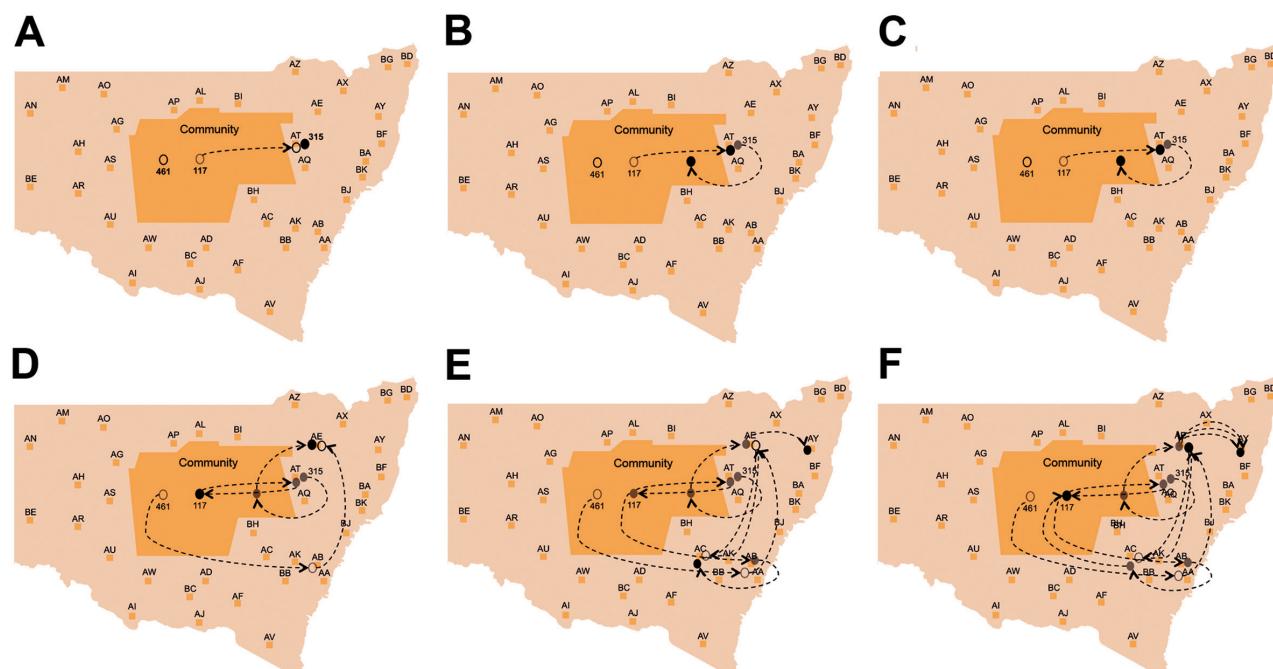


Figure 4. Reconstruction of the likely hepatitis C virus transmission dynamics among prisoners in New South Wales, Australia, 2005–2012. Geographic representation of the transmission dynamics among 3 participants identified in cluster A over a 12-month period and co-location dynamics of these participants during October 2007–October 2008 between the prisons in New South Wales are shown. Participants moved between 4 prisons and between prisons and the outside community (arbitrarily located in the center of the map of New South Wales). A) Time 0 (earliest record of location of the cluster members before co-location events occurred between any of the pairs within the cluster); B) 2 months after time 0; C) 4 months after time 0; D) 8 months after time 0; E) 10 months after time 0; F) 12 months after time 0. Prisons are de-identified, indicated with a 2-letter code and random locations. Arrows represent the movement of participants between 2 prisons. Filled ovals indicate viremic participants; empty ovals indicate nonviremic patients; gray indicates previous location (past movements) of each participant.

HCV transmission. The analysis of the HITS-p cohort showed that opioid substitution therapy uptake reaches only 20% of the population (12,24), despite 64% reporting having ever injected heroin. A recent study on a cohort of PWID in NSW has identified a strong protective effect of opioid substitution therapy (38). The combination of needle and syringe exchange programs and opioid substitution therapy programs is the most effective approach for mitigating HCV transmission, reducing incidence by a substantial amount (30%–80%) (39,40). However, needle and syringe exchange programs remain prohibited in NSW prisons. By identifying ongoing HCV transmission in prisons, this study advocates for new strategies for reducing risk behavior, such as increasing opioid substitution therapy use and eventually introducing needle and syringe programs in prison settings.

The HITS-p investigators include Kate Dolan, Paul Haber, William Rawlinson, Carla Treloar, Greg Dore, Lisa Maher, and authors Andrew Lloyd and Fabio Luciani.

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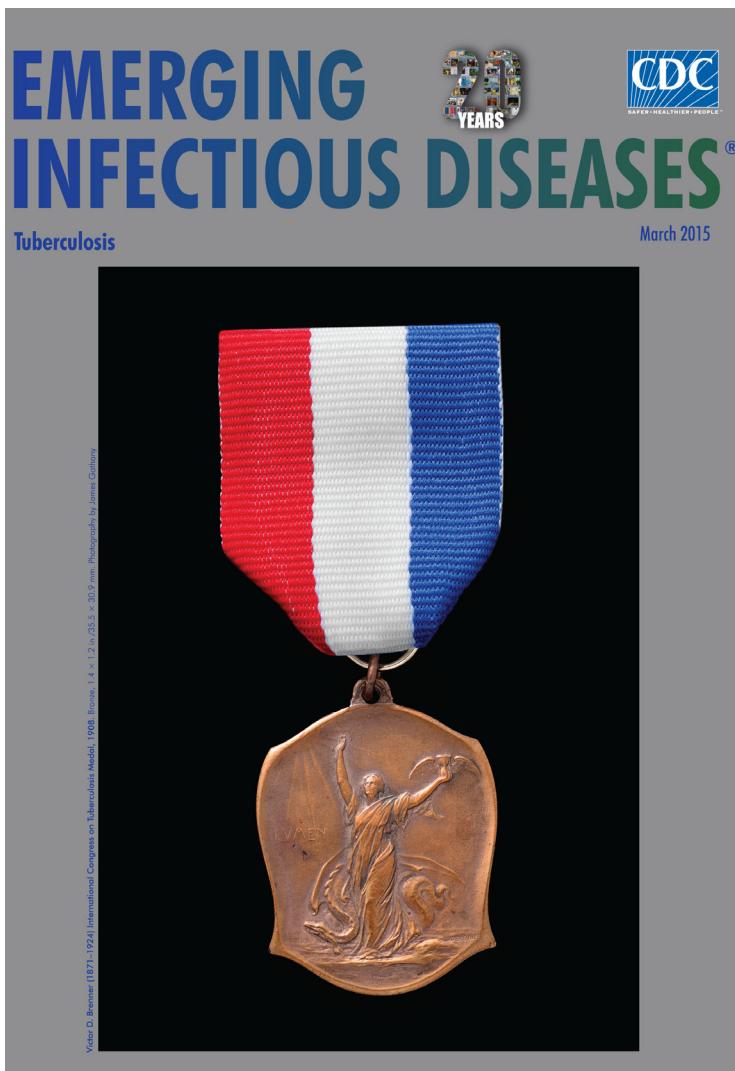
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Pathologic Changes in Wild Birds Infected with Highly Pathogenic Avian Influenza A(H5N8) Viruses, South Korea, 2014

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In January 2014, an outbreak of infection with highly pathogenic avian influenza (HPAI) A(H5N8) virus began on a duck farm in South Korea and spread to other poultry farms nearby. During this outbreak, many sick or dead wild birds were found around habitats frequented by migratory birds. To determine the causes of death, we examined 771 wild bird carcasses and identified HPAI A(H5N8) virus in 167. Gross and histologic lesions were observed in pancreas, lung, brain, and kidney of Baikal teals, bean geese, and whooper swans but not mallard ducks. Such lesions are consistent with lethal HPAI A(H5N8) virus infection. However, some HPAI-positive birds had died of gunshot wounds, peritonitis, or agrochemical poisoning rather than virus infection. These findings suggest that susceptibility to HPAI A(H5N8) virus varies among species of migratory birds and that asymptomatic migratory birds could be carriers of this virus.

Highly pathogenic avian influenza (HPAI) A virus infection of gallinaceous birds (e.g., poultry) is associated with high morbidity and mortality rates (1). Wild waterfowl, including ducks, are natural reservoir hosts for influenza A viruses and play a role in virus ecology and propagation. However, since 2003, repeated outbreaks of HPAI virus subtype H5N1 infection have occurred in poultry flocks in several Southeast Asia countries, resulting in high mortality rates among domestic ducks and wild migratory birds (2). Several studies have raised concern about the spread of HPAI virus by migratory birds (3–5).

Within the past 10 years, 4 outbreaks of HPAI A(H5N1) have occurred in South Korea (during winter and spring); migratory birds were identified as putative vectors (6–9). In 2014, an outbreak of HPAI A(H5N8) in South Korea led to the culling of millions of domestic poultry.

Hundreds of sick and dead wild birds were collected and tested, and the results confirmed HPAI A(H5N8) virus infection (10,11). Examining the pathologic changes caused by H5N8 virus infection in different wild bird species is essential for understanding their role in the spread of this highly infectious virus. We therefore examined many of the dead or sick wild birds collected during an outbreak of HPAI A(H5N8) virus during 2014 and report the gross and histologic findings and the patterns of virus antigen expression. We examined 8 Baikal teals, 3 bean geese, 1 whooper swan, and 2 mallard ducks naturally infected with HPAI A(H5N8) virus.

Materials and Methods

Samples

During January–June 2014, a total of 771 wild bird carcasses were submitted to the Animal and Plant Quarantine Agency in Anyang, South Korea (Table 1). On January 17, many dead or sick wild birds were found around Donglim Reservoir in southwestern Korea. Three sick Baikal teals showing neurologic symptoms, including torticollis, ataxia, and limb paresis, were captured and euthanized (Figure 1, panel A). Over a 5-day period, the bodies of 119 Baikal teals, 9 bean geese, and 1 coot were collected from near the Donglim Reservoir for necropsy. On January 22 and January 27, a total of 5 dead Baikal teals were found near the Geumgang River in midwestern South Korea (Table 2). Another 634 dead birds were found in other parts of the country. Necropsies were performed on all dead birds; trachea, kidney, cecal tonsil, pancreas, liver, intestine, heart, and lung were collected for virus isolation. Parenchymal tissues were collected for histopathologic analysis from 8 Baikal teals, 2 bean geese, and 1 whooper swan showing gross lesions and 3 bean geese and 2 mallard ducks not showing gross lesions. Collected tissues were fixed for 24 hours in 10% buffered neutral formaldehyde and processed for paraffin embedding. Bacterial culture was performed by

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Table 1. Results of necropsy of 771 bird carcasses collected January–June 2014, South Korea

Cause of death	Diagnosis	No. birds*
Pathogen (18.5%)	Highly pathogenic avian influenza A virus infection	167 (29)
	Bacterial infection	29
	Parasite infection	9 (1)
	Fungal infection	3
Nonpathogen (81.5%)	Agrochemical poisoning	222
	Gunshot	12
	Trauma	103
	Miscellaneous	32
	Putrefaction	52
	Unknown	142

*Parentheses indicate number of birds in which a pathogenic organism was detected in combination with agrochemical poisoning.

using standard methods. The stomach contents were subjected to toxicology testing, as described previously (12).

Virus Isolation and Identification

Tissue samples from wild birds were inoculated into specific pathogen free embryonated chicken eggs (9–11 days of gestation), and influenza viruses were identified by using a hemagglutination assay and reverse transcription PCR. Virus identification was confirmed by sequence analysis, as described previously (10). In addition, molecular pathotyping

was performed by nucleotide sequence analysis of the hemagglutinin cleavage site within the H5 subtype.

Histopathology and Immunohistochemistry

Paraffin-embedded sections were cut (5 μ m), dewaxed, and stained with hematoxylin and eosin. Duplicate sections were immunohistochemically analyzed to determine the distribution of influenza virus antigens in individual tissues. Briefly, sections were stained with a mouse monoclonal antibody against influenza A virus nucleoprotein (MCA-400; AbD Serotec, Duesseldorf, Germany), followed by a biotinylated goat anti-mouse IgG secondary antibody. Bound antibodies were detected with an avidin-biotin detection system (Ventana Medical Systems, Tucson, AZ, USA). The RedMap kit (Ventana Medical Systems) served as the substrate chromogen.

Results

Wild Bird Carcasses

Of a total of 771 wild birds, HPAI A(H5N8) viruses were isolated from 167. For the other 604 birds, test results for other avian influenza viruses were negative (Table 1). Bacterial (*Escherichia coli*, *Staphylococcus aureus*, and

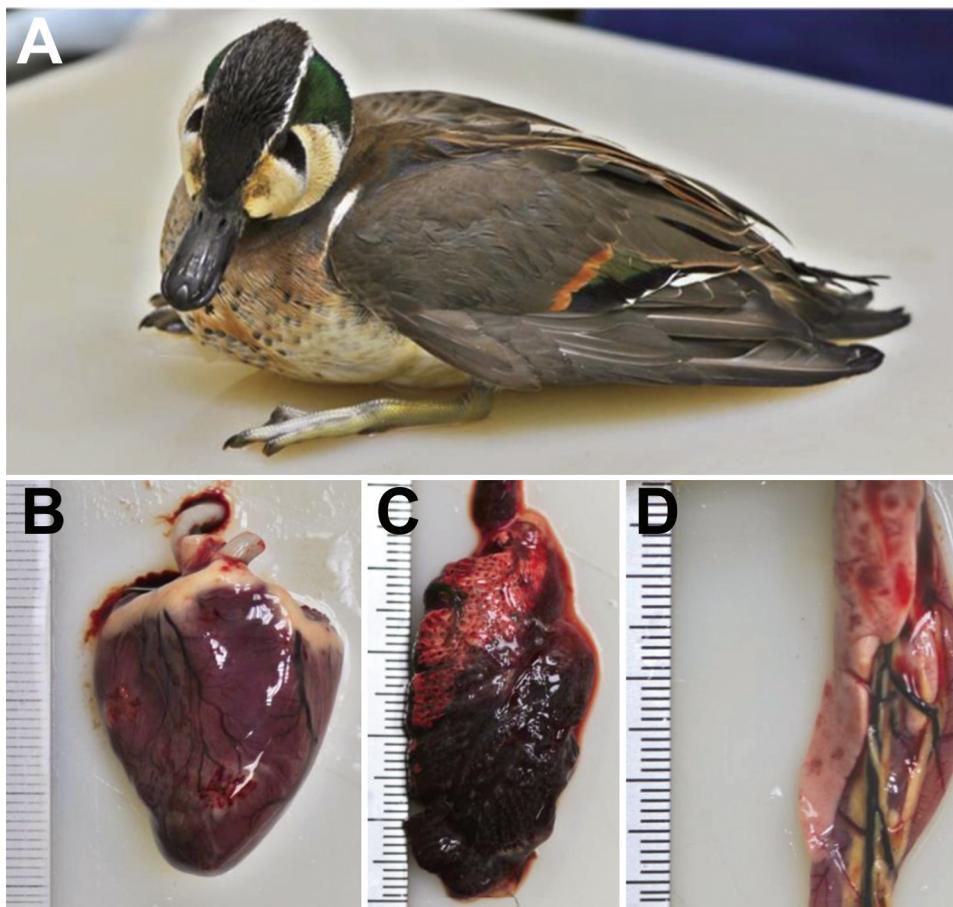


Figure 1. Baikal teal captured at Donglim Reservoir, showing A) neurologic signs of torticollis, ataxia, and limb paresis; B) hemorrhage and necrosis in heart muscle; C) edema and congestion of lung; and D) necrosis of pancreas.

Table 2. Wild birds infected with highly pathogenic avian influenza A(H5N8) virus, South Korea, 2014*

Family/species	No. birds, n = 167	Region	Date	Gross lesions	Infected organs	Other cause of death
Anatidae						
Baikal teal (<i>Anas formosa</i>)	122	Donglim Reservoir	Jan 17–22	Y	T, C, K, L, Lu, P	None
	1	Jeonbuk	Jan 21	Y	P	None
	5	Geumgang River	Jan 22, 27	Y	T, C, K, Lu, P	None
	20	Chungnam	Jan 23	Y	T, K, P, H	Monocrotophos poisoning
Bean goose (<i>Anser fabalis</i>)	9	Donglim Reservoir	Jan 19–21	N	T (K)	Monocrotophos poisoning (3)
	1	Incheon	Feb 1	Y	UK	None
	1	Gyeonggi	Mar 9	Y	T, C, P	None
Mallard (<i>Anas platyrhynchos</i>)	1	Jeonnam	Jan 27	N	UK	Peritonitis
	1	Jeonnam	Jan 29	N	UK	Gunshot, parasite infection
White-fronted goose (<i>Anser albifrons</i>)	1	Gyeonggi	Jan 28	N	UK	None
Whooper swan (<i>Cygnus cygnus</i>)	1	Jeonbuk	Feb 6	Y	UK	Renal failure
Other (not Anatidae)						
Coot (<i>Fulica atra</i>)	1	Donglim Reservoir	Jan 22	None	I, K	Postmortem change
Little grebe (<i>Podiceps ruficollis</i>)	2	Gyeonggi	Feb 27	None	T, C, K	Postmortem change
Great egret (<i>Egretta alba alba</i>)	1	Jeonbuk	Mar 8	N	UK	Peritonitis

*C, cecal tonsil; H, heart; I, intestine; K, kidney; L, liver; Lu, lung; N, no; T, trachea; P, pancreas; UK, unknown (pooled trachea, cecal tonsil, and kidney); Y, yes.

Salmonella Typhimurium), parasitic (nematodes, cestodes), and fungal infections were diagnosed for 29, 9, and 3 birds, respectively. We found that 73% of birds died of noninfectious causes. Agrochemicals, including monocrotophos, phosphamidon, carbofuran, diazinon, carbosulfan, endosulfan, parathion, dichlorvos, and methomyl, were found in the stomach contents of 222 birds; gunshot wounds, trauma (road kill or fracture), or miscellaneous (cachexia, dehydration, or suffocation) were the cause of death for 12, 103, and 32 birds, respectively. For 194 wild birds, the cause of death could not be determined because of postmortem autolysis, putrefaction, or both.

Observation of Gross Lesions and Isolation of HPAI Virus

During January–March 2014, a total of 167 wild birds of 8 species were infected with HPAI A(H5N8) virus. All 148 infected Baikal teals showed evidence of multifocal necrosis in the pancreas and liver, pulmonary congestion and edema, subepicardial hemorrhage, and myocarditis (Figure 1, panels B–D), and H5N8 virus was isolated from the trachea, cecal tonsil, kidney, liver, lung, pancreas, and heart. Monocrotophos poisoning was also diagnosed for 20 Baikal teals collected in Chungnam Province. Although no lesions were visible in the organs of 9 bean geese found near Donglim Reservoir, H5N8 virus was identified in the trachea and kidney of all 9. These birds also contained high concentrations of monocrotophos. Necropsy of 2 bean geese and 1 whooper swan found during February–March revealed distinct lesions in the pancreas and kidney; H5N8 virus was isolated from the trachea, cecal tonsil, and

pancreas. However, no gross lesions associated with HPAI virus infection were found in the organs from a mallard and a white-fronted goose; for these birds, the cause of death seemed to have been peritonitis and gunshot wounds. HPAI A(H5N8) virus infection was found in 1 coot, 2 little grebes, and 1 great egret; however, because of postmortem changes, no gross lesions associated with HPAI virus infection were identified (Table 2). All H5N8 virus isolates showed an HPAI virus motif (LREK[R]RRKR/GLF) at cleavage sites of hemagglutinin.

Histopathologic and Immunohistochemical Findings

Baikal Teals

Histologic examination revealed lesions in the pancreas, kidney, brain, and lung of all 8 birds examined. The pancreas showed moderate to severe, multifocal to confluent acinar necrosis, and virus antigen was detected in necrotic cells (Figure 2, panels A, B). Glomerular capillaries showed evidence of diffuse thrombosis and mild necrosis of tubules along with crystalline urate; virus antigen was detected in the tubular epithelium and glomerular capillary endothelium (Figure 2, panels C, D). Mild lymphocytic perivascular cuffing and loss of Purkinje cells were observed in the cerebrum and cerebellum, and virus antigen was detected in ependymal cells and epithelium of the choroid plexus and in cerebellar Purkinje cells. The lungs showed evidence of marked congestion, edema, and hemorrhage, and thrombosis was found in the alveolar capillaries. Influenza virus antigen was observed in a few capillary endothelial cells and macrophages in the alveolar lumen. Mild multifocal necrosis of hepatocytes and a lymphocytic infiltrate

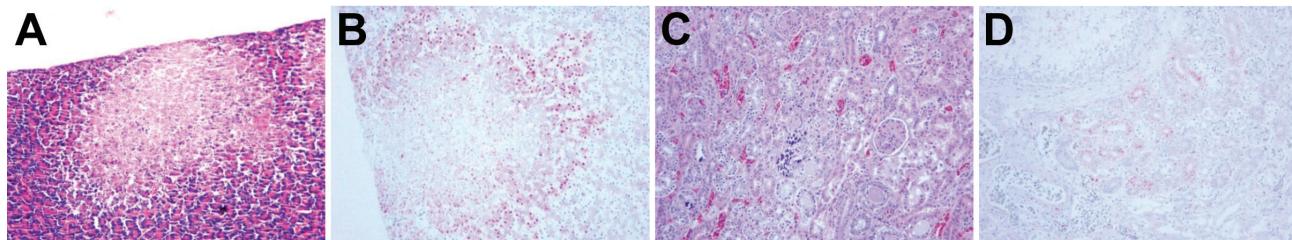


Figure 2. Histopathologic and immunohistochemical (IHC) testing results for Baikol teal. A) Focal necrosis in pancreas (hematoxylin and eosin [H&E] stain). B) Avian influenza virus antigen in necrotic pancreatic acini (IHC stain). C) Gout and renal tubular necrosis (H&E stain). D) Avian influenza virus antigen in renal tubule cells (IHC stain). Original magnifications $\times 100$.

were also observed, and massive amounts of virus antigen were distributed within the sinusoidal endothelium and in necrotic hepatocytes within the liver. No lesions were visible in the trachea, intestine, muscle, spleen, or heart, and no antigen-positive cells were found (Table 3).

Bean Geese

In 2 of 3 bean geese examined, major histopathologic lesions were found in the same organs as in the Baikol teals. Moderate multifocal pancreatic necrosis was also observed. Myocardial myofibers showed evidence of segmental necrosis, and mildly swollen nuclei, focal necrosis, and virus antigen were detected in the heart (Figure 3, panels C, D). Also observed were randomly distributed foci of neuronal necrosis and mild to moderate lymphocytic perivascular cuffing in the cerebrum and a paucity of cerebellar Purkinje cells and focal necrosis in the cerebellum. Staining was positive for virus antigen in neurons, glial cells, dendritic cells, granule cells, and Purkinje cells (Figure 3, panels E–H). Moreover, renal tubular necrosis and crystalline urinary casts were observed in the kidney, and virus antigen was detected in the tubular epithelium. No lesions were evident in intestine, skeletal muscle, or spleen (Table 3).

Whooper Swan

In the 1 bird examined, lesions were found mainly in the pancreas, kidney, and brain. The distribution of the lesions and the antigenic staining patterns were similar to those observed for bean geese.

Mallard Duck

Of the 2 mallard ducks examined, a heterotopic parasite was observed in the pancreas of 1 and fibrinous peritonitis affecting the pancreas and intestine was observed in the other. No virus antigen was detected in the intestine or pancreas of either bird (Tables 2, 3).

Discussion

The 2014 outbreak of HPAI A(H5N8) in South Korea was unexpected because the H5N8 subtype is uncommon in this area. A genetic characterization study suggests that this H5N8 virus (clade 2.3.4.6) was introduced into South Korea by migratory birds and spread from there to poultry farms (10).

Infection with H5N8 virus was found in all 148 Baikol teals, 2 bean geese, and 1 whooper swan. Necrotic lesions and avian influenza virus antigen staining were observed in multiple visceral organs, suggesting that the H5N8 virus causes a systemic infection. It also seems that the neurotropism of the H5N8 virus was the key factor contributing to death in these migratory birds of 3 species. The results of this study are consistent with those of other studies of HPAI pathogenicity in experimentally infected waterfowl (3–5,13). The gregarious behavior and migratory patterns of Baikol teals may underlie the mass mortality event that occurred at Donglim Reservoir.

Although a few Baikol teals were sick but not dead, the infection was clinically severe, and gross and histopathologic lesions were found. In addition, in 9 bean

Table 3. Histopathologic lesions and immunohistochemical results for avian influenza virus antigen in 11 wild birds infected with highly pathogenic avian influenza virus

Organ	Positive result by histopathology/immunohistochemistry*			
	Baikol teal, 8/8	Bean goose, 2/2	Whooper swan, 1/1	Mallard, 0/0
Trachea	–/–	NT	NT	NT
Lung	+/+	+/+	NT	NT
Heart	–/±	+/+	–/–	–/–
Brain	+/+	+/+	+/+	NT
Kidney	+/+	+/+	+/+	NT
Skeletal muscle	–/–	NT	NT	NT
Intestine	–/–	–/–	–/–	–/–
Pancreas	+++/>+++	+++/>+++	+++/>+++	–/–
Liver	++/>++	NT	±/±	NT
Spleen	–/–	–/–	+/–	NT

*Histopathologic results: –, no lesions; +, mild lesions; ++, moderate lesions; +++, severe lesions. Immunohistochemistry results: –, no antigen; ±, faint antigen; +, mild antigen; ++, moderate antigen; +++ severe antigen; NT, not tested.

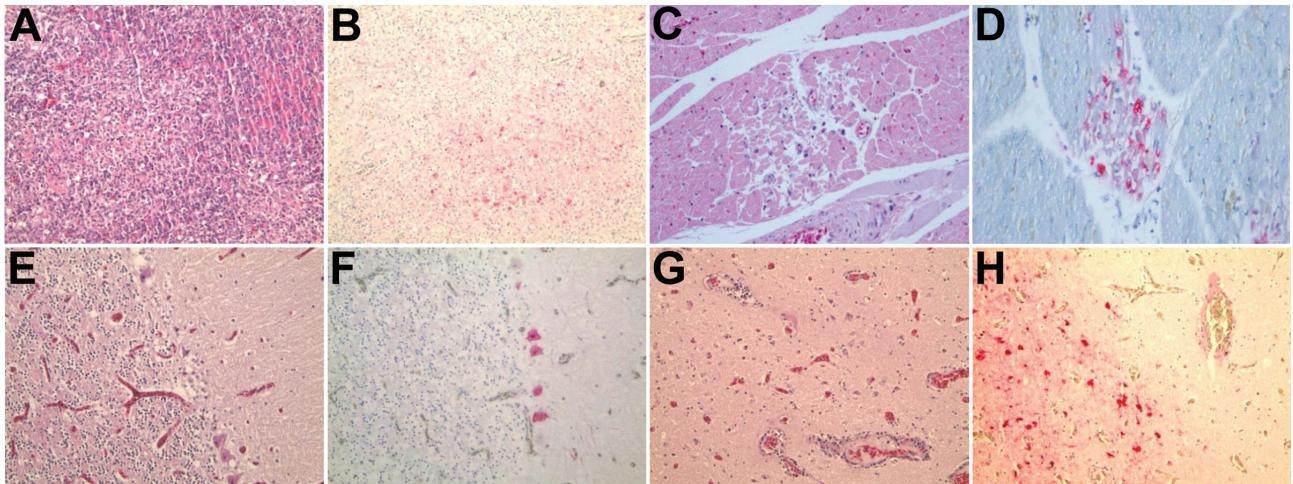


Figure 3. Histopathologic and immunohistochemical (IHC) testing results for bean goose. A) Diffuse necrotizing pancreatitis (hematoxylin and eosin [H&E] stain). B) Avian influenza virus antigen in necrotic pancreatic cells (IHC stain). C) Segmental necrosis of myofibers with mildly swollen nuclei focal necrosis (H&E stain). D) Avian influenza virus antigen in necrotic myofiber of the heart (IHC stain). E) Paucity of Purkinje cells, cerebellum (H&E stain). F) Avian influenza virus antigen in Purkinje cells (IHC stain). G) Neuronal necrosis and perivascular cuffing, cerebrum (H&E stain). H) Avian influenza virus antigen in neuron and glial cells (IHC stain). Original magnifications $\times 100$.

geese (all found in the same location at the same time), no evidence of lesions was found; however, the H5N8 virus was identified in the trachea and kidneys. Thus, the H5N8 virus did not cause sudden death in these waterfowl, despite their infection with the virus. This finding suggests that the infection is not peracute during the early stages.

By contrast, although mallard ducks and white-fronted geese were asymptotically infected with H5N8 HPAI, these birds died of other causes, including gunshot wounds or peritonitis. Experimental infection studies show that some wild ducks, geese, and swans shed H5N1 virus despite being asymptomatic (5,14–16). Also, HPAI subtype H5 viruses have been isolated from healthy wild waterfowl, providing evidence of nonlethal infection (17,18). Thus, these species of migratory bird may be long-distance vectors for the H5N8 virus.

The histopathologic findings and the localization of H5N8 virus antigen associated with renal failure and gout in Baikal teals, bean geese, and whooper swans were unusual. Experimental infection studies have shown that although HPAI (H5N1) infects the tubular epithelium in the kidneys of various waterfowl, no evidence of gross or histopathologic lesions has been found in the kidneys (4,5). A few studies report that low pathogenicity H9 and H10 influenza viruses are nephrotropic in chickens (19,20) and that HPAI subtype H5 causes acute renal lesions in mammals and primates (including humans) (21–23). The results of our study suggest that the HPAI A(H5N8) virus affects waterfowl differently than do other HPAI viruses; therefore, further studies are needed to fully understand the pathology of H5N8 in waterfowl.

In summary, we report the pathogenicity of HPAI A(H5N8) virus (clade 2.3.4.6) in various species of waterfowl in South Korea. Baikal teals, bean geese, and whooper swans are susceptible to this virus, which causes high mortality rates; however, infection in mallard ducks is asymptomatic. Although many questions regarding HPAI A(H5N8) virus pathogenesis remain, the results reported herein suggest that susceptibility to HPAI A(H5N8) virus differs among different species of migratory birds. Thus, these birds may be susceptible to or carriers of this infectious virus.

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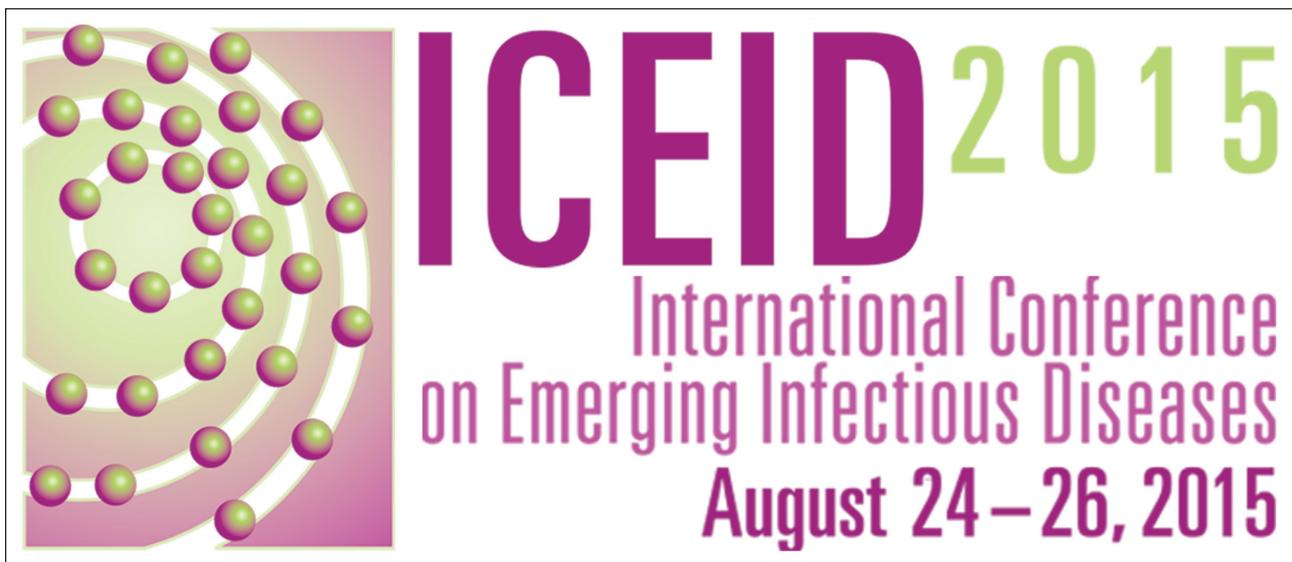
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Itaya virus, a Novel *Orthobunyavirus* Associated with Human Febrile Illness, Peru

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Our genetic analyses of uncharacterized bunyaviruses isolated in Peru identified a possible reassortant virus containing small and large gene segment sequences closely related to the Caraparu virus and a medium gene segment sequence potentially derived from an unidentified group C orthobunyavirus. Neutralization tests confirmed serologic distinction among the newly identified virus and the prototype and Caraparu strains. This virus, named Itaya, was isolated in 1999 and 2006 from febrile patients in the cities of Iquitos and Yurimaguas in Peru. The geographic distance between the 2 cases suggests that the Itaya virus could be widely distributed throughout the Amazon basin in northeastern Peru. Identification of a new *Orthobunyavirus* species that causes febrile disease in humans reinforces the need to expand viral disease surveillance in tropical regions of South America.

The *Orthobunyavirus* genus, part of the group of viruses known as arboviruses, comprises several human and zoonotic pathogens known to be transmitted by mosquitoes, culicoides midges, nest bugs, and ticks and is the largest of the 5 genera within the *Bunyaviridae* family. Orthobunyaviruses, like other members of the *Bunyaviridae* family, have a trisegmented (large [L], medium [M], and small [S] segments) negative-sense RNA genome. The L RNA segment encodes for the RNA-dependent RNA polymerase, the M segment encodes for the glycoproteins Gn and Gc, and the S segment encodes for the nucleocapsid

protein. Many orthobunyaviruses also encode the nonstructural proteins NSm and NSs within the M and S segments, respectively; however, these proteins are not encoded in all orthobunyaviruses described (1,2).

Because of the segmented nature of their genome, bunyaviruses, like other segmented genome viruses, can undergo genetic reassortment. In recent years, increasing numbers of reassortant bunyaviruses have been identified by using sequencing and phylogenetic analyses, and novel reassortant bunyaviruses with increased pathogenicity have been documented (3,4). Evidence that genetic reassortment appears to be the driving force in bunyavirus evolution (5) strongly supports the possibility that novel reassortant bunyaviruses will continue to be identified. Therefore, efforts to characterize existing and recently isolated bunyavirus strains are needed.

Some of the viruses within the genus *Orthobunyavirus*, including Oropouche, Iquitos, Guaroa, Jamestown Canyon, La Crosse, Cache Valley, Wyeomyia, and members of the group C viruses such as Caraparu and Murutu-cu, have been documented as causes of clinical disease in humans in the Americas (6–10). These orthobunyaviruses cause many symptoms, primarily febrile illness that has potential to be severely debilitating and that is sometimes accompanied by neurologic manifestations requiring intensive care (10). Human group C viruses infections, largely associated with mild febrile illness, are indistinguishable from dengue fever (9), and recent studies on the genetic characterization of reference strains have described their genetic relationship (11).

Since 1999, the US Naval Medical Research Unit No. 6 (NAMRU-6) in Lima, Peru, has collaborated with the Peruvian Ministry of Health to investigate the etiology of febrile illnesses in Peru and greater Latin America (9). As part of these activities, >54 orthobunyaviruses, including group C, Guaroa, Maguari, and Oropouche viruses, were isolated, and some have been genetically characterized in an effort to understand their relationships to other strains identified in South America (8,11,12). These efforts have already resulted in identification of Iquitos virus as a proposed reassortant bunyavirus in the Simbu serogroup that causes febrile illness in Peru (8).

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A recent study that examined some clinical isolates of group C viruses in South America isolated during 2003–2008 showed that the strain FSL2923, isolated from a febrile patient in Yurimaguas in 2006, had complete L and S RNA segments of Caraparu virus; however, the M segment was only 75.3% identical to that of Caraparu (11). No attempts were made to antigenically characterize the strain to confirm differences from the Caraparu virus. Here, we report the identification of this strain as a possible novel reassortant group C virus, which we named Itaya virus after the Itaya River that surrounds Iquitos, where this virus was isolated. We demonstrate that the Itaya virus causes clinical disease in humans similar to that of other group C viruses. We also describe the genetic relationship of Itaya virus to other group C serogroup viruses and the clinical manifestations among persons infected with the viruses.

Methods

Viruses

The viral isolates used in this study are summarized in Table 1. The first strain of Itaya virus (IQT9646) was isolated in 1999 from samples from a 25-year-old man in Iquitos, Peru. The second strain (FSL2923) was isolated in 2006 from a 59-year-old febrile man in Yurimaguas, Peru (11). The origin of the Caraparu strain BeAn3994 was described by Causey et al. (16). The source and isolation of group C virus prototype strains have been described elsewhere (9,17,18).

Study Sites

The locations of the study sites where IQT9646 and FSL2923 were isolated are depicted in Figure 1. Iquitos is a city of ≈380,000 inhabitants located 120 meters above sea level in the Amazon Basin of northeastern Peru. Yurimaguas is a city of ≈63,000 inhabitants located ≈184 meters above sea level and ≈388 km southwest of Iquitos.

Febrile Surveillance Study Population

The human use study protocols were approved by the Peruvian Ministry of Health and by the NMRC Institutional Review Board (protocol NMRC.2000.0006). Study subjects enrolled were ≥5 years of age and sought treatment for an acute, febrile, undifferentiated illness at military or civilian outpatient clinics at predetermined study sites. The criteria for inclusion of patients were fever ≥38°C of no more than 5 days in duration and nonspecific symptoms, such as headache, fatigue, or myalgia. Demographic and clinical information were obtained from each patient at the time of voluntary enrollment, and patients ≥18 years of age signed individual consent forms. Paired blood samples were collected, the first during the acute phase of illness and the second 2–4 weeks after symptom onset.

Virus Isolation and Serologic Assays

Serum samples collected during the acute phase of illness were used to isolate viruses by cell culture techniques, and samples collected during both acute and convalescent phases of illness were assayed by using IgM ELISA for evidence of arboviral infections (9). The procedure used for virus isolation was described by Aguilar et al. (8). In brief, serum samples were inoculated into flasks containing either confluent monolayers of African green monkey kidney cells of the Vero lineage or *Aedes albopictus* mosquito (C6/36) cells and maintained at 37°C and 28°C, respectively. The cell cultures were examined daily for 10 days for evidence of viral cytopathic effects (CPE); on the appearance of CPE, spot-slides were prepared and an immunofluorescence assay was done by using a polyclonal antibody against specific arboviruses that are known to circulate in Peru (9).

Prototype group C viruses were inoculated into flasks containing confluent monolayers of African green monkey Vero kidney cells and maintained at 37°C, as described previously. On the appearance of CPE, cell culture supernatants were harvested and clarified by centrifugation, and viral RNA was extracted.

Table 1. Viruses analyzed to determine their genetic relationship to Itaya virus, a novel *Orthobunyavirus*, Peru*

Strain	Country	Year isolated	Host species	Age, y/sex	Occupation	Virus	Reference
IQT9646	Peru	1999	Human	25/M	Worker	Itaya	This study
FSL2923	Peru	2006	Human	59/M	Teacher	Itaya	(11)
FPI2066	Peru	2011	Human	29/M	Farmer	Caraparu	This study
BeAn 3994	Brazil	1956	Sentinel monkey	NA	NA	Caraparu	(13,14)
BeAn 974	Brazil	1955	<i>Cebus apella</i>	NA	NA	Murutucu	This study
TRVL 51144	Trinidad	1963	<i>Culex (Melanoconium) portesi</i>	NA	NA	Restan	This study
TRVL 18462	Trinidad	1957	<i>Culex (Aedinus) accelerans</i>	NA	NA	Nepuyo	This study
77V-74814	Brazil	1977	Sentinel mouse	NA	NA	Broconha	This study
Fe3-71H2	United States	1963	<i>Culex (Melanoconium)</i>	NA	NA	Gumbo Limbo	This study
BeAn 848	Brazil	1955	<i>Cebus apella</i>	NA	NA	Apeu	(15)
BeAn15	Brazil	1954	<i>Cebus apella</i>	NA	NA	Marituba	(11)
BeAn17	Brazil	1954	<i>Cebus apella</i>	NA	NA	Oriboca	(11)
BT4075	Panama	1961	Human	36/M	Unknown	Madrid	(11)

*NA, not applicable.



Figure 1. Geographic distribution of the confirmed Itaya virus human cases (arrows) identified as part of the febrile disease surveillance project in Loreto, Peru during 1999 and 2006

Extraction of Viral RNA

For the extraction of RNA, cell culture supernatants were harvested and clarified by low-speed centrifugation ($2,000 \times g$, 10 min at 4°C), filtered through a 0.45- μm pore size filter (EMD Millipore, Billerica, MA, USA), and treated with a combination of DNases: 14 U Turbo DNase (Ambion, Austin, TX, USA); 20 U Benzonase (EMD Millipore); and 20 U RNase One (Promega, Madison, WI, USA) for 1 h at 37°C . Next, 24 mL of supernatant was loaded on top of 8 mL 30% sucrose (in TEN, pH 7.4), and centrifuged at $15,000 \times g$ for 4 h at 4°C . Finally, the pellet was resuspended in 250 mL RNase/DNase and protease-free water (Ambion), and viral RNA was extracted using the Trizol reagent (Invitrogen, Carlsbad, CA, USA) by using the manufacturer's protocols.

Next-Generation Sequencing and Phylogenetic Analyses

Viral RNA ($\approx 0.9 \mu\text{g}$) was fragmented by incubation at 94°C for 8 min in 19.5 mL of Illumina fragmentation buffer 15016648 (Illumina, San Diego, CA, USA). A sequencing library was prepared from the sample RNA by using an Illumina TruSeq RNA Sample Preparation Kit v2 using the manufacturer's protocol (Illumina). The sample was sequenced on a HiSeq 1000 by using the 2×50 paired-end protocol. Reads in fastq format were quality filtered, and any adaptor sequences were removed by using Trimmomatic software (19). The de novo assembly program ABySS (20) was used to assemble the reads into contigs, using several different sets of reads and k values from 20 to 40. In all samples, host reads were filtered

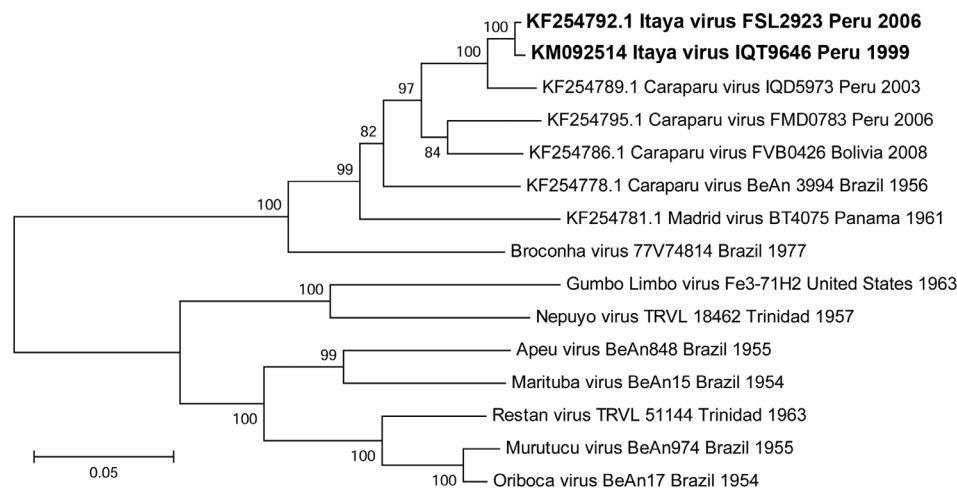


Figure 2. Neighbor-joining phylogenetic tree of group C orthobunyaviruses constructed by using MEGA5 (23) on the basis of the small gene segments of published virus sequences and Itaya virus strains isolated in Peru in 1999 and 2006 (boldface). The Itaya strain segments show a close relationship to Caraparu virus. Virus strains are labeled by code designation. Numbers indicate bootstrap values for the clades to the right. Bootstrap values were obtained based on 1,000 replicates. Scale bar indicates nucleotide substitutions per site.

out before de novo assembly. The longest contigs were selected, and reads were mapped back to the contigs by using bowtie2 (21) and visualized with the Integrated Genomics Viewer (22) to verify that the assembled contigs were correct. Total reads ranged from 1.5 to 12 million; the percentage of reads mapping to the virus genome in each sample ranged from 12% to 33%. (Details are available upon request from the authors.)

We deposited the complete genome sequences of Itaya virus and other group C viruses obtained for this study in GenBank under accession numbers KM092512–KM092514 and KM280924–KM280938. We used the neighbor-joining method available in MEGA5 (23) for phylogenetic analysis. The support for each node was determined by using 1,000 bootstrap replicates.

Antigenic Characterization

We used established methods to obtain hyperimmune ascitic fluid for classic cross-neutralization tests (24). Mice received 4 weekly intraperitoneal injections of 10% virus-infected newborn mouse brain suspension with Freud's adjuvant. Sarcoma 180 cells were also given intraperitoneally with a final injection to induce ascites formation. Antigenic differences among the viruses were then investigated by using cross-neutralization assays (25).

Results

Identification of IQT9646 as a Novel Reassortant *Orthobunyavirus*

In 1999, the virus strain IQT9646 was isolated from a 25-year-old male febrile patient who resided in Belen, Iquitos, Peru. The patient had an illness with symptoms of fever, headache, retro-orbital pain, arthralgia, chills, cough, and nasal congestion. These clinical symptoms are also

characteristic of dengue, malaria, and other tropical infectious diseases common in the region (26,27). The strain was initially classified as a Maguari isolate based on the results of serologic reactivity in an indirect immunofluorescence test. Maguari virus has been previously isolated from mosquitoes of the *Aedes*, *Mansonia*, and *Psorophora* spp. in Brazil; a variety of other mosquito species in Ecuador, Brazil, Trinidad, Colombia, Argentina, and French Guiana; and from horses in Guyana and Colombia (28,29). Nevertheless, evidence of human infection with Maguari virus is lacking. Therefore, we attempted to further identify and genetically characterize this strain using primers specific for different orthobunyaviruses, including Maguari; however, our attempts to amplify partial or complete genomic segments were unsuccessful. We therefore sought to obtain the complete sequences of the S, M, and L segments using an unbiased sequencing approach, then phylogenetic analyses to determine the relationship of the IQT9646 isolate to other viruses within the *Orthobunyavirus* genus.

Phylogenetic trees based on the S and L gene segments placed the IQT9646 virus among isolates of Caraparu virus, a member of the group C virus serogroup (Figures 2, 3), a pathogen known to cause febrile illness in humans and animals in the Amazon region of Peru (9,11). However, the M segment phylogenetic tree indicated that the IQT9646 virus had an M segment sequence divergent from Caraparu. In an attempt to determine the source of the M segment, we used a comprehensive full-genome sequence approach of other group C viruses and determined that the M segment of the IQT9646 strain was not closely related to other group C viruses but instead was most similar to the Caraparu and Apeu viruses (Figure 4). The S and L segment fragment sequences of the IQT9646 strain exhibited 97.6% and 96.6% nucleotide identity, respectively, to the prototype Caraparu strain BeAn3994 and to the Peruvian Caraparu strain IQD5973, whereas the M segment sequence

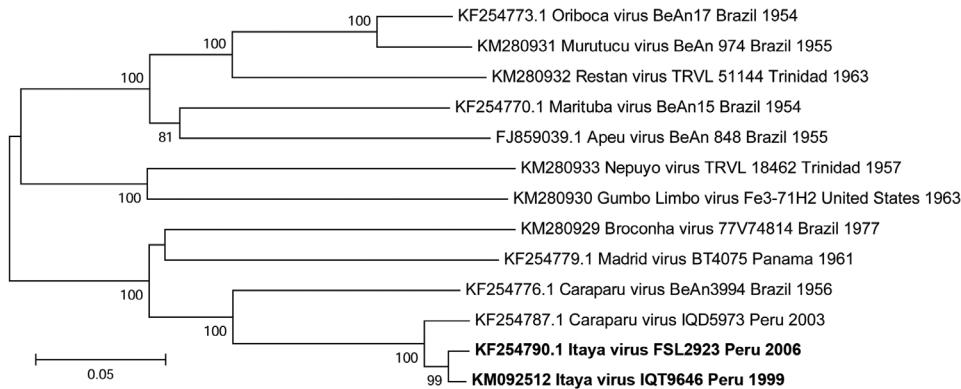


Figure 3. Neighbor-joining phylogenetic tree of group C orthobunyaviruses constructed by using MEGA5 (23) on the basis of the large gene segments of published virus sequences and Itaya virus strains isolated in Peru in 1999 and 2006 (boldface). The Itaya strain segments show a close relationship to Caraparu virus. Virus strains are labeled by code designation. Numbers indicate bootstrap values for the clades to the right. Bootstrap values were obtained based on 1,000 replicates. Scale bar indicates nucleotide substitutions per site.

displayed $\approx 75\%$ nucleotide identity to the prototype and Peruvian Caraparu strains. In summary, these results suggest that IQT9646 is possibly a group C reassortant virus.

Antigenic Characterization of IQT9646

To determine if the IQT9646 strain is antigenically distinct from Caraparu virus, we investigated the serologic relationships of IQT9646 using cross-neutralization tests. Mouse antisera were prepared against the IQT9646 strain and the prototype Caraparu strain BeAn 3994. These antisera displayed a minimum 4-fold difference in neutralization titers between these viruses, indicating that IQT9646 is serologically distinct from the prototype Caraparu virus. Because some genetic variation exists between the prototype Caraparu and recent Caraparu isolates from Peru (11) that may translate into minor antigenic differences, we included in our analyses a recent Caraparu strain isolated in Peru. The results were consistent with those obtained with the prototype Caraparu strain (Table 2). In summary, we found that the IQT9646 strain is antigenically distinct from Caraparu virus.

IQT9646 Virus in the Amazon Region of Peru

Genomic characterization of some group C prototype viruses and other isolates found in South America were recently reported (11). The strain FSL2923, isolated from a febrile patient residing in Yurimaguas, Peru, in 2006 was found to possess L and S RNA segments closely related to Caraparu virus; the M segment was only $\approx 75\%$ identical to the M segment of Caraparu virus (11). However, this strain was not characterized antigenically, causing uncertainty of whether this virus strain was distinct from Caraparu virus.

Considering our findings with the IQT9646 strain, which, like FSL2923, possesses the S and L RNA segment sequences closely related to Caraparu virus and the M RNA segment sequence derived from an unidentified group

C virus, we made genetic comparison and observed that the S, M, and L RNA segments of IQT9646 and FSL2923 shared $>98\%$ nucleotide and $>99\%$ amino acid sequence homology for all 3 viral RNA segments. These findings indicate that the FSL2923 isolate is the same virus as strain of IQT9646. The data also suggest that this reassortant virus is widely distributed throughout the Amazon basin in northeastern Peru, because it was isolated in Iquitos and Yurimaguas, in the Department of Loreto, within a 7-year time period (Figure 1).

Discussion

Arboviral diseases continue to be a frequent cause of illness and death worldwide. In recent years, a considerable number of novel arboviruses associated with outbreaks of human or livestock disease have been identified, and the expansion of known arboviruses into new geographic areas have also been reported (8,30–33). Of great concern is the potential that arboviruses will spread into novel geographic regions with completely naive human and animal populations, changing their patterns of illness as they move across the globe. Another major concern is the possibility that relatively benign viruses with segmented genomes could reassort, resulting in increased pathogenicity. Therefore, because of the public health impact that arboviral diseases continue to have around the globe, there is an urgent need to reinforce surveillance systems to identify the emergence of novel arboviruses and to monitor the activity of existing viruses and their potential expansion across geographic areas.

Surveillance studies performed by NAMRU-6 over the past 2 decades have been instrumental in characterizing the extent of many endemic tropical diseases throughout Latin America (8,34–37). Among the bunyaviruses, Oropouche, Iquitos, Guaroa, and members of the group C viruses were found to account for $\approx 2.5\%$ of all febrile cases (8,9).

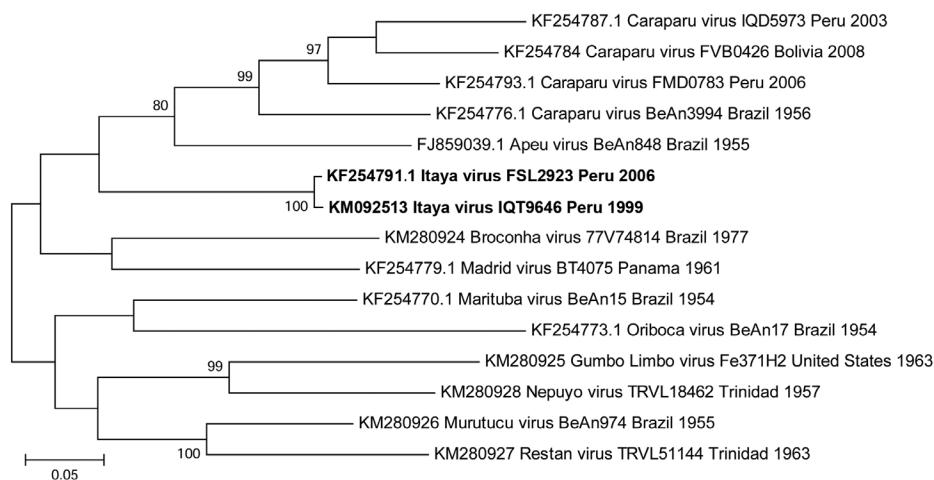


Figure 4. Neighbor-joining phylogenetic tree of group C orthobunyaviruses constructed by using MEGA5 (23) on the basis of the large gene segments of published virus sequences and Itaya virus strains isolated in Peru in 1999 and 2006 (boldface). The Itaya strain segments show a more distant relationship to Caraparu virus than for the small and large segments, indicating that Itaya virus is a novel reassortant strain. Virus strains are labeled by code designation. Numbers indicate bootstrap values for the clades to the right. Bootstrap values were obtained based on 1,000 replicates. Scale bar indicates nucleotide substitutions per site.

Although the percentage of cases appears relatively low, it is likely that most of human cases are undoubtedly going unrecognized or misdiagnosed as dengue, malaria, or other common acute tropical infectious diseases. This scenario is likely related to laboratory testing limitations and lack of extensive surveillance networks.

Among the bunyaviruses, the average symptomatic rates in Iquitos for group C and Iquitos viruses were 14.3/100,000 and 14.2/100,000, respectively, based on participants enrolled in NAMRU-6's febrile surveillance protocol (8,9). Because group C viruses include several human pathogens, recent studies have attempted to genetically characterize a few group C viruses isolated from humans. These efforts identified Caraparu and Marituba viruses and associated them with febrile human disease in Peru (9,11). However, ≥ 10 of the 13 distinct group C viruses have been associated with human disease elsewhere, including Oriboca, Itaqui, Nepuyo, Apeu, Murutucu, Restan, Ossa, Madrid, Caraparu, and Marituba viruses (38).

In this study, we aimed to expand our knowledge and understanding of bunyaviruses associated with febrile human illness in Peru, and these efforts focused on Maguari-like viruses, which were provisionally identified by using indirect immunofluorescence assays. Although indirect immunofluorescence assay is a procedure commonly used in laboratories to tentatively identify viruses, there is a degree of cross-reactivity among viruses from the same family; therefore, additional testing is needed to properly identify the viral agent. Therefore, we used a genetic and antigenic approach to characterize and identify the Maguari-like virus isolated from samples of febrile patients. These efforts yielded the identification of the group C reassortant virus that we named Itaya virus. Furthermore, we generated complete genome sequence information for prototype group C viruses, and as noted (13), we also

observed discrepancies with the Nepuyo, Restan, Murutucu, and Gumbo Limbo virus sequences when compared to those described by Nunes et al. (39). We still do not know the reasons for these discrepancies; however, Nunes et al. have acknowledged errors in their sequences and their plans to revise them (40), which should eventually clarify these discrepancies.

The prototype strain of Itaya virus was isolated from a patient who resided in Iquitos. The patient visited the health post in January 1999 with a mild febrile illness characterized by fever, headache, retro-orbital pain, arthralgia, and chills, among other symptoms. During February 1999, a patient with similar signs and symptoms visited the same health post in Iquitos, and subsequent studies determined that the patient had been infected with a novel reassortant orthobunyavirus, which we subsequently named Iquitos virus (8). The emergence of 2 novel reassortant orthobunyaviruses raises some questions as to what ecologic and environmental conditions favored the reassortment events leading to an emergence of novel human pathogens and their recognition in close succession. Because our limited serologic data suggests that the current arbovirus diagnostic tests fail to detect IgM antibodies produced in response to Itaya virus (data not shown), retrospective studies of febrile cases using Itaya virus specific tests may help determine the public health effects this pathogen may have in the area before and after its first isolation. The fact that another strain of Itaya virus was isolated in 2006 from another region within the northeastern Amazon Basin suggests that the virus may have caused more febrile human cases than previously recognized. Therefore, Itaya virus should be included in the list of potential pathogens that may account for a percentage of the 67% febrile cases enrolled in ongoing passive surveillance that are currently undiagnosed (9). Additional epidemiologic and ecologic studies are also

Table 2. Neutralization titers for IQT9646 and Caraparu viruses by using mouse antisera to determine antigenic differences, Peru*

Strain (virus)	Anti-IQT9646	Anti-BeAn3994
IQT9646 (Itaya)	2,560	80
FPI 2066 (Caraparu)	<40	2,560
BeAn3994 (Caraparu)	40	1,280

*Neutralization titers are expressed as the dilution of mouse immune ascitic fluid antiserum inhibiting 80% of plaque forming units.

needed to determine how widespread the virus is within the Amazon region and in neighboring areas and to identify potential vectors and reservoirs involved in the transmission of Itaya and other group C viruses.

In conclusion, our report expands upon the list of known arboviruses associated with febrile illness in Peru and raises awareness about the continuous emergence of reassortant bunyaviruses with human pathogenic potential. Future studies designed to genetically characterize existing and recently isolated bunyaviruses may be able to identify the viral donor of the Itaya M segment.

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The study protocol was approved by the Naval Medical Research Center Institutional Review Board (Protocol NMRCD.2000.0006) in compliance with all applicable federal regulations governing the protection of human subjects. The experiments reported herein were conducted in compliance with the Animal Welfare Act and in accordance with the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996.

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Isolation of *Onchocerca lupi* in Dogs and Black Flies, California, USA

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In southern California, ocular infections caused by *Onchocerca lupi* were diagnosed in 3 dogs (1 in 2006, 2 in 2012). The infectious agent was confirmed through morphologic analysis of fixed parasites in tissues and by PCR and sequencing of amplicons derived from 2 mitochondrially encoded genes and 1 nuclear-encoded gene. A nested PCR based on the sequence of the cytochrome oxidase subunit 1 gene of the parasite was developed and used to screen *Simulium* black flies collected from southern California for *O. lupi* DNA. Six (2.8%; 95% CI 0.6%–5.0%) of 213 black flies contained *O. lupi* DNA. Partial mitochondrial 16S rRNA gene sequences from the infected flies matched sequences derived from black fly larvae cytotaxonomically identified as *Simulium tribulatum*. These data implicate *S. tribulatum* flies as a putative vector for *O. lupi* in southern California.

Onchocerca lupi is a zoonotic parasite capable of infecting dogs, cats, and humans. Human infection was first suspected in 2002, when a case of human subconjunctival filariasis was found to have a worm with morphology similar to that of *O. lupi* (1). Human infection was confirmed in 2011, when a subconjunctival nematode in the eye of a young woman in Turkey was identified by molecular methods as *O. lupi* (2). Overall, ≈10 confirmed or

suspected human cases have been reported in Turkey (3,4), Tunisia (4), Iran (5), the southwestern United States (6), Crimea (1), and Albania (1). In most cases, clinical findings were similar, with a single immature worm found within a periocular mass. In the US case, a mature, gravid female worm was found within a mass in the cervical spinal canal of a young child in Arizona (6). The Centers for Disease Control and Prevention recently confirmed 5 additional cases in humans in the southwestern United States (M.L. Eberhard, unpub. data).

Several parasites of the genus *Onchocerca* are known to occur in North America, including 2 in cattle (*O. gutturosa* and *O. lienalis*) (7) and 1 in horses (*O. cervicalis*) (8). In addition, at least 2 parasites of the native cervid species (9) are known to be endemic to North America; at least 1 of these (*O. cervipedis*) has been identified in deer in California (10). Although most *Onchocerca* species are associated with ungulates, *O. lupi* is unique in that it is primarily associated with canids. The first report of *O. lupi* infection was in a wolf in Russia (11). In the past 20 years, ≈70 cases of *O. lupi* infection have been reported in domestic dogs in the United States, Greece, and Portugal (12–17). Probable cases also have been reported in Germany, Hungary, Switzerland, and Canada (16,18,19). Many affected dogs contained gravid female worms, presenting the possibility that canids may be a reservoir host for the parasite. The only additional species reported to have been infected were cats: 2 cases were documented in Utah, USA (20). Both cats were infected with gravid female worms, suggesting that cats also might be reservoir hosts. However, both cats also were infected with feline leukemia virus and probably were immunosuppressed and therefore not representative of most cats.

In the United States, confirmed and probable *O. lupi* infection has been documented in at least 12 dogs (17) and 2 cats (20) since 1991. That 6 of the 12 cases in dogs were in southern California (17,21) highlights this area as a focus of infection. Clinical signs in dogs typically involve

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0.3–0.7-cm periocular masses that contain adult worms. Infections may be associated with additional ocular pathology (Figure 1). The masses are typically subconjunctival or episcleral but can be found anywhere in the orbit (22).

The life cycle of *O. lupi*, including the vector and its primary reservoir host, remains unknown. Determining the vector is the critical step in preventing exposure. Black flies (*Simulium* spp.) and biting midges (*Culicoides* spp.) are vectors for other species of *Onchocerca* (23) and might be vectors for *O. lupi*. Black flies are routinely detected in certain areas of Los Angeles County, including a 29-km stretch of the Los Angeles River (<http://www.glacvcd.org/>), in the San Gabriel Valley area (<http://sgvmosquito.org/>), and in western areas of the county (<http://www.lawestvector.org/>). We report 3 additional *O. lupi* infections in dogs in southern California and present molecular evidence implicating the black fly species *S. tribulatum* as the possible vector for this parasite.

Materials and Methods

Identification of Cases and Parasites

In Los Angeles County, the Los Angeles County Department of Public Health conducts animal disease surveillance. Private practice veterinarians report diseases in all species, including companion animals. Veterinarians are required to report infectious diseases, particularly those listed as being of priority (<http://www.publichealth.lacounty.gov/vet/docs/AnimalReportList2013.pdf>), as well as any unusual diseases.

In March 2012, a local veterinarian reported a case of onchocerciasis in a local dog (dog B). Discussions with the veterinary ophthalmologist and the laboratory examining ocular tissue from the dog revealed an earlier case (dog A) and a concurrent case (dog C).

In May 2006, a 10-year-old, spayed female Labrador Retriever mix (dog A) was examined by a veterinary ophthalmologist in Los Angeles, California. The dog had a brown, lobulated 16-mm episcleral mass in the lateral temporal area of the left eye. One week of topical ophthalmologic antimicrobial and corticosteroid therapy failed to shrink the mass, and it was surgically removed. No other abnormalities were found. The mass contained mixed inflammatory cells surrounding 2 fragments of a cuticle with 2 striae per ridge, characteristic of *O. lupi*. The dog was from the Hollywood Hills area of Los Angeles, ≈3 km south of the 29-km black fly control zone of the Los Angeles River. A travel history was not available.

In February 2012, the same veterinarian examined an 8-year-old female spayed Boxer (dog B). The dog had severe bilateral corneal ulcerations, a 10-mm conjunctival mass in the nasodorsal area of the right eye, and persistent mydriasis in the left eye. No other abnormalities were found. Corneal ulceration is a common disorder in Boxers,



Figure 1. Right eye of a dog with *Onchocerca lupi* infection, southern California, USA, 2004. The dog had severe conjunctival inflammation, corneal degeneration, and an elevated intraocular pressure of 31 mm Hg. Ultimately, enucleation was performed, and histology revealed *Onchocerca* adult worms.

but the mass was considered to be unrelated to the ulcers (24). The mass was surgically removed and the ulcers treated surgically and medically. The mass contained multifocal granulomatous nodules with central necrosis surrounding sections of partially mineralized nematodes with 2 striae per ridge, characteristic of *O. lupi*. The dog lived in the San Fernando Valley area of Los Angeles, within 5 km of the black fly control zone of the Los Angeles River. It had lived in the Los Angeles area all of its life.

In January 2012, a 4-year-old pit bull mix (dog C) was examined by a veterinary ophthalmologist in San Diego. The dog had 2 episcleral masses (10 mm and 5 mm) at the lateral limbus of the left eye. The masses were immediately adjacent to each other and were associated with the lateral rectus muscle. No other abnormalities were found. The masses were surgically removed, and *O. lupi* was identified morphologically in the tissues. The dog was living at a humane society in San Diego, and further history on the dog was not available.

Molecular Characterization of Parasite Samples

Unstained, formalin-fixed, paraffin-embedded tissues containing the parasite were obtained from each of the 3 dogs. DNA was extracted by using a kit designed for purification of DNA from formalin-fixed paraffin-embedded tissue (QIAamp DNA FFPE Tissue Kit; QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. DNA was then amplified by using each of 3 sets of primers targeting highly conserved nematode or filarial species. This set included primers for the internal transcribed spacer (ITS) in the 5S rRNA gene: (S2: 5'-GTTA-AGCAACGTTGGCCTGG-3'; S16: 5'-TTGACAGATC-GGACGAGATG-3') the 12S small subunit rRNA gene of

the mitochondrion (12SF: 5'-GTTCCAGAATAATCG-GCTA-3', 12SR: 5'-ATTGACGGATGRTTTGTACC-3'), and cytochrome oxidase I (COI; COIF: 5'-TGATTGGTG-GTTTTGGTAA-3', COIR: 5'-ATAAGTACGAGTAT-CAATATC-3'), as described previously (25,26). PCR products were cloned into the TOPO-TA plasmid vector (Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions, and plasmids were prepared and sequenced in both directions by using TOPO-specific primers as described previously through a commercial service (Macrogen, Gaithersburg, MD, USA). DNA sequences were initially analyzed using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). Sequence alignments were performed using ClustalW (27), and an unrooted phylogenetic reconstruction was performed using the neighbor-joining method followed by bootstrap analysis using the MegAlign V11 routine of the DNASTar program package (DNASTar, Madison, WI, USA). Sequences were submitted to GenBank with the following accession numbers: KC763786, KC763785, KC763784, KC763783, KC763782, KC763781, KC763780, KC763779, and JX489168.

Black Fly Collection and Processing

During April–August 2013, we collected 248 black flies from 13 locations in the San Gabriel Valley in Los Angeles County throughout an area ≈ 380 km². This area is ≈ 40 – 50 km east of the veterinary clinic that diagnosed *O. lupi* infection in dogs A and B and ≈ 180 km north of the clinic that diagnosed it in dog C. The area contains the watershed of the San Gabriel River. The convenience sample of black flies was caught in CO₂-baited encephalitis virus surveillance traps that had been set for mosquito collection. Each fly was identified as belonging to the *Simulium* genus by standard taxonomic keys and was fixed in 70% ethanol.

We prepared DNA from the individual flies by using the DNeasy Blood and Tissue Kit (QIAGEN) following the manufacturer's instructions. Flies were prepared in batches of 12 samples; each batch contained 10 individual flies and 2 sham extractions that served as negative controls. A total of 2 μ L of the purified genomic product was then used as a template in a nested PCR targeting the *O. lupi* COI gene. All PCRs were conducted in a total volume of 50 μ L. The initial amplification reaction was conducted in a solution of 300 mmol/L Tris-HCl (pH 9.0); 75 mmol/L (NH₄)₂SO₄; 10 mmol/L MgCl₂; 200 μ mol/L each of dATP, dCTP, dGTP, and dTTP; 0.5 mM of each primer, and 2.5 U of Taq DNA polymerase (Invitrogen). Primer sequences employed in the initial reaction were 5'-TGTTGCCTTT-GATGTTGGGG-3' and 5'-GGATGACCGAAAAC-CAAAACAAG-3', and amplification conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 52°C for 30 s, and 72°C for 90 s, with a final extension of 72°C for 10 min. This reaction produced an amplicon of

475 bp. A total of 1 μ L of the product of the first reaction was used as the template in the nested reaction, which used the buffer conditions described above and primers with the sequences 5'-TCAAATATGCGTTCTACTGCTGTG-3' and 5'-CAAAGACCCAGCTAAAACAGGAAC-3'. Cycling conditions consisted of 94°C for 4 min, followed by 40 cycles of 94°C for 45 s, 50°C for 45 s, and 72°C for 90 s, and a final extension of 72°C for 10 min. This reaction produced an amplicon of 115 bp. Products from the nested reaction were analyzed by electrophoresis on a 1.5% agarose gel. Samples producing a band of the appropriate size in the initial screens (115 bp) were subjected to a second independent PCR. Samples producing products of the expected size were considered as putative positives. Amplicons of putative positives were subjected to DNA sequence analysis to confirm the identity of the product, using a commercial service (Genewiz, South Plainfield, NJ, USA). We calculated 95% CIs surrounding the estimate of the proportion of infected flies using standard statistical methods (28).

Fly Identification

We amplified a portion of the mitochondrial 16S rRNA gene from the DNA prepared from the infected flies, following previously published protocols (29). The primers used in the amplification reaction were 16S F: 5'-CGCCT-GTTTATCAAAAACAT-3' and 16S R: 5'-CTCCGGTTT-GAACTCAGATC-3'. The resulting amplicons were subjected to DNA sequence analysis, as described above. The DNA sequences obtained were submitted to the GenBank sequence database under accession numbers KP233211 and KP233212.

Black fly larvae were collected from 4 sites near the locations where the infected flies were trapped. The isolated larvae were cut in half horizontally immediately upon collection. The anterior end of each larva (head) was fixed in 70% isopropanol (rubbing alcohol), and the posterior end (abdomen) was fixed in Carnoy's solution (3 parts 95% ethanol and 1 part glacial acetic acid by volume).

DNA was prepared from the heads of the fixed larvae and used to amplify the portion of the mitochondrial 16S rRNA gene, as described above. The abdomen of each larva was opened ventrally with fine needles and stained with the Feulgen method (30). Salivary glands with stained nuclei and 1 gonad for sex determination were dissected from the abdomen, placed in a drop of 50% acetic acid, flattened under a coverslip, and examined with oil immersion. Identifications were based on diagnostic species-specific rearrangements of the polytene chromosomes (31,32).

Results

Unstained, formalin-fixed, paraffin-embedded tissue from the 3 dogs was used to amplify parts of 2 mitochondria-

encoded (*CO1* and 12S rRNA) and 1 nuclear-encoded (rRNA ITS1) genes. On the basis of the alignments and phylogenetic analyses, each parasite isolated from the 3 dogs was shown unequivocally to be *O. lupi*. For example, the sequences obtained from the ITS1 amplicon from the parasites from each dog were close to 100% identical to an *O. lupi* isolate from Hungary (Figure 2). Identical relationships were obtained when the 12S rDNA and *CO1* PCR amplicons were analyzed (online Technical Appendix Figures 1, 2, <http://wwwnc.cdc.gov/EID/article/21/5/14-2011-Techapp1.pdf>).

Of the 248 individual black flies collected, 213 were screened using the nested PCR targeting the *O. lupi CO1* gene. Of these, 6 (2.8%; 95% CI 0.6%–5.0%) produced nested amplicons of the expected size of 115 bp. The sequences of all 6 amplicons exactly matched the published *O. lupi CO1* sequence (data not shown). We then attempted to recover the amplicon from the first reaction and determine

the DNA sequence of this larger fragment. This attempt was successful in 4 of the 6 positive flies, resulting in 399 bp of sequence between the primer sites. The sequence of the recovered amplicons matched that of the GenBank reference sequence almost exactly in all 4 samples (Figure 3). We noted single-nucleotide polymorphisms in 3 of the 4 amplicons when we compared them with published sequence; 2 of the isolates shared 1 polymorphism (Figure 3).

Of the 13 locations sampled, 5 contained positive flies (online Technical Appendix Figure 3); 1 location had 2 positive flies (Table). These 5 locations spanned ≈270 km², covering most of the sampled area, except its northwest corner. Of the 5 positive sites, 4 were within a circle with a radius of 17.5 km (online Technical Appendix Figure 3). Three of the 5 positive collection sites were located along the San Gabriel River (online Technical Appendix Figure 3). All positive flies were collected during the spring (April 22–June 4, 2013).

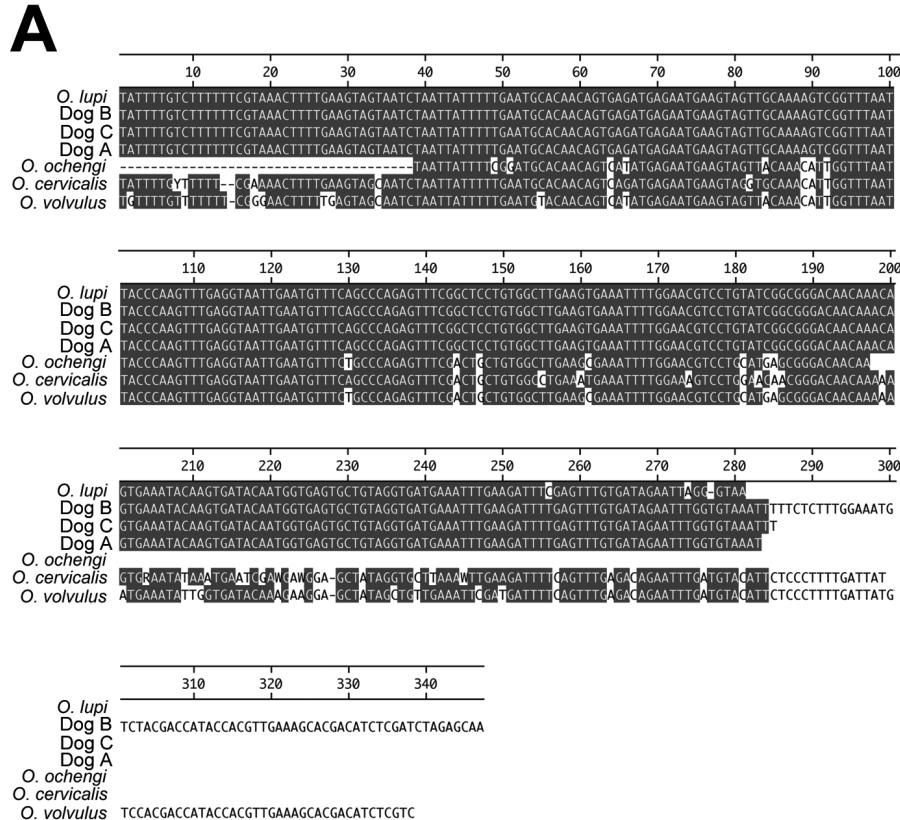


Figure 2. Molecular identification of parasites collected from 3 dogs in southern California, USA, by using sequence from the rRNA internal spacer (ITS). A) Multiple sequence alignment of ITS sequences from the 3 dogs and sequences from various *Onchocerca* parasites. Gray shading indicates areas of sequence identity. B) Unrooted phylogeny of the sequences shown in panel A. Numbers refer to the percentage of times the grouping distal to the number was supported in a bootstrap analysis of 1,000 replicate datasets. GenBank accession numbers for the sequences used in the alignment and phylogeny were as follows: *O. lupi*, JX489168; dog B, KC763782; dog C, KC763781; dog A, KC763779; *O. ochengi*, DQ523781; *O. cervicalis*, U13678; *O. volvulus*, AF325546. Scale bar indicates nucleotide substitutions per 100 residues.

		10	20	30	40	50
<i>O. lupi</i>	1	TCTTTTGGT	TTACTTTTGT	ATCTTTGTTG	ATGGTTTATC	AGTCTTTTTT
LAC 7-45	1	TCTTTTGGT	TTACTTTTGT	ATCTTTGTTG	ATGGTTTATC	AGTCTTTTTT
LAC 12-26	1	TCTTTTGGT	TTACTTTTGT	ATCTTTGTTG	ATGGTTTATC	AGTCTTTTTT
LAC 17-5	1	TCTTTTGGT	TTACTTTTGT	ATCTTTGTTG	ATGGTTTATC	AGTCTTTTTT
LAC 17-18	1	TCTTTTGGT	TTACTTTTGT	ATCTTTGTTG	ATGGTTTATC	AGTCTTTTTT
		60	70	80	90	100
<i>O. lupi</i>	51	TATTGGAGGC	GGTCCTGGTA	GTAGTTGAAC	TTTTTATCCT	CCTCTTAGAG
LAC 7-45	51	TATTGGAGGC	GGTCCTGGTA	GTAGTTGAAC	TTTTTATCCT	CCTCTTAGAG
LAC 12-26	51	TATTGGAGGC	GGTCCTGGTA	GTAGTTGAAC	TTTTTATCCT	CCTCTTAGAG
LAC 17-5	51	TATTGGAGGC	GGTCCTGGTA	GTAGTTGAAC	TTTTTATCCT	CCTCTTAGAG
LAC 17-18	51	TATTGGAGGC	GGTCCTGGTA	GTAGTTGAAC	TTTTTATCCT	CCTCTTAGAG
		110	120	130	140	150
<i>O. lupi</i>	101	TAGAGGGTCA	GCCTGAGTTA	TCTTTGGATA	CTATAGTTTT	GGGTTTGCAT
LAC 7-45	101	TAGAGGGTCA	GCCTGAGTTA	TCTTTGGATA	CTATAGTTTT	GGGTTTGCAT
LAC 12-26	101	TAGAGGGTCA	GCCTGAGTTA	TCTTTGGATA	CTATAGTTTT	GGGTTTGCAT
LAC 17-5	101	TAGAGGGTCA	GCCTGAGTTA	TCTTTGGATA	CTATAGTTTT	GGGTTTGCAT
LAC 17-18	101	TAGAGGGTCA	GCCTGAGTTA	TCTTTGGATA	CTATAGTTTT	GGGTTTGCAT
		160	170	180	190	200
<i>O. lupi</i>	151	ACTGTTGGTG	TTGGTTCTTT	GTTGGGTGCT	ATTAATTTTA	TGTTACTAC
LAC 7-45	151	ACTGTTGGTG	TTGGTTCTTT	GTTGGGTGCT	ATTAATTTTA	TGTTACTAC
LAC 12-26	151	ACTGTTGGTG	TTGGTTCTTT	GTTGGGTGCT	ATTAATTTTA	TGTTACTAC
LAC 17-5	151	ACTGTTGGTG	TTGGTTCTTT	GTTGGGTGCT	ATTAATTTTA	TGTTACTAC
LAC 17-18	151	ACTGTTGGTG	TTGGTTCTTT	GTTGGGTGCT	ATTAATTTTA	TGTTACTAC
		210	220	230	240	250
<i>O. lupi</i>	201	TCAAAAATATG	CGTCTACTG	CTGTGACTTT	AGATCAAATT	AGTATGTTTG
LAC 7-45	201	TCAAAAATATG	CGTCTACTG	CTGTGACTTT	AGATCAAATT	AGTATGTTTG
LAC 12-26	201	TCAAAAATATG	CGTCTACTG	CTGTGACTTT	AGATCAAATT	AGTATGTTTG
LAC 17-5	201	TCAAAAATATG	CGTCTACTG	CTGTGACTTT	AGATCAAATT	AGTATGTTTG
LAC 17-18	201	TCAAAAATATG	CGTCTACTG	CTGTGACTTT	AGATCAAATT	AGTATGTTTG
		260	270	280	290	300
<i>O. lupi</i>	251	TCTGGACTTC	TTATTAACT	TCTTTTTTAT	TAGTATTATC	TGTTCCGTGT
LAC 7-45	251	TCTGGACTTC	TTATTAACT	TCTTTTTTAT	TAGTATTATC	TGTTCCGTGT
LAC 12-26	251	TCTGGACTTC	TTATTAACT	TCTTTTTTAT	TAGTATTATC	TGTTCCGTGT
LAC 17-5	251	TCTGGACTTC	TTATTAACT	TCTTTTTTAT	TAGTATTATC	TGTTCCGTGT
LAC 17-18	251	TCTGGACTTC	TTATTAACT	TCTTTTTTAT	TAGTATTATC	TGTTCCGTGT
		310	320	330	340	350
<i>O. lupi</i>	301	TTAGCTGGGT	CTTTGTATT	TTTGTGTTG	GATCGTAATT	TTAGTACTTC
LAC 7-45	301	TTAGCTGGGT	CTTTGTATT	TTTGTGTTG	GATCGTAATT	TTAGTACTTC
LAC 12-26	301	TTAGCTGGGT	CTTTGTATT	TTTGTGTTG	GATCGTAATT	TTAGTACTTC
LAC 17-5	301	TTAGCTGGGT	CTTTGTATT	TTTGTGTTG	GATCGTAATT	TTAGTACTTC
LAC 17-18	301	TTAGCTGGGT	CTTTGTATT	TTTGTGTTG	GATCGTAATT	TTAGTACTTC
		360	370	380	390	400
<i>O. lupi</i>	351	TTTTTATGAT	ACTAAAAAAG	GTGGTAATCC	TTTGTGTGAT	CAGCACTTG
LAC 7-45	351	TTTTTATGAT	ACTAAAAAAG	GTGGTAATCC	TTTGTGTGAT	CAGCACTTG
LAC 12-26	351	TTTTTATGAT	ACTAAAAAAG	GTGGTAATCC	TTTGTGTGAT	CAGCACTTG
LAC 17-5	351	TTTTTATGAT	ACTAAAAAAG	GTGGTAATCC	TTTGTGTGAT	CAGCACTTG
LAC 17-18	351	TTTTTATGAT	ACTAAAAAAG	GTGGTAATCC	TTTGTGTGAT	CAGCACTTG

Figure 3. Sequence of the *Onchocerca lupi* cytochrome oxidase subunit 1 gene amplicons recovered from infected flies, southern California, USA, 2012. Gray shading indicates areas of sequence identity. Labels refer to the sample number of the individual infected flies from which the sequences were obtained. *O. lupi* GenBank accession no. KC763786. LAC, Los Angeles County.

To determine the identity of the infected flies, we amplified a portion of the black fly mitochondrial 16S rRNA gene from the remaining DNA samples. This sequence has previously been shown to be phylogenetically informative in distinguishing several North American black fly species (29). A comparison of the sequence data obtained from the amplicons with the GenBank sequence database showed that the sequences were most similar to members of the genus *Simulium*; however, an exact match was not obtained to any of the sequences in GenBank, which

precluded identification of the infected flies to the species level (data not shown). To identify the flies to the species level, we collected black fly larvae from sites near the locations from which the infected flies were trapped (online Technical Appendix Figure 3). These larvae were bisected and preserved for molecular and cytotoxic analyses. The diagnostic portion of the 16S rRNA gene was then amplified from the collected larvae and compared with the sequences obtained from the infected flies. Larvae that had sequences matching those of the infected flies exactly were

Table. Species identification of *Onchocerca lupi*-infected flies, southern California, USA, 2012

Infected fly no.	Collection date	Location	Latitude	Longitude	Species and genotype*
LAC 3-2	2013 Apr 22	Monterey Park City Yard	34.05341	-118.116537	<i>Simulium tribulatum</i> A
LAC 5-1	2013 Apr 24	Bernard Biostation	34.116667	-117.7125	<i>S. tribulatum</i> A
LAC 7-45	2013 Apr 29	Walnut Coop	34.029101	-117.854616	<i>S. tribulatum</i> A
LAC 12-26	2013 May 7	Rainbow Canyon Ranch	34.144731	-117.935686	ND
LAC 17-5	2013 Jun 4	Santa Fe Dam	34.116667	-117.95	<i>S. tribulatum</i> B
LAC 17-18	2013 Jun 4	Santa Fe Dam	34.116667	-117.95	<i>S. tribulatum</i> B

*LAC, Los Angeles County; ND, definitive identification not successful.

then identified by cytotaxonomy. Of the 6 infected flies, 5 were identified as *S. tribulatum* using this process (Table). Two 16S mitochondrial alleles were identified in the population of larvae and infected flies identified as *S. tribulatum*. These were designated *S. tribulatum* A and *S. tribulatum* B, which were 97% similar to one another (online Technical Appendix Figure 4). *S. tribulatum* B was identified in the infected flies from the Santa Fe Dam, whereas infected flies from the Monterey Park City Yard, Bernard Biostation, and Walnut Coop contained *S. tribulatum* A (Table). The sequence of the infected fly from Rainbow Canyon Ranch matched that of 1 larva in the collection; however, definitive cytotaxonomic identification of this larva was not successful because of poor fixation.

Discussion

Our data imply that *O. lupi* infection in dogs is ongoing in southern California. The possibility that dogs might be serving as sentinels for this infection suggests that humans and cats in the area also could be at risk for infection.

Several other *Onchocerca* species are endemic to North America. However, except for *O. lupi*, all of these are known to use ungulates as their primary hosts. Thus, isolation of these parasites from dogs, together with the phylogenetic analysis of 3 different gene sequences that all group the isolates with *O. lupi*, strongly support the identification of these parasites as *O. lupi*.

The nested PCRs detected *CO1*-derived amplicons with sequences 99.5%–100% identical to the published *O. lupi* *CO1* sequence in 6 flies. Some of these sequences could be derived from other *Onchocerca* because *Simulium* spp. flies are known to be vectors for several *Onchocerca* species for which sequence data are not available (33,34). However, previous studies have suggested that sequence variation in the mitochondrial genome varies from 7% to 15% among *Onchocerca* species (9,35), and intraspecies variation within the mitochondrial genome is limited in the genus *Onchocerca* (9,35). Therefore, the sequences detected in the flies are unlikely to have derived from a species other than *O. lupi*.

Our data suggest that the black flies collected frequently fed on a host species that was infected with *O. lupi*, a host that remains unidentified. However, our data implicate *S. tribulatum* flies as the vector for *O. lupi* in southern California. *S. tribulatum*, a member of the *S. vittatum* species

complex, is one of the most abundant and widespread species of *Simulium* flies in North America (36). *S. tribulatum* flies generally feed on large mammals (e.g., cattle or horses) and rarely bite humans (36).

The implication of *S. tribulatum* flies as a possible vector for *O. lupi* also might provide insight about the reasons that *O. lupi* cases have primarily been found in the southwestern United States. Cities and human settlements there typically rely on anthropogenic water sources, such as aquifers, reservoirs, and other water impoundments. The *S. vittatum* complex (of which *S. tribulatum* is a member) includes some of the few black fly species in North America that prosper in these environments (36).

The assay we used to detect *O. lupi* in the black flies cannot distinguish between viable and nonviable parasites or immature and infective larvae. Thus, although our data implicate *S. tribulatum* flies as the vector, additional studies are needed to confirm this hypothesis. Laboratory colonies of *S. vittatum*, a sibling species of *S. tribulatum*, could prove useful in confirming that flies of this species complex are actually competent vectors for this parasite (37).

The black flies that tested positive for *O. lupi* came from geographic locations adjacent to the San Gabriel River and its watershed. During the past 20 years, southern California has tried to restore natural watershed and wetland habitats, including those in the San Gabriel Valley area (38). Black flies rely on rivers and other bodies of water, often with aquatic vegetation for egg laying and larval development, all of which the San Gabriel River and Los Angeles River watersheds provide.

The San Gabriel Mountains are directly upstream of the sites from which we collected the larvae and positive flies. The San Gabriel River, its watershed, and its recreational areas are likely to be providing a wildlife corridor that enables an easily accessible transmission interface. Although most cases in canids have been described in domestic dogs, the relative rarity of infections in domestic animals suggests that the parasite uses a different species as its primary reservoir. The ubiquitous presence of coyotes and other nondomestic canids in the San Gabriel watershed might provide a convenient natural reservoir for the parasite. Additional studies involving sampling of the coyote population in the area, coupled with molecular identification of the blood meals taken by the local black flies (39), would be useful in resolving these questions.

Prevention of *O. lupi* infection ultimately might rely on effective *Simulium* control programs, which must address black fly breeding in a variety of settings. The most effective control methods used for the past 20 years in the San Gabriel Valley have been applications of VectoBac 12AS (*Bti*) (K. Fujioka, San Gabriel Valley Mosquito and Vector Control District, pers. comm.) and occasionally stopping the flow of water for a minimum of 48 hours because the larvae are vulnerable to desiccation (40). The role of ivermectin, milbemycin, and other heartworm preventive medications commonly used in dogs and cats is unknown. These medications would probably kill microfilariae, but their efficacy against infective L3 larvae of *O. lupi* is unknown. These medications in pets may play a role in preventing infection or in preventing infected pets from serving as reservoir hosts, reducing transmission of this infection.

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Mr. Hassan is a research associate in the Department of Global Health at the University of South Florida. His research interests focus on the development of novel diagnostic tests for, and the ecology of, vector-borne diseases, with an emphasis on human infections with *Onchocerca* spp.

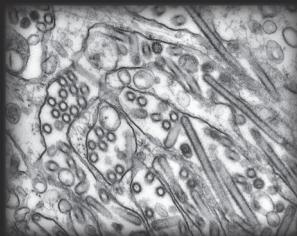
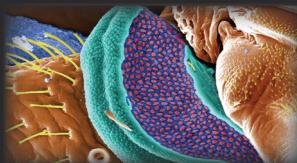
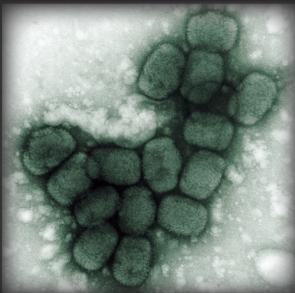
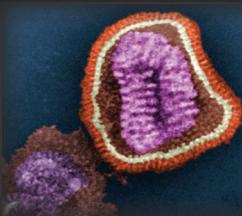
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Molecular Epidemiology of *Plasmodium falciparum* Malaria Outbreak, Tumbes, Peru, 2010–2012

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During 2010–2012, an outbreak of 210 cases of malaria occurred in Tumbes, in the northern coast of Peru, where no *Plasmodium falciparum* malaria case had been reported since 2006. To identify the source of the parasite causing this outbreak, we conducted a molecular epidemiology investigation. Microsatellite typing showed an identical genotype in all 54 available isolates. This genotype was also identical to that of parasites isolated in 2010 in the Loreto region of the Peruvian Amazon and closely related to clonnet B, a parasite lineage previously reported in the Amazon during 1998–2000. These findings are consistent with travel history of index case-patients. DNA sequencing revealed mutations in the *Pf dhfr*, *Pf dhps*, *Pf crt*, and *Pf mdr1* loci, which are strongly associated with resistance to chloroquine and sulfadoxine/pyrimethamine, and deletion of the *Pf hrp2* gene. These results highlight the need for timely molecular epidemiology investigations to trace the parasite source during malaria reintroduction events.

During the past decade, remarkable progress in malaria control has been achieved globally (1). As low-risk areas progress toward the preelimination phase of malaria elimination (<http://www.who.int/malaria/areas/elimination/overview/en/>), new challenges are posed by risk for reintroduction of parasites into areas where malaria transmission was interrupted (2). Human movement from malaria-endemic regions could facilitate outbreaks in areas where malaria had been eliminated (2,3). Molecular epidemiology tools have been used to investigate the sources of malaria reintroduction (4,5). Use of these tools

enables rapid characterization of potentially pathogenic or multidrug-resistant strains before they become adapted and expand to other non-malaria-endemic areas where anopheline vectors are present (6–9).

In Peru, malaria reemerged in the 1990s and the number of cases peaked at ≈160,000 cases in 1998 (10). Most reported cases had occurred in the Amazon Basin (Loreto region) and areas in the northern Pacific coast of Peru, including the Tumbes and Piura regions. In vivo efficacy studies conducted during 1998–2000 revealed different patterns of drug resistance between parasites in the Amazon region and coastal areas (11,12). Although parasites from the eastern Amazon region were resistant to chloroquine and sulfadoxine/pyrimethamine, parasites from the northern Pacific coast were resistant to chloroquine but remained sensitive to sulfadoxine/pyrimethamine (11–13). In 2001, artesunate/mefloquine combination therapy was introduced in the Peruvian Amazon while artesunate-sulfadoxine/pyrimethamine remained in use in the northern Pacific coastal region (12).

After 2005, changes in drug policy and increased vector control efforts in Peru led to a drastic reduction in the number of malaria cases in the country. A major accomplishment was the interruption of *Plasmodium falciparum* transmission in the northern Pacific coast; no autochthonous malaria case has been reported since 2006. However, in October of 2010, the Regional Health Directorate in Tumbes received reports of 2 cases of *P. falciparum* malaria. An outbreak investigation confirmed the *P. falciparum* malaria epidemic in Tumbes. This outbreak continued to spread through 2012, when the last case of *P. falciparum* malaria was reported. Epidemiology investigations identified 2 index case-patients among military personnel stationed in Tumbes; surveillance activities conducted during the outbreak investigation suggested that these patients potentially acquired *P. falciparum* infection while in the Peruvian Amazon. We therefore hypothesized that a detailed genetic characterization of the parasite populations isolated during this outbreak might provide a better understanding of the source and main biological features of the parasite responsible for the reintroduction of malaria into Tumbes.

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Previous genetic analyses of *P. falciparum* strains collected at the peak of the malaria epidemic, 1999–2000, revealed at least 5 distinct clonal lineages (clonets A–E), as defined by genotyping of 7 neutral microsatellite loci (14). These clonets, which were distributed in different areas of Peru, exhibited distinct patterns of mutations based on sequencing of the *Pfprt*, *Pfmdr1*, *Pfdhps*, and *Pfdhfr* genes (14). Considering these historical data, we tested the following hypotheses. First, if the *P. falciparum* outbreak in Tumbes was caused by bottlenecked parasites from the coastal region, the parasites causing this outbreak would be genetically similar or closely related to clonet E, which was the only lineage found in the northern Pacific coast during 1999–2000. Second, if the parasite was introduced from the Peruvian Amazon, then the parasites causing this outbreak would be related to clonets A, B, C, or D. Third, if these parasites were introduced from outside Peru, they may have different molecular signatures.

Materials and Methods

Study Area and Sample Collection

The Tumbes region is located in the Pacific northwestern part of Peru near the border with Ecuador (Figure 1, panel A). Tumbes is divided into 13 districts with a total surface of 4,670 km² and a population of ≈228,227 (Figure 1, panel B). In the late 1990s, malaria transmitted by *P. vivax* and *P. falciparum* was highly endemic to the region (11,15). In Tumbes, *Anopheles albimanus* mosquitoes predominate, unlike in the Amazon region, where *An. darlingi* mosquitoes predominate. In the 2000s, malaria incidence was drastically reduced, and the parasite predominance shifted to *P. vivax*, which is seasonal in this area, peaking during the rainy season (February–June). The last autochthonous case of *P. falciparum* malaria in Tumbes was reported in 2006.

In October 2010, after the Regional Health Directorate received reports of 2 cases of *P. falciparum* malaria,

an outbreak response team led by the Ministry of Health of Peru with the support of the US Naval Medical Research Unit No. 6 (NAMRU-6), the US Centers for Disease Control and Prevention (CDC), and the US Agency for International Development conducted an outbreak investigation and response. As part of these activities, malaria cases were detected by passive surveillance of febrile patients seeking treatment at local health facilities or at the regional referral hospital. Additional cases were detected by various case-finding activities conducted in areas where laboratory-confirmed *P. falciparum* malaria cases were found. For all patients, thick and thin smears stained with Giemsa 10% were examined for parasites. Slides were read at the local health facility and sent to the National Institute of Health in Peru (INS) and NAMRU-6 for species confirmation and quality control. Blood was spotted onto Whatman 3MM filter paper (GE Healthcare, Atlanta, GA, USA) and sent to INS and NAMRU-6. For some cases, whole blood was collected by venipuncture and shipped to INS and NAMRU-6. All biological samples were collected exclusively for the purpose of diagnosis, case investigation, and patient management as part of a public health intervention led by the Ministry of Health of Peru.

DNA Isolation and PCR Analysis

DNA was isolated from filter paper blood spots or whole blood samples by use of the QIAamp DNA Blood Mini Kit (QIAGEN, Valencia, CA, USA) as described elsewhere (16). Nested PCR was used to confirm *P. falciparum* infection for all patients in this study (17). Molecular analysis was performed at the NAMRU-6 laboratory in Lima, Peru. Selected samples were sent to CDC for further genetic characterization.

Microsatellite Analysis

Whole-genome amplified DNA (REPLI-g; QIAGEN) was used for microsatellite characterization. All *P. falciparum*–

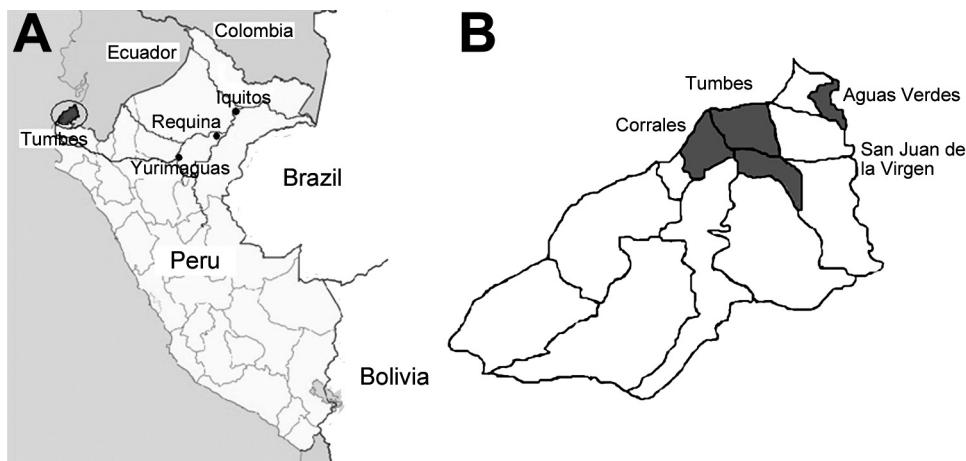


Figure 1. Peru, showing the department of Tumbes (dark gray shading), the city of Iquitos, and the Requena district located in the Loreto region of the Peruvian Amazon (A) and the 13 districts in the department of Tumbes (B). Gray shading indicates the 4 districts (Tumbes, Corrales, Aguas Verdes, and San Juan de la Virgen) where the 210 cases were reported during the 2010–2012 outbreak of *Plasmodium falciparum* malaria; blue lines indicate travel routes by river; red line indicates travel route by road.

confirmed samples were assayed for 7 putatively neutral microsatellite loci. In studies in South America, 5 microsatellite loci have been used: TA1 (chromosome 6); poly a (chromosome 4); PfkPK2 (chromosome 12); TA109 (chromosome 6), and 2490 (chromosome 10) (18–20). In addition to these markers, we also amplified the loci C2M34 (chromosome 2) and C3M69 (chromosome 3) (21). Primer sequences and PCR parameters for these loci have been described (14,16). Furthermore, all samples were assayed for 5 microsatellite loci that span \approx 11 kb on chromosome 4 around *Pfdhfr*, 9 loci that span 17 kb on chromosome 8 around *Pfdhps*, 4 loci that span 11 kb on chromosome 7 around *Pfcrt*, and 6 loci that span 8 kb on chromosome 5 around *Pfmdr1*. The primers used to amplify microsatellite loci have been described (16,22). The amplification products were labeled with fluorescent dyes (HEX or FAM) and assayed for size on an 3130xl sequencer (Applied Biosystems, Foster City, CA, USA). The fragments were then scored by using GeneMapper software version 3.7 (Applied Biosystems) with default microsatellite settings, whereby bands of <500 relative fluorescence units were defined as background. Samples for which we obtained no amplification in some loci were reanalyzed to complete the haplotypes. Earlier, we had created haplotype identifiers for each of these genes on the basis of microsatellite loci that were nearby (16).

Genotyping of Markers of Drug Resistance

DNA isolates were sequenced for point mutations in *Pfdhfr*, *Pfdhps*, chloroquine resistance transporter (*Pfcrt*), and *Pfmdr1*. The methods used are described elsewhere (14,16,21,23,24).

Detection of *P. falciparum* Histidine-Rich Protein 2 Deletions

Two sets of primers were designed to amplify a 228-bp fragment of *P. falciparum* histidine-rich protein 2 (*Pfhrp2*) in a nested PCR. The outward forward primer was 5'-GGTTTCCTTCTCAAAAATAAAG-3', and the outward reverse primer was 5'-TCTACATGTGCTTGAGTTTCG-3'. The secondary reaction used 5'-GTATTATCCGCTGCCGTTTTTGGC-3' (forward) and 5'-CTACACAAGTTATTATTAATGCGGAA-3' (reverse) primers. The cycling conditions were as follows: primary reaction at 95°C for 5 min; 30 cycles of 95°C for 30 s, 60°C for 30 s, 68°C for 30 s; and 68°C for 5 min; and secondary reaction at 95°C for 5 min; 30 cycles of 95°C for 30 s, 65°C for 30 s, 68°C for 30 s; and 68°C for 5 min.

Ethics Considerations

The activities were conducted in compliance with all applicable federal and international regulations governing the protection of human subjects. No informed consent was requested from the patients because all biological samples

were collected as part of a public health intervention led by the Ministry of Health of Peru. All samples received by INS, CDC, or NAMRU-6 were coded, and no access to personal identifiable data was provided.

Results

During October 2010–June 2012, a total of 210 cases of *P. falciparum* malaria were reported in Tumbes. Filter paper or whole blood samples were available for laboratory testing for 57 (27%) of the 210 patients. Figure 2 shows the temporal distribution of all 210 cases reported during the outbreak and the 57 cases that were included in this study. PCR confirmed *P. falciparum* mono-infection in 54 patients; *P. vivax/P. falciparum* mixed infections in the other 3 patients led to their exclusion from further analysis. To determine the clonal composition of the isolates, we genotyped 7 neutral microsatellite markers located in 6 different chromosomes. We observed virtually the same clonal lineage across all 54 isolates with the exception of 3 isolates that had alleles that were 3 bp shorter in loci TA1 and PfkPK2 (Table 1). These results suggested that the outbreak originated from a single parasite source or from various sources of the same parasite population.

In a previous study, we found 5 distinct clonal lineages (clonets A–E) among *P. falciparum* isolates collected in the Peruvian Amazon (14). We compared the genotype of the outbreak parasite to genotypes in a historical microsatellite database and found that the genotype of the isolates from Tumbes closely resembled clonet B, a lineage found in the eastern Peruvian Amazon during 1999–2006 (14). However, the outbreak haplotype differed at 2 loci: C2M34 (232 vs. 226 bp) and C3M69 (134 vs. 149 bp) (Table 1; 14). On the basis of these results, we postulated that the isolates from Tumbes might be related to clonet B. Surveys and available patient records revealed that 7 malaria cases occurred among members of a military facility in Tumbes. At least 1 of these patients reported having traveled to

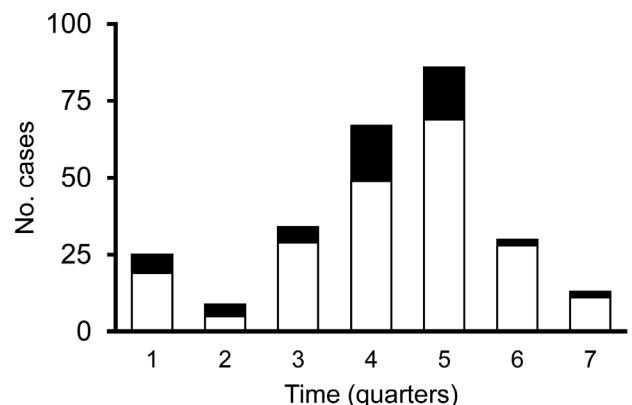


Figure 2. Temporal distribution of cases of falciparum malaria reported to the Tumbes Regional Health Directorate, Peru, October 2010–June 2012. Black bar sections indicate number of cases with samples available for analysis.

Table 1. Genetic lineage based on 7 neutral microsatellite markers of representative *Plasmodium falciparum* isolates from malaria outbreak in Tumbes and Amazon Region, Peru, 2010–2012*

Code	Collection Date	Isolation area	Genetic lineage	Chromosome location, locus name						
				6 TA1	4 Poly α	7 PfPK2	6 TA109	10 2490	2 C2M34	3 C3M69
MIS 0760	2010 Oct 25	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS 0761	201 Oct 26	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS 1142	2011 Mar 31	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS 1147	2011 Jul 19	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS 1146	2011 Jul 26	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS 1168	2011 Aug 19	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS1280	2011 Aug 27	Tumbes	B _{V1}	172	183	172	164	84	232	134
TI-435	2011 Sep 22	Tumbes	B _{V1}	172	183	172	164	84	232	134
TIA-4156	2011 Sep 28	Tumbes	B _{V1}	169	183	172	164	84	232	134
T3-861	2011 Oct 4	Tumbes	B _{V1}	169	183	169	164	84	232	134
TIA-4333	2011 Oct 14	Tumbes	B _{V1}	172	183	172	164	84	232	134
MIS1278	2011 Nov 1	Tumbes	B _{V1}	172	183	172	164	84	232	134
T3-989	2011 Nov 18	Tumbes	B _{V1}	172	183	172	164	84	232	134
M01L1615	2011 Dec 1	Tumbes	B _{V1}	169	183	172	164	84	232	134
TIA-434	2011 Dec 1	Tumbes	B _{V1}	172	183	172	164	84	232	134
PSSI-205	2011 Dec 27	Corrales	B _{V1}	172	183	172	164	84	232	134
Z1-22	2012 Mar 14	Zarumilla	B _{V1}	172	183	ND	164	84	232	134
PR-12†	Dec 2009	Requena (C)	B _{V1}	172	183	172	164	84	232	134
PY013†	Mar 2010	Yurimaguas (E)	B _{V1}	172	183	172	160	84	232	134
Clonet B†	1999–2000	Loreto (E)	B	172	183	172	164	84	226	149
Clonet A†	1999–2000	Loreto (E)	A	169	172	166	164	84	240	132
Clonet C†	1999–2000	Loreto (E, W)	C	178	164	163	160	80	246	136
Clonet D†	1999–2000	Loreto (E, W)	D	178	161	175	160	80	233	122
Clonet E†	1999–2000	Tumbes	E	172	148	175	160	74	226	138

*Microsatellite genotypes of all 11 isolates from Requena and 1 of 12 isolates from Yurimaguas were identical to those of the Tumbes outbreak. Only 17 of 54 isolates processed are shown. Isolates were selected to cover the extent of the duration of the outbreak (October 2010–March 2012). ND, not determined.

†Historical microsatellite alleles of clonets A–E found in Peru in 1999–2000 and the B_{V1} variant found in Loreto in 2009–2010. C, Central Amazon; E, eastern Amazon; W, western Amazon.

Yurimaguas, a small town in the Peruvian Amazon ≈400 km southwest of the city of Iquitos. For this reason, we compared the genotype of the parasites from Tumbes with those from 23 samples collected during 2009–2010 from Yurimaguas (12 samples) and from the Requena District (11 samples), which is 250 km northeast of Yurimaguas in the Peruvian Amazon (Figure 1). For all 11 isolates from Requena and 1 from Yurimaguas, the microsatellite genotypes were identical to that of the isolates from Tumbes—we named this parasite B_{V1} variant (Table 1).

To gain further insight into the drug-resistance pattern of the *P. falciparum* B_{V1} variant, we performed DNA sequencing to characterize point mutations in *Pfdhfr*, *Pfdhps*, *Pfprt*, and *Pfmdr1*, which have been associated with resistance to sulfadoxine/pyrimethamine and chloroquine. Similar to clonet B, all outbreak samples shared 437/540/581 *Pfdhps*, SVMNT *Pfprt*, and 184/1034/1042/1246 *Pfmdr1* genotypes. However, clonet B and the B_{V1} lineage differed in *Pfdhfr* haplotype. Whereas clonet B was 51I/108N/164L, the B_{V1} outbreak lineage and parasite isolate from Requena had the 50R/51I/108N *Pfdhfr* haplotype (Table 2; 14). Typing of microsatellite loci near the boundaries of the *Pfdhfr* and *Pfmdr1* genes showed that the B_{V1} variant belonged to the haplogroup DHFR-D1, which in a previous study was found to be a rare haplogroup in Iquitos in 2006–2007 (only found in 3

[5%] of 62 samples); and MDR-A1, which in a previous study was found to be abundant in Iquitos in 2006–2007 (frequency >50%) (16).

Because 40% of *P. falciparum* isolates from the Peruvian Amazon have major deletions of the genes coding for HRP2 and HRP3 (25), the most commonly used targets of rapid diagnostic tests (25), we investigated the presence of these deletions in these samples. All isolates in this outbreak lacked the *Pfhrp2* gene (Table 2).

Discussion

Our molecular epidemiology investigation identified a possible source of the *P. falciparum* parasites causing a major outbreak of malaria in Tumbes, Peru, a region that had been free of falciparum malaria since 2006. Our results suggested that this outbreak was caused by a single introduction of a parasite population that originated in the Loreto region of the Peruvian Amazon. These parasites had a chloroquine- and sulfadoxine/pyrimethamine-resistant mutation pattern. This study illustrates the value of molecular epidemiology tools during malaria outbreak investigations and malaria reintroduction events.

All *P. falciparum* samples available for testing belonged to a single genetic lineage, according to their nearly identical microsatellite genotypes. For 2 isolates, 2 alleles were slightly different sizes (169 vs. 172 bp). This variation

Table 2. Drug-resistant allele haplotypes of representative *Plasmodium falciparum* isolates from the Tumbes outbreak, clonets A–E, and isolates from the Amazon region, Peru, 2010–2012*

Code	Collection date	Source	Gene haplotypes				
			<i>Pfdhfr</i>	<i>Pfcr</i>	<i>Pfdhps</i>	<i>Pfmdr1</i>	<i>Pfhrp2</i>
MIS 0760	2010 Oct 25	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS 0761	2010 Oct 26	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS 1142	2011 Mar 31	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS 1147	2011 Jul 19	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS 1146	2011 Jul 26	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS 1168	2011 Aug 19	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS1280	2011 Aug 27	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
TI-435	2011 Sep 22	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
TIA-4156	2011 Sep 28	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
T3-861	2011 Oct 4t	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
TIA-4333	2011 Oct 14t	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
MIS1278	2011 Nov 1	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
T3-989	2011 Nov 18	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
M01L1615	2011 Dec 1	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
TIA-434	2011 Dec 1	Tumbes	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
PSSI-205	2011 Dec 27	Corrales	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
Z1-22	2012 Mar 14	Zarumilla	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
PR-12	2009 Dec 17	Requena	50R/51I/108N	SVMNT	437G/540E/581G	184F/1034C/1042D/1246Y	Neg
PY013	2010 Mar	Yurimaguas	ND	ND	ND	ND	Neg
Clonet B	1999–2000	Loreto (E)	51I/108N/164I	SVMNT	437G/581G	184F/1042D and 184F/1034C/1042D/1246Y	4/18
Clonet A	1999–2000	Loreto (E)	51I/108N/164I	SVMNT	WT, 437G/581G, and 437G/540E/581G	184F/1034C/1042D and 184F/1034C/1042D/1246Y	1/24
Clonet C	1999–2000	Loreto (E, W)	108N	CVMNT	WT	184F/1034C/1042D	5/25
Clonet D	1999–2000	Loreto (E, W)	108N	CVMNT	540-SYN	184F/1034C/1042D and 184F/1034C/1042D/1246Y	9/15
Clonet E	1999–2000	Zarumilla	WT and 108N	CVMNT	WT	ND	Pos

*Only representative alleles are included in this table. Gene haplotypes are indicated by numbers where a mutated amino acid was found. C, central Amazon; E, eastern Amazon; W, western Amazon; neg, *Pfhrp2* negative; pos, *Pfhrp2* positive, 1/24, 1 of 24 isolates positive for *Pfhrp2*; ND, not determined; WT, wild type.

could represent microevolution of parasites during this outbreak, which has been observed during outbreaks involving other microsporidia parasites (26).

Because one of the first patients reported in October 2010 in Tumbes had a history of travel to Loreto, we hypothesized that the parasite population causing this outbreak could be associated with parasites from Loreto. The genotype of the parasites from Tumbes was identical to that of 11 of 11 *P. falciparum* isolates collected from the Requena District in Loreto in 2010 and 1 of 12 *P. falciparum* isolates collected from Yurimaguas (V. Udhayakumar, pers. comm.). Further analysis revealed that the Tumbes genotype was closely related to clonet B, which we previously reported to have been introduced to Loreto from Brazil before the major malaria epidemic in the 1990s (14,16). Furthermore, the parasite from Tumbes was highly unrelated to clonet E, which was the only parasite population found in the northern coast of Peru before falciparum malaria was eliminated in this region (14). These results demonstrate that the outbreak of falciparum malaria in Tumbes was most likely caused by a single event, the introduction of a parasite population from Loreto.

Consistent with the neutral microsatellite findings, all other genetic markers tested supported the aforementioned conclusion. Included were the chloroquine-resistant

SVMNT genotype, pyrimethamine-resistant genotype (*Pfdhfr* 50R, 51I, and 108N), sulfadoxine-resistant genotype (437G, 540E, and 581G), and deletion of the *Pfhrp2* gene.

Several observations are relevant to the recent resurgence of malaria in Tumbes. First, this lineage has a new *Pfdhfr* triple mutant genotype (50R, 51I, 108N) that was mainly found outside of Peru in South America (14,16,21,27). This genotype seems to have been introduced into Peru during or after peak transmission of malaria within Peru because such genotypes were only reported in, not before, 2006 (14,16). Second, in the northern coast of Peru, artesunate-sulfadoxine/pyrimethamine combination therapy was being used for the treatment of *P. falciparum* malaria, while this newly introduced parasite strain had mutation patterns consistent with resistance to chloroquine and sulfadoxine/pyrimethamine. Coincidentally, malaria cases reported during this outbreak were treated with artesunate and mefloquine to decrease the risk for treatment failure. Therefore, the use of artesunate-sulfadoxine/pyrimethamine for the treatment of *P. falciparum* in the northern coast of Peru, as is currently recommended by treatment guidelines published by the Ministry of Health of Peru, may not be appropriate in this region because of the risk for malaria reintroduction from the Amazon region. Third, a region with no documented evidence of *Pfhrp2*-deleted

parasites suddenly became populated with such a parasite strain, thereby making HRP2-based rapid tests inadequate diagnostic tools for this investigation. Misdiagnosis could have occurred if HRP2-based rapid tests were used as the primary diagnostic tool in this region.

In summary, this study provides experimental evidence of the value of timely molecular epidemiology investigations for pinpointing the source of *P. falciparum* re-introduction in areas working toward malaria elimination. Additionally, these data point out that future screening of military recruits (or any other migrant population from malaria-endemic areas such as the Amazon region) for the presence of malaria and provision of appropriate treatment can help prevent future re-introduction of malaria in areas from which it had been eliminated.

Acknowledgments

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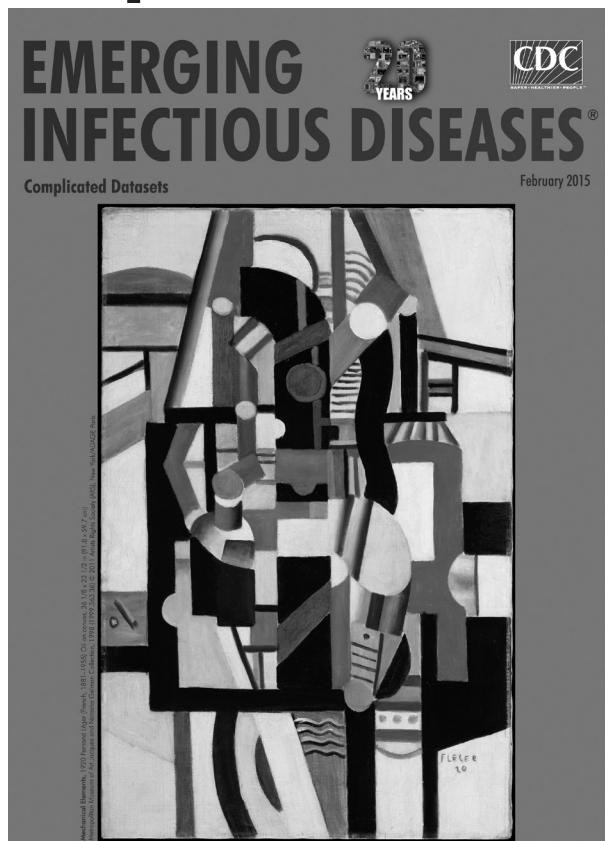
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February 2015: Complicated Datasets



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Delayed-Onset Hemolytic Anemia in Patients with Travel-Associated Severe Malaria Treated with Artesunate, France, 2011–2013

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Artesunate is the most effective treatment for severe malaria. However, delayed-onset hemolytic anemia has been observed in $\approx 20\%$ of travelers who receive artesunate, $\approx 60\%$ of whom require transfusion. This finding could discourage physicians from using artesunate. We prospectively evaluated a cohort of 123 patients in France who had severe imported malaria that was treated with artesunate; our evaluation focused on outcome, adverse events, and postartesunate delayed-onset hemolysis (PADH). Of the 123 patients, 6 (5%) died. Overall, 97 adverse events occurred. Among the 78 patients who received follow-up for >8 days after treatment initiation, 76 (97%) had anemia, and 21 (27%) of the 78 cases were recorded as PADH. The median drop in hemoglobin levels was 1.3 g/dL; 15% of patients with PADH had hemoglobin levels of <7 g/dL, and 1 required transfusion. Despite the high incidence of PADH, the resulting anemia remained mild in 85% of cases. This reassuring result confirms the safety and therapeutic benefit of artesunate.

Intravenous (IV) artesunate has been the recommended first-line treatment for severe malaria worldwide since 2010 (1). Two large randomized trials showed a 35.0% reduction (from 22.0% to 15.0%) in death rates among

adults in Asia and a 22.5% (from 10.9% to 8.5%) reduction among children in Africa when artesunate was compared with parenteral quinine in the treatment of severe malaria (2,3). Four case series performed in Western countries reported death rates of $<4\%$ (4–7).

Artesunate is generally considered safe (8). However, its use in Western countries has shown that delayed hemolytic events occur in $\approx 20\%$ of patients with severe imported malaria, and 60% of these patients require blood transfusion (4,6,7,9–11). Delayed-onset anemia (herein referred to as postartesunate delayed-onset hemolysis [PADH] pattern of anemia) has been observed to occur 2–3 weeks after initiation of IV artesunate, after complete clearance of parasites, and to resolve during weeks 3–6 (7). The mechanism of this anemia is hemolytic, as demonstrated by high serum lactate dehydrogenase (LDH) and low plasma haptoglobin levels. Across several studies, no common conventional cause of hemolysis was identified (4,6,12–14). In a comparative study, PADH anemia was described in 5 of 8 patients with hyperparasitemia treated with artesunate alone or combined with quinine; it was not seen in patients treated with quinine alone. This finding supports the assumption that this side effect is associated with artesunate (11). PADH anemia has not been reported in meta-analyses (8) nor observed in large clinical trials (2,3). However PADH has been reported recently in children in Africa (15).

This PADH is a matter of concern for the medical community. Without a systematic assessment of the incidence and outcome of artesunate-associated PADH anemia, a slowdown may occur in the ongoing change toward favoring treatment with artesunate rather than quinine, a less-efficient treatment for severe malaria. The World Health Organization recently recommended increased vigilance

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¹Members of the French Artesunate Working Group are listed in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/5/14-1171-Techapp1.pdf>).

for PADH anemia and called for a more precise description of its incidence, time course, and severity (16). To determine the effectiveness and safety of artesunate in patients with severe imported malaria, we focused on PADH anemia cases detected through an existing artesunate surveillance program in France.

Materials and Methods

Temporary Use Authorization Program and Treatment

In May 2011, IV artesunate (60-mg vial of powder and solvent) became available in France through the Agence Nationale de Sécurité du Médicament, the French national drug agency. The product, manufactured by Guilin Laboratories in China, was imported to Europe by ACE Pharmaceuticals. Within the framework of a temporary use authorization program, data were prospectively collected during May 2011–May 2013 from medical charts and by using Agence Nationale de Sécurité du Médicament forms that were completed by attending physicians at the beginning and end of treatment. A dedicated team at the National Reference Center for Malaria (NRCM) retrieved the data. Additional data were obtained from the national pharmacovigilance system and an NRCM database, as described (17). Retrieved data included age, sex, native country, place of malaria acquisition, immunocompromised status, pregnancy status, appropriateness of chemoprophylaxis, purpose of travel, duration of disease before treatment, location where artesunate was prescribed, drug used as first-line treatment, median duration/dose of artesunate treatment, duration of hospitalization, outcome, clinical and biologic criteria for severe malaria, and duration of follow-up. The artesunate database was implemented and informed consent was obtained from patients in accordance with a procedure common to all French National Reference Centers (<http://www.legifrance.gouv.fr/affichTexte.do?cidTexte=JORFTEXT000000810056&dateTexte=&categorieLien=id>). Data were collected and analyzed anonymously. After diagnosis, all patients received IV artesunate (2.4 mg/kg) at 0, 12, and 24 hours and daily thereafter until oral antimalarial treatment could be administered to complete treatment as recommended in France (18). For patients who received a full 7-day course of IV artesunate, no other treatment was administered.

Case Definitions

Severe malaria was defined as malaria in persons with blood smears positive for asexual forms of *Plasmodium falciparum* parasites and at least 1 criterion of severity according to the definition of severe malaria used in France (19,20). Patients with mixed-species infections were excluded from analysis.

Anemia was defined as a blood hemoglobin level of <12 g/dL in female and <13 g/dL in male patients (reference

values 12.0–16.5 and 13.0–17.5 g/dL, respectively). Hemolysis was defined as a plasma haptoglobin level of <0.1 g/L (reference value 0.55–2.50 g/L), plasma lactic dehydrogenase (LDH) level of >390 IU/L (reference value 190–390 IU/L), or both. We defined 3 patterns of anemia as previously described (7,10,11): PADH, non-PADH, and indeterminate. The PADH pattern was defined by 1) a new drop in the hemoglobin level after day 8 of treatment initiation and the appearance or reappearance of hemolytic markers (>10% drop in hemoglobin or >10% rise in LDH levels) occurring any time between day 8 and the end of follow-up and/or 2) by any information in the medical chart referring to acute hemolysis occurring after day 8. The non-PADH pattern was defined by a hemoglobin nadir and a hemolysis peak occurring before day 8, with or without positive markers of hemolysis after day 8 and without a nadir or sudden drop of hemoglobin after day 8 as defined for the PADH pattern. The indeterminate pattern was defined as all other cases of anemia for which information was lacking or with an evolution pattern that did not fit the other patterns.

Sample Collection

Blood samples were routinely collected from the patients on days 0, 2 (± 1), 7 (± 2), 14 (± 3), 21 (± 3), and 28 (± 3) after treatment initiation (18,21). Samples were analyzed to determine the levels of hemoglobin, total bilirubin, glucose, plasma bicarbonate, lactate, serum creatinine, blood urea nitrogen, LDH, haptoglobin, and parasitemia and the reticulocyte count.

Cure, Evolution, and Side Effects

The death rate at day 28 was the main clinical endpoint. Parasitological cure was defined as a *P. falciparum*-negative blood smear on day 7, with possible confirmation later. Relapse was defined as the reappearance of fever and a blood smear positive for asexual *P. falciparum* parasite forms at any time after a first negative result during the 28-day follow-up period. All side effects reported on medical charts were recorded and graded by using the National Institutes of Health grading system (<http://www.niaid.nih.gov/LabsAndResources/resources/DAIDSClinRsrch/Documents/daidsaegradingtable.pdf>).

Statistical Analyses

Travel characteristics and demographic, clinical, and laboratory variables were evaluated. Quantitative variables were expressed as medians (quartiles 1–3 [Q1–3]) or, when appropriate, as means (SEMs). Qualitative variables were expressed as percentages. Differences between groups (survivors vs. nonsurvivors, patterns of anemia) were analyzed by using the Fisher exact test for categorical variables and Mann-Whitney test for continuous variables. Statistical analyses were performed by using IBM SPSS Statistics

version 20 (IBM, Armonk, NY, USA). All reported *p* values are 2-tailed.

Results

General Presentation of Cohort

A study flowchart is provided in Figure 1. Demographic and clinical characteristics of the 123 patients who received artesunate treatment are summarized in online Technical Appendix Table 1 (<http://wwwnc.cdc.gov/EID/article/21/5/14-1171-Techapp1.pdf>).

Effectiveness

Among the 123 patients with severe malaria treated with artesunate, 117 fully or partially recovered and 6 died from malaria (death rate 4.9%, 95% CI 2.0–10.8). All deaths were related to severe multiorgan failure and occurred within 3 days of receiving artesunate.

The following characteristics were seen more frequently at admission in patients who died versus those who survived: lower median Glasgow Coma Scale (median score 10 [Q1–3: 3–13] vs. 14 [Q1–3: 14–15], *p* = 0.001; reference score 15); respiratory distress (50% [3/6] vs. 9% [11/117], *p* = 0.019); higher median parasitemia level (11% [Q1–3: 8–26] vs. 6% [Q1–3: 2–10], *p* = 0.05); higher median lactate level (10 mmol/L [Q1–3: 3–12] vs. 2 mmol/L [Q1–3: 2–3], *p* = 0.002; reference value <1.8 mmol/L); higher total bilirubin concentration (98 μmol/L [Q1–3: 98–209] vs. 49 μmol/L [Q1–3: 26–75], *p* = 0.007; reference value 2–17 mmol/L); lower glucose level (<2.2 mmol/L in 50% [3/6] vs. <2.2 mmol/L in 3% [4/117], *p* = 0.002; reference value 3.9–5.8 mmol/L); and renal insufficiency (80% [5/6] vs. 9% [11/117], *p* < 0.001). Age, sex, immunocompromised state, place of malaria acquisition, cardiocirculatory impairment (*p* = 0.098), and severe anemia at day 0 were not significantly associated with death. The median time between symptom onset and initiation of artesunate treatment was 1.5 days (Q1–3: 1–5) in the 6 patients who died versus 4.0 days (Q1–3: 2–5) in those who survived (*p* = 0.13). Artesunate was used as second-line treatment after quinine in 2 of 6 patients who died versus 49 of 117 patients who survived (*p* = 1). All survivors had complete parasite clearance before treatment day 7. Only 1 relapse was observed; it occurred 26 days after a 3-day course of IV artesunate that was not followed by the recommended oral course of antimalarial drug therapy.

Safety

Safety data were available for days 0–8 and 9–28 for 123 and 78 patients, respectively. All reported adverse events resolved during follow-up. A summary of the severity of reported adverse events possibly associated with artesunate is shown in Table 1.

Rash, telogen effluvium, and mild pruritus were recorded for 1 patient each. The rash occurred several days after the end of artesunate administration and was considered unrelated to artesunate. Telogen effluvium was diagnosed

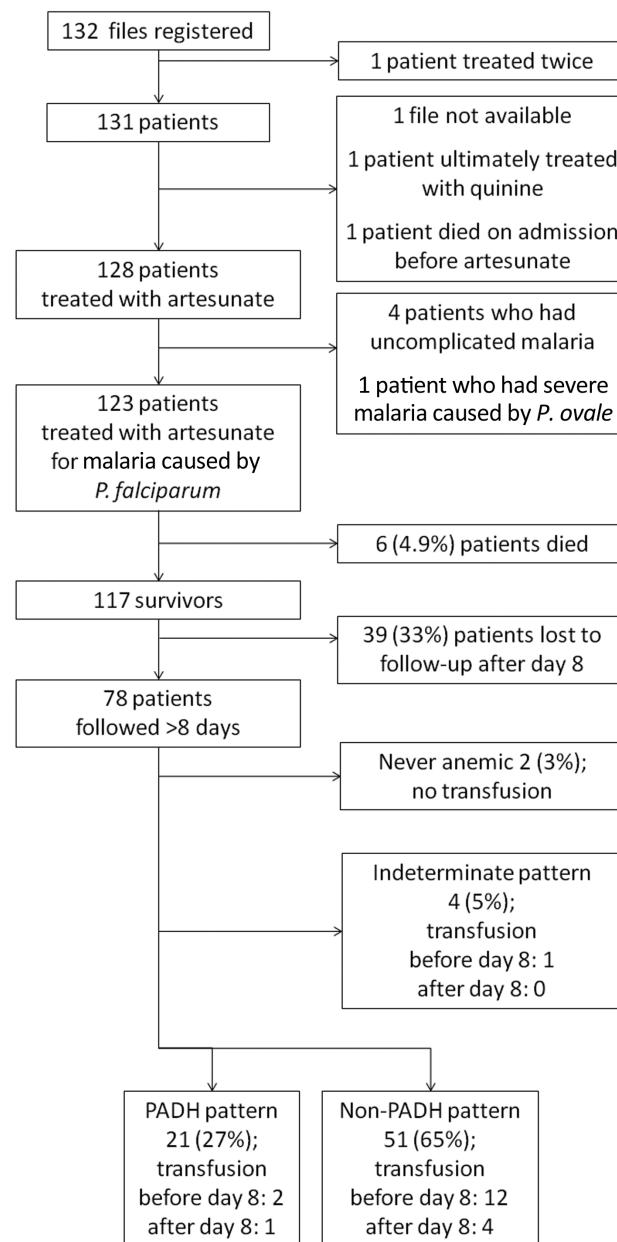


Figure 1. Distribution of PADH and non-PADH patterns of anemia in a prospective analysis of delayed-onset hemolytic anemia in patients with severe imported malaria treated with artesunate, France, 2011–2013. Of 123 patients who received treatment, 6 died and 39 were lost to follow-up after day 8, leaving a total of 78 patients with sufficient clinical and/or biologic information to fulfill the anemia definition criteria for PADH or non-PADH classification. Indeterminate pattern, cases of anemia for which information was lacking or with an evolution pattern that did not fit PADH or non-PADH patterns; PADH, postartesunate delayed-onset hemolysis.

Table 1. Severity of reported adverse events possibly associated with artesunate treatment of severe imported malaria in 117 patients, France, 2011–2013*

Adverse event	Grade 1, mildly severe	Grade 2, moderately severe	Grade 3, severe	Grade 4, life threatening	Total
Cutaneous	2†	1‡	0	0	3
Ataxia, tremor, CNS ischemia	0	2	1	1	4
Tinnitus	1	0	0	0	1
Cardiac and arterial ischemia	1	1	1	1	4
Hypertension	0	0	1§	0	1
Elevated level of ALT	2	2	3	1	8
Hyperkalemia	1	0	0	0	1
Anemia¶	20	11	27	17	75
Total	27	17	33	20	97

*Adverse events were obtained from medical charts and graded by using the National Institutes of Health classification for side effects (<http://www.niaid.nih.gov/LabsAndResources/resources/DAIDSClinRsrch/Documents/daidsaegradingtable.pdf>). ALT, alanine transaminase; CNS, central nervous system.

†Mild pruritus and rash.

‡Telogen effluvium.

§Regressive retinopathy.

¶Grading was done during the follow-up by using available nadir of hemoglobin. For HIV-negative patients, the severity of anemia was graded as follows: grade 1, 10.0–10.9 g/dL; grade 2, 9.0–9.9 g/dL; grade 3, 7.0–8.9 g/dL; grade 4, <7.0 g/dL. For HIV-positive patients, the severity of anemia was graded as follows: grade 1, 8.5–10.0 g/dL; grade 2, 7.5–8.4 g/dL; grade 3, 6.5–7.4 g/dL; grade 4, <6.5 g/dL.

during the 28 days following treatment initiation. The pruritus occurred during artesunate treatment and disappeared without intervention.

Liver enzyme levels increased in 8/117 patients who survived, including 6 who concurrently received ≥ 1 medication(s) with liver toxicity as a potential side effect (paracetamol [acetaminophen] or nonsteroidal antiinflammatory drug). All these episodes occurred before day 8. Vision loss occurred in 1 patient and was considered by the attending ophthalmologist to be associated with hypertensive retinopathy. Two cases of acute cerebellar syndrome occurred; both were thought to be associated with a postmalaria neurologic syndrome. Tinnitus was reported in 1 patient who received quinine just before artesunate. One patient experienced continuous tremors that resolved spontaneously.

QTc lengthening (i.e., corrected lengthening of the interval between start of the Q wave and end of the T wave in the heart's electrical cycle) and transient bradycardia were recorded for 1 and 2 patients, respectively. One transient bradycardia (54 bpm) episode occurred between 2 artesunate injections and resolved spontaneously. In the patient with QTc lengthening (460 ms; reference value <440 ms), artesunate treatment (total dose 480 mg) was changed to artemether/lumefantrine (4 tablets, each with 20 mg artemether and 120 mg lumefantrine) and then to atovaquone/proguanil (4 tablets/d for 3 d, each with 250 mg atovaquone and 100 mg proguanil) because of persistent QTc lengthening (560 ms) accompanied by low potassium levels (<3 mmol/L; reference range 3.5–5.0 mmol/L). These 2 patients were 13 and 15 years of age and weighed 62 kg and 40 kg, respectively. Another patient experienced severe disseminated intravascular coagulation that led to arterial ischemia of extremities and central nervous system ischemia (caudate nuclei, corona radiata, and white matter).

Amputation of fingers and legs was necessary. All sequelae in this patient were considered related to severe malaria.

One patient with myasthenia gravis received artesunate and experienced no worsening of the disease (22). Four patients were pregnant; 1 of the pregnancies was discovered during therapy. One miscarriage occurred; hemorrhage led to a blood transfusion. Artesunate was well tolerated in 2 women during the second and third trimesters and in 1 during labor. Hypoglycemia was not recorded during artesunate treatment.

Anemia, Hemolysis, and Transfusion

Anemia commonly occurred, was slow to resolve, and followed variable patterns (Figures 1, 2). Of the 78 patients with appropriate follow-up, 76 (97.4%, 95% CI 91.0%–99.7%) experienced anemia (Figure 1). The non-PADH and PADH patterns were observed in 51 (65.4%, 95% CI 53.8%–75.8%) and 21 (26.9%, 95% CI 17.5%–38.2%) patients, respectively, with or without transfusion, who received clinical and/or laboratory follow-up beyond day 8. In the PADH group, hemoglobin levels dropped a median of 1.3 g/dL from day 7 (± 2) to day 14 (± 3), but levels ranged from 4.6 g/dL to 12.9 g/dL on day 14 (± 3). During days 11–27, a total of 8 patients had severe anemia, of whom 4 experienced typical PADH anemia ($p = 0.4$) (Figure 2). Of the 21 total patients with PADH, 3 (14.3%, 95% CI 3.1%–36.3%) had blood hemoglobin levels of <7 g/dL (6.2, 4.6, and 6.3 g/dL) during week 2 after treatment initiation.

With the exception of hemoglobin level, no parameters were significantly associated with a particular anemia pattern (Table 2; online Technical Appendix Table 2); median hemoglobin levels at day 0 were estimated to be 11.3 g/dL (Q1–Q3: 9.6–13.1) and 13.6 g/dL (Q1–Q3: 11.6–15.4), respectively, for patients with non-PADH and PADH anemia

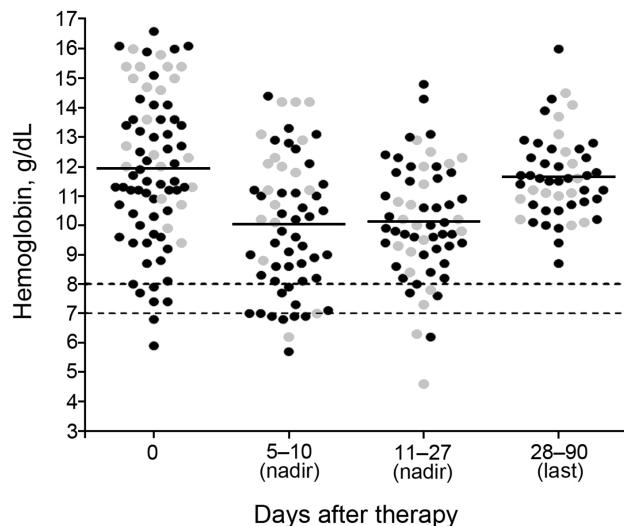


Figure 2. Nadir and last hemoglobin levels for 78 patients in a prospective analysis of delayed-onset hemolytic anemia in patients with severe imported malaria treated with artesunate, France, 2011–2013. Gray dots, hemoglobin level for patients with the postartesunate delayed-onset hemolysis (PADH) pattern of anemia; black dots, hemoglobin level for patients with non-PADH pattern of anemia, indeterminate pattern and nonanemic patients. Dotted lines represent hemoglobin level thresholds of 8 or 7 g/dL.

($p = 0.002$) (Tables 2, 3). The incidence of the PADH anemia did not differ between patients who received artesunate as first-line treatment and those who received quinine before being switched to artesunate ($p = 0.38$, Fisher exact test). During week 2, the rise in LDH levels and the

drop in haptoglobin levels were consistent with hemolytic anemia in the PADH group (Figure 3); these changes lasted ≈ 1 –2 weeks. Median LDH levels remained high at days 21 (724 IU/L [range 344–1,564] and 28 (497 IU/L [range 177–922])). Haptoglobin remained undetectable during weeks 2 and 3 (Table 3; Figure 3). During subsequent weeks, hemoglobin levels rose slowly in the non-PADH group (Table 3). Maximum reticulocyte production occurred during weeks 2 and 3 for the non-PADH and PADH groups, respectively (Figure 3; Table 3). At day 28, patients in both groups had hemoglobin levels >11 g/dL.

Of the 78 patients, 15 (19.3%, 95% CI 10.5%–28.0%) received a total of 20 blood transfusions, 15 (75%) of which were performed before day 8, during the acute phase of the disease (Figure 1). Among the 21 patients with delayed hemolysis, 1 (4.8%, 95% CI 0.1%–23.8%) had a blood transfusion after day 8 (hemoglobin nadir 6.3 g/dL). No deaths were related to any side effects, including anemia.

Discussion

According to NRCM data, each year in France, ≈ 250 patients are treated for severe imported malaria. In this cohort of 123 patients treated for severe malaria in high-care settings, IV artesunate was effective and generally safe. The death rate was 5%, and blood transfusion was necessary for $<20\%$ of all patients and for $<5\%$ of patients with PADH anemia. Compared with retrospective case series, our prospective approach reduced bias toward severe anemia cases and provided a robust evaluation of artesunate safety, particularly as concerns PADH anemia.

Table 2. Association between selected variables and non-PADH and PADH patterns of anemia in 72 patients with severe imported malaria treated with artesunate, France, 2011–2013*

Variable	Pattern		p value
	Non-PADH, n = 51†	PADH, n = 21‡	
Sex, no. (%)			
M	29 (57)	11 (52)	0.8§
F	22 (43)	10 (48)	
Age, y, median (Q1–Q3)	42 (27–52)	41 (31–53)	0.7¶
Location of birth, no. (%)			
Africa	31 (61)	10 (48)	0.3§
Europe, North America	18 (35)	11 (52)	
South, Central America	2 (4)	0	
Asia	0	0	
Duration of illness before artesunate treatment, median d (Q1–Q3)	4 (3–5)	4 (3–5)	0.8¶
Hyperparasitemia, no. (%)			
$>4\%$ infected erythrocytes	28 (55)	16 (76)	0.1§
$>10\%$ infected erythrocytes	13 (25)	5 (24)	1§
Parasitemia level at day 0, median % infected erythrocytes (Q1–Q3)	5.0 (1.4–10.1)	7.1 (3.75–14.5)	0.1¶
Hemoglobin level, median g/dL (Q1–Q3), at day 0	11.3 (9.6–13.1)	13.6 (11.6–15.4)	0.002¶
Total dose of artesunate, median mg (Q1–Q3)	840 (540–1035)	800 (676–955)	0.8¶
Artesunate first-line treatment for current severe malaria episode, no. (%)	29 (57)	15 (71)	0.3§

*PADH, postartesunate delayed-onset hemolysis; Q1–Q3, quartiles 1–3.

†Non-PADH pattern was defined by a hemoglobin nadir and a hemolysis peak occurring before day 8, with or without positive markers of hemolysis after day 8 and without a nadir or sudden drop of hemoglobin after day 8.

‡PADH pattern was defined by 1) a new drop in the hemoglobin level after day 8 of treatment initiation and the appearance or reappearance of hemolytic markers occurring any time between day 8 and the end of follow-up and/or 2) by any information in the medical chart referring to acute hemolysis occurring after day 8.

§Fisher exact test.

¶Mann-Whitney test.

Table 3. Laboratory values for 72 patients with artesunate-treated severe imported malaria and a PADH or non-PADH pattern of anemia during days 0–28 after treatment initiation, France, 2011–2013*

Patient group, laboratory test	Median value (range), no. results available				
	Day 0	Day 7	Day 14	Day 21	Day 28
Non-PADH†					
Hemoglobin level, g/dL‡	11.3 (5.9–16.6), 51	9.2 (5.7–13.1), 62	9.9 (6.2–14.3), 32	10.6 (7.7–13.0), 22	11.5 (8.2–13.9), 30
Reticulocyte count, G/L	61 (3–183), 16	60 (2–444), 25	156 (75–412), 20	108 (56–204), 12	73 (34–100), 12
LDH level, IU/L	777 (161–3,003), 24	803 (312–2,722), 28	537 (261–1,139), 17	521 (201–905), 12	464 (240–798), 15
Haptoglobin level, g/L	0.05 (0.00–2.20), 8	0 (0.00–2.90), 25	0 (0.00–2.50), 20	0 (0.00–2.00), 10	0.40 (0.00–1.40), 12
PADH§					
Hemoglobin level, g/dL¶	13.6 (9.4–16.0), 21	11.2 (6.2–14.2), 20	9.9 (4.6–12.9), 24	10.0 (6.7–13.4), 15	11.1 (10–15), 17
Reticulocyte count, G/L	61 (61–61), 1	34 (8–132), 12	124 (63–315), 15	162 (90–431), 12	127 (63–223), 13
LDH level, IU/L	846 (293–1,195), 9	634 (510–793), 14	1,128 (554–4,000), 17	724 (344–1,564), 13	497 (177–922), 14
Haptoglobin level, g/L	0 (0.00–0.20), 5	0 (0.00–1.50), 15	0 (0.00–0.60), 18	0 (0.00–0.00), 13	0 (0.00–1.50), 15

*G/L, Giga/L; LDH, lactate dehydrogenase; PADH, postartesunate delayed-onset hemolysis.

†Non-PADH pattern was defined by a hemoglobin nadir and a hemolysis peak occurring before day 8, with or without positive markers of hemolysis after day 8 and without a nadir or sudden drop of hemoglobin after day 8.

‡Median drop in hemoglobin during the first week of treatment was 2.1 g/dL.

§PADH pattern was defined by 1) a new drop in the hemoglobin level after day 8 of treatment initiation and the appearance or reappearance of hemolytic markers (defined as >10% drop in hemoglobin or >10% rise in LDH levels) occurring any time between day 8 and the end of follow-up and/or 2) by any information in the medical chart referring to acute hemolysis occurring after day 8.

¶Median drop in hemoglobin during the first week of treatment was 2.4 g/dL.

The 5% death rate in this cohort is lower than rates observed among artesunate-treated adults who received treatment in malaria-endemic countries (2,3) but similar to rates among smaller cohorts of travelers from non-malaria-endemic countries whose treatment was managed in facilities with high levels of care (23,24). A retrospective study in the United Kingdom that compared 143 quinine-treated patients with 24 artesunate-treated patients reported no deaths, fewer intensive care unit admissions, and shorter durations of hospitalization for artesunate-treated patients (25). In a study of 400 severe imported *P. falciparum* malaria cases treated with quinine (10% death rate), the major factors associated with death were low Glasgow Coma Scale score, respiratory failure, severe renal impairment, hyperlactemia, or hypoglycemia (23) during the first 24 hours after admission. Despite our use of different data-capture methods and severity scores, we found the same factors associated with death in this cohort of artesunate-treated patients.

PADH occurred in 27% of patients in this study, but it was rarely associated with severe anemia and was never fatal. Previous observations (7) may have been partially biased toward the most severe cases; in contrast, our prospective approach efficiently captured asymptomatic mild or moderate anemia cases. In our study, the median delayed drop in hemoglobin levels was 1.3 g/dL. Although PADH accounted for most of the severe anemia cases (Figure 2), only 3 (15%) patients had hemoglobin levels of <7 g/dL, and only 1 received a transfusion. This transfusion rate (<5%) is markedly lower than that previously reported for patients with severe imported malaria and delayed-onset

anemia (~60%) (4,6,7,11,13). Taken together, our results demonstrate that, in the setting of severe imported malaria, delayed hemolysis does not alter the life-saving effect of IV artesunate, but it does need focused medical attention and follow-up.

With the exception of the pretreatment hemoglobin level, no parameters, including the cumulative dose of artesunate and initial parasitemia levels, were correlated with the risk for delayed hemolysis in our study. Some authors have associated high parasitemia levels with delayed-onset hemolysis (6,7,11); others have demonstrated that not all patients with high parasitemia levels experience late-onset hemolysis (4). Other factors are related to the peculiar mode of action linked to artemisinin derivatives and are probably involved in delayed-onset hemolysis (4,26). Indications pointing to the involvement of pitting (27,28), a process whereby dead parasites are expelled from infected erythrocytes, has been reported (29,30). We have shown that the risk for PADH is linked to the peak number of pitted erythrocytes rather than the absolute initial level of parasitemia (31).

Side effects of artesunate frequently include gastrointestinal disturbances, neutropenia (1.3%), reticulocytopenia (0.6%), and elevated liver enzymes (1.1%) (32–34). In studies of patients with travel-associated malaria treated in non-malaria-endemic countries, no severe hemodynamic, cardiac, or allergic reactions were attributed to artesunate (4,5,7,11). Artesunate is considered potentially cardiotoxic at doses >15 mg/kg (32). However, in our study, mild to moderate cardiotoxicity developed in 3 patients treated with the recommended 2.4-mg/kg dose. The patients were

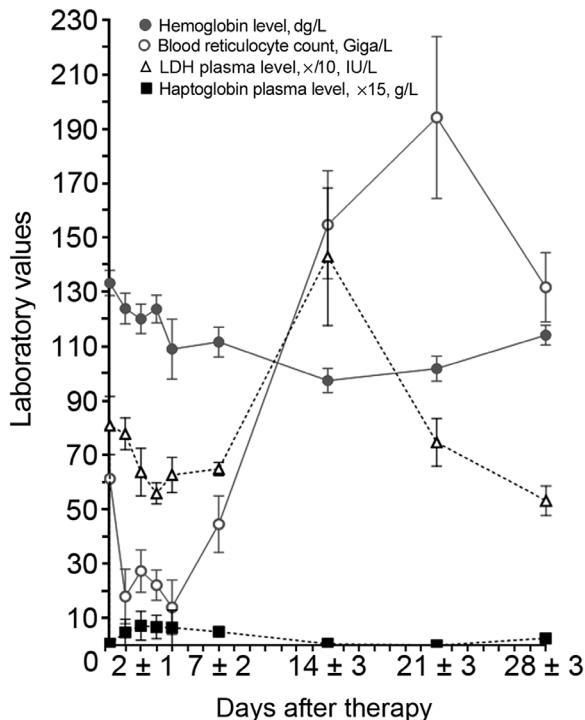


Figure 3. Typical features of postartesunate delayed-onset hemolysis and anemia for 21 patients followed in a prospective analysis of delayed-onset hemolytic anemia in patients with severe imported malaria treated with artesunate, France, 2011–2013. During the second and third weeks of late hemolytic anemia, a drop in hemoglobin occurred along with a reoccurrence of markers of hemolysis (defined as >10% drop in hemoglobin level or >10% rise in LDH concentration). Reticulocyte regeneration occurred during week 3 (delayed in comparison with other patterns of anemia, in which regeneration usually occurs during week 2 [not shown]). Values are means (SEMs). LDH, lactate dehydrogenase.

rapidly switched to another therapy, and the signs and symptoms of cardiotoxicity disappeared. Whether these cardiac episodes were related to artesunate is not clear. In a study performed in Bangladesh involving 21 adults with severe malaria treated with artesunate, 2 patients experienced QTc lengthening (>500 ms), but, as observed in 1 of our patients, hypokalemia was present (35). None of the other side effects reported as possibly attributable to artesunate in other studies (e.g., dizziness, nausea, diarrhea, anorexia, metallic taste in the mouth) were recorded in this cohort, and the neurologic episodes were considered to be related to malaria rather than artesunate by the attending physicians. As reported for artesunate-associated side effects in other studies, those in our study were generally mild.

This study has limitations. The prospective surveillance system implemented in France relies on the motivation of attending physicians and parasitologists to report data to the NRCM team because the reporting of imported

malaria cases is not compulsory in metropolitan France. Thus, some adverse events might have been overlooked. In addition, patients with PADH whose anemia was well tolerated may have gone unreported or been lost to follow-up. Furthermore, symptoms of slight delayed anemia may be confused with slow clinical recovery from severe malaria and thus remain undetected. In addition, it is likely that the 39 patients who were lost to follow-up recovered without problems instead of remaining as postinfectious patients with continuing problems. A rapid analysis, excluding patients who died, did not show any differences in demographic variables or length of hospital/intensive care unit stay between the group that was followed for 28 days and the group that was followed <8 days (data not shown). Nevertheless, it is unlikely that severe cases of delayed hemolysis or other severe side effects would be overlooked in a temporary use authorization program implemented at facilities with high levels of care, and, as mentioned, this study captured a fairly high proportion of mild to moderate cases of delayed hemolysis. Furthermore, the study was not randomized, but in the setting of severe imported malaria, it is considered unethical to repeat artesunate versus quinine trials already performed in malaria-endemic countries (3,36). The decision to use artesunate or quinine was left to the attending physician, but use of artesunate was mostly related to its availability at the hospital. It is unlikely that the decision to use/not use artesunate as a first-line treatment was made according to the clinical severity of disease in a patient.

Our prospective analysis joins other reports (16,37) in confirming the very favorable risk-to-benefit ratio of IV artesunate in the treatment of severe imported malaria, despite PADH anemia. Our results show that, in this setting, delayed hemolysis did not alter the life-saving effect of IV artesunate. Delayed hemolysis was a common occurrence among the patients but resulted in very low levels of hemoglobin in $\approx 15\%$ of cases. To ensure the appropriate diagnosis and treatment of severe anemia, the World Health Organization and national entities recommend that hemoglobin levels be assessed weekly for 1 month after artesunate administration (16,18,21). Further studies are needed to find predictive markers of hemolysis and anemia to facilitate posttreatment follow-up in travelers to and children in malaria-endemic countries (10–12,31).

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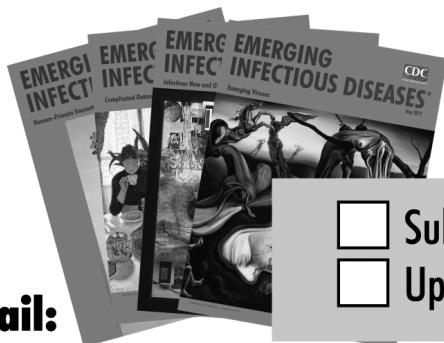
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Protective Antibodies against Placental Malaria and Poor Outcomes during Pregnancy, Benin

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Placental malaria is caused by *Plasmodium falciparum*-infected erythrocytes that bind to placental tissue. Binding is mediated by VAR2CSA, a parasite antigen coded by the *var* gene, which interacts with chondroitin sulfate A (CSA). Consequences include maternal anemia and fetal growth retardation. Antibody-mediated immunity to placental malaria is acquired during successive pregnancies, but the target of VAR2CSA-specific protective antibodies is unclear. We assessed VAR2CSA-specific antibodies in pregnant women and analyzed their relationships with protection against placental infection, preterm birth, and low birthweight. Antibody responses to the N-terminal region of VAR2CSA during early pregnancy were associated with reduced risks for infections and low birthweight. Among women infected during pregnancy, an increase in CSA binding inhibition was associated with reduced risks for placental infection, preterm birth, and low birthweight. These data suggest that antibodies against VAR2CSA N-terminal region mediate immunity to placental malaria and associated outcomes. Our results validate current vaccine development efforts with VAR2CSA N-terminal constructs.

Tissue sequestration of *Plasmodium falciparum*-infected erythrocytes drives malaria-related pathologic changes (1). Tissue sequestration is primarily mediated by members of the parasite variant antigen family of *P. falciparum* erythrocyte membrane protein 1, which is expressed on the membrane of infected erythrocytes. These proteins display extensive antigenic variation, concurrently changing receptor recognition, and tissue tropism of infected erythrocytes (2). Accumulation of infected erythrocytes in placental intervillous spaces characterizes malaria during

pregnancy (3). This sequestration of infected erythrocytes results in maternal anemia and low birthweight (LBW) (4–6), as well as consequences for child health (7–10).

Sequestration of infected erythrocytes in the placenta is mediated by VAR2CSA, the *P. falciparum* erythrocyte membrane protein 1 variant that binds to chondroitin sulfate A (CSA) on the syncytiotrophoblast (11,12). VAR2CSA is a multidomain protein (~350 kDa). Acquisition of antibodies against VAR2CSA occurs during pregnancy after exposure to infected erythrocytes sequestering in the placenta. Concentrations of these antibodies and those of antibodies that inhibit binding of infected erythrocytes to CSA (13,14) increase with parity. Furthermore, women with VAR2CSA-specific antibodies give birth to babies with higher birthweights (15). VAR2CSA-expressing parasites are the primary cause of placental malaria (16,17), which suggests that parasites can escape preexisting immunity (i.e., that naturally acquired immunity against pre-erythrocytic or erythrocytic stages of malaria does not protect against this syndrome).

The demonstration that parasites that have the *var2c-sa* knockout gene irreversibly lose the ability to adhere to CSA (18), as well as the ability of VAR2CSA to induce antibodies that inhibit adherence of placental infective erythrocytes to CSA in vitro, strongly argue for use of VAR2CSA as a vaccine against placental malaria. However, VAR2CSA-based vaccine research is challenged by the size and polymorphism of this protein and requires identification of smaller functional domains that combine an ability to induce strain-transcending antibody responses with a facility of production in a recombinant protein form. Therefore, identifying the region of VAR2CSA that induces antibodies associated with protection in multigravid women in malaria-endemic regions is a priority. The VAR2CSA critical CSA binding site is located in its N-terminal region (19–21), but the characteristics of naturally acquired antibodies against this region remain to be defined.

The Strategies to Prevent Pregnancy-associated Malaria Project, a cohort study of pregnant women enrolled early in pregnancy and followed up until delivery, was conducted during 2008–2011 in Comé in southern Benin. In

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this substudy, we assessed the effect of antibody response to placental infected erythrocytes, measured early in pregnancy and at delivery, on major pregnancy outcomes.

Materials and Methods

Study Site and Population

A detailed description of the Comé area has been reported (22). The Strategies to Prevent Pregnancy-associated Malaria Project was approved by the Comité Consultatif de Déontologie et d'Éthique of the Research Institute for Development (Paris, France) and the ethical committee of the Faculty of Health Sciences (University of Abomey-Calavi, Cotonou, Benin). Pregnant women were provided information about the study at the first antenatal visit during their first or second trimester of pregnancy (gestational age <24 weeks).

Women from whom informed consent was obtained were included in the study and followed up until delivery. A clinical examination was conducted, and 10 mL of venous blood was collected at inclusion and during each antenatal or emergency visit. Ultrasonography was performed by using a portable ultrasound system (Titan Ultrasound System; SonoSite Inc., Bothell, WA, USA) to determine exact gestational age and to plot fetal growth. Fetal growth alterations were used to define children born small-for-gestational age (SGA) (23). At delivery, peripheral and perfused placental blood samples were collected along with clinical data for the newborn.

Women were given 2 doses of intermittent preventive treatment in pregnancy with sulfadoxine/pyrimethamine (IPTp-SP) (Stichting International Dispensary Foundation, Amsterdam, the Netherlands) at least 1 month apart in the second–third trimesters of pregnancy under supervision of midwives according to national guidelines in Benin. When women had clinical symptoms between antenatal visits, they were encouraged to report to health facilities. Any participant with fever (axillary temperature $\geq 37.5^{\circ}\text{C}$) and malaria, as assessed by a rapid diagnostic test, received quinine or SP if this treatment coincided with scheduled IPTp intake.

Diagnosis of Infection with *P. falciparum*

At each visit, a rapid diagnostic test for *P. falciparum* was performed, and thick and thin blood smears were prepared and double-read according to standard procedures. At delivery, blood smears were prepared from placental blood.

Plasma Antibody against *P. falciparum*–Infected Erythrocyte Surface

Plasma samples collected at inclusion and delivery were analyzed by using *P. falciparum* strain FCR3. Parasite

cultures were selected by panning (enriching) on BeWo cells as described (24). The ability of plasma to label the surface of late-stage infected erythrocytes was tested as described (25,26). Antibody surface-labeling of ethidium bromide–positive infected erythrocytes was quantified by using flow cytometry, and data were analyzed by using CellQuest Pro or FlowJo version 7.6 (TreeStar, Ashland, OR, USA). Median fluorescence intensity was converted into relative fluorescence intensity as described (27).

Antibody-Mediated Inhibition of Infected Erythrocyte Adherence to Chondroitin Sulfate Proteoglycan

For an inhibition of binding assay (IBA), a petri dish was coated overnight with phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA) and 5 $\mu\text{g}/\text{mL}$ decorin (chondroitin sulfate proteoglycan [CSPG]; Sigma, St. Louis, MO, USA) and blocked with 3% BSA in PBS for 30 min. Late-stage FCR3 Bewo-selected infected erythrocytes were blocked in BSA/RPMI medium for 30 min. A 20% parasite suspension was incubated with plasma (1:5 dilution) or 500 $\mu\text{g}/\text{mL}$ soluble CSA for 30 min at room temperature, added to the ligand, and incubated for 15 min at room temperature (28). Nonadherent cells were removed by using an automated washing system. Cells were fixed with 1.5% glutaraldehyde in PBS and stained with Giemsa. Adherent infected erythrocytes were quantified by microscopy as number of infected erythrocytes bound per square milliliter (16).

Plasma Antibody Levels against Recombinant *P. falciparum* VAR2CSA

The full-length ectodomain of VAR2CSA (FV2) from the FCR3 strain and the truncation corresponding to Duffy binding-like (DBL) antigen (DBL1–DBL2 encompassing 2 domains, DBL3, DBL4, DBL5, and DBL6 domains) were produced in baculovirus-infected SF9 cells as described (11,19,29). Recombinant protein of *P. falciparum* apical membrane antigen 1 (PfAMA1) from the FVO strain was also used.

Levels of specific IgG against VAR2CSA were measured in plasma samples by using an ELISA as described (13). In brief, microtiter plates were coated with 0.5 $\mu\text{g}/\text{mL}$ of each protein and incubated overnight at 4°C with 100 μL of plasma at dilutions of 1:100 (for antibodies against VAR2CSA) or 1:1,000 (for antibodies against PfAMA1). Plates were washed 3 times with 0.1% PBS-Tween 20, and a 1:15,000 dilution of horseradish peroxidase–conjugated antibody against human IgG (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and incubated at room temperature for 1 h. After plates were washed 4 times, antibody reactivity was visualized at 450 nm after addition of tetramethylbenzidine (Sigma-Aldrich). The negative control pool consisted of plasma samples from

pregnant women in France who had no history of travel to malaria-endemic areas. The positive control pool consisted of plasma samples from multigravid women from Benin who had known high levels of surface reactivity to infected erythrocytes from placental isolates. Optical density values were converted into arbitrary absorbance units as described (30). Threshold of positivity was defined for each antigen from the mean \pm 3 SD response of 30 unexposed pregnant women from France.

Statistical Analysis

Categorical variables were compared by using the Fisher exact test. Comparisons between groups were made by using nonparametric tests (Kruskal-Wallis test for unpaired comparisons and paired Wilcoxon test to compare levels between inclusion and delivery within the same persons). Correlations between antibody levels and different antigens and with binding inhibition capacity of plasma were studied by using the Spearman rank correlation test.

For association between antibody levels and protection against infection or poor birth outcomes, we first considered antibody levels in plasma samples at inclusion to address a cause-effect chronology. Association between antibody levels was sought with key pregnancy outcomes, including number of peripheral *P. falciparum* infections, placental infection, LBW, maternal anemia at delivery, and preterm birth (PTB). Multivariate logistic regression modeled the effect of each antibody (defined in quartiles) on the outcome after adjustment for study center, gravidity (primigravidae versus multigravidae), and *P. falciparum* infection at inclusion.

To study the effect of antibody levels early in pregnancy on the number of infections occurring during the follow-up period, we adjusted a binomial negative model for the same covariates and offset by the duration of the follow-up period. The binomial negative distribution was used instead of a Poisson distribution to account for data overdispersion. In all models, interaction between infection at inclusion and antibody levels was tested, and results were stratified when appropriate. Type 1 error for significance was 0.05. To account for multiple testing, we applied the Holm-Bonferroni method (31); corrected *p* values are given when necessary.

Given the potential for antibody maturation during the follow-up period and a subsequent increase in specificity, association between increases in plasma binding inhibitory capacity and protection against infection was also analyzed at delivery. The same modeling approach was used with placental infection, LBW, PTB, and SGA in an appropriate subgroup of women (i.e., women with ≥ 1 documented infection during the follow-up period were classified as truly exposed).

Results

Study Profile and Population

The study site and population have been reported (22). In brief, 854 women took both doses of IPTp-SP and were followed up until delivery. One fourth of them slept under bed nets. Antibody assays were performed with samples from the 710 women for whom clinical data and plasma samples were available. A total of 326 (46%) women had ≥ 1 parasitemia throughout the follow-up period, including 116 (16%) women who were infected at inclusion. Eighty-two women who had fever received curative treatment with quinine, and 6 received other antimalarial drugs. A total of 546 microscopically detectable parasitemias were recorded. At delivery, placental infection was observed in 70 (12%) women; this infection was the only parasitemia recorded for 17 women. Prevalence of placental infection was highest in primigravid women (19%) and decreased to 12% in those with second or third pregnancies and to 9% in those with fourth or more pregnancies ($p = 0.025$, by Fisher exact test). Mean (\pm SD) birthweight was 3,002 (486) g. A total of 10% of the babies had LBW, and 8% had PTB (Table 1).

Modification of Acquisition of VAR2CSA-Specific IgG during Pregnancy by *P. falciparum* Infection

All 6 recombinant VAR2CSA proteins were detected by ELISA in plasma samples from pregnant women (Figure 1). Specific antibodies were present at high levels at inclusion and delivery, and responses to the 6 VAR2CSA recombinant proteins were correlated with each other ($0.28 < r < 0.77$, $p < 0.0001$ for all comparisons). Between inclusion and delivery, responses to all VAR2CSA proteins decreased, except for those to DBL6 and the full-length construct (FV2). The IPTp-SP that women received effectively reduced contact with blood-stage parasites.

Women were assigned to 2 subgroups: those who had ≥ 1 parasitemia during the follow-up period and those who did not (Figure 2). At delivery, IgG responses to all VAR2CSA proteins were higher for women infected during follow-up period than in the other women. In infected women, antibody responses between inclusion and delivery increased ($p < 0.001$ for all comparisons) or were unchanged (DBL5 and PfAMA-1). Conversely, for women who were not infected, antibody levels decreased, except those against DBL6 and FV2 (Figure 2). Women infected at inclusion (at blood sampling) had higher antibody responses to all VAR2CSA proteins than those who were uninfected.

Effect of Gravidity on VAR2CSA-Specific Antibody Levels

Antibody responses to VAR2CSA proteins other than DBL4 and DBL6 increased with gravidity. Plasma levels of

Table 1. Characteristics of 710 women and their infants in study of protective antibodies against placental malaria and poor outcomes during pregnancy, Benin*

Characteristic	Value
At enrollment	
Study center, no. (%)	
Akodeha	279 (39)
Comé	266 (38)
Wedeme Pedah	165 (23)
Age, y, mean \pm SD, n = 698	26.7 \pm 6.3
Gravidity, no. (%)	
Primigravidae	115 (16)
Secundigravidae	154 (22)
Multigravidae	441 (62)
Gestational age, wk, mean \pm SD	16.6 \pm 4.8
HIV status	
Positive, no. (%)	13 (1.8)
Negative, no. (%)	697 (98.2)
Hb level, g/dL, mean \pm SD, n = 704	10.6 \pm 1.3
Anemia (Hb level <11 g/dL), no. (%), n = 704	439 (62)
Malaria infection, no. (%)†	116 (16)
During follow-up	
No. antenatal visits, median (IQR)	5 (4–6)
Anemia during follow-up, no. (%), n = 708	619 (87)
No. malaria infections during follow-up, no. (%)‡	
0	384 (54)
1	183 (26)
2	89 (12)
\geq 3	54 (8)
Treatment for malaria other than IPTp-SP, no. (%)‡	88 (12)
At delivery	
Twin delivery, no. (%)	16 (2)
Birthweight, g, mean \pm SD, n = 679§	3,002 \pm 486
Low birthweight infant (<2,500 g), no. (%), n = 679§	71 (10)
Gestational age, wk, mean \pm SD, n = 680§	39.6 \pm 2.0
Small for gestational age, no. (%), n = 612§	100 (16)
Preterm birth (age <37 wk), no. (%), n = 680§	53 (8)
Hb level, g/dL, mean \pm SD, n = 649	11.0 \pm 1.4
Anemia (Hb level <11 g/dL), no. (%), n = 649	289 (45)
Placental malaria detected by blood smear, no. (%), n = 60	70 (12)

*Hb, hemoglobin; IQR, interquartile range; IPTp-SP, intermittent preventive treatment in pregnancy with sulfadoxine/pyrimethamine.

†Defined by a positive blood smear regardless of symptoms.

‡82 women received quinine and 6 received other antimalarial medications.

§Excluding twins and stillbirths.

antibodies against VSA (reactive with erythrocyte surface) showed similar profiles of gravidity dependence at inclusion and at delivery (Figure 1). Proportions of women seropositive for different antigens at inclusion and delivery are shown in Table 2. Relationships with gravidity remained for all proteins except for DBL6.

Antibody Levels at Inclusion and Association with Protection against Infection

Antibodies were tested in separate models after adjustment for study site, gravidity, and infection at inclusion. Results are summarized in Table 3. We first investigated the relationship between antibody responses at inclusion (divided into quartiles) and number of infections during the follow-up period by using binomial negative regression modeling. The independent variable was the number of infections during the follow-up period, excluding infection at inclusion, which was used as an adjustment covariate. High responses to particular VAR2CSA antigens (FV2, DBL3X)

at inclusion were associated with a lower subsequent risk for *P. falciparum* parasitemia.

We then tested whether antibody responses at inclusion were predictive of placental infection at delivery by using multivariate logistic regression. High responses to DBL3X at inclusion were associated with reduced prevalence of placental infection at delivery ($p = 0.011$). A trend between strong antibody responses against VSA and placental infection was observed.

We investigated relationships between antibody responses at inclusion and LBW. Strong IgG responses against DBL1–DBL2 were associated with reduced prevalence of LBW babies ($p = 0.003$); a similar trend was observed for responses to DBL3X ($p = 0.02$) (Table 3). High levels of these antibodies were also associated with increased mean birthweight. In addition, high levels of DBL3X-specific antibodies showed a trend toward protection against being born with SGA ($p = 0.013$). No relationships were observed for PTB or maternal anemia at delivery.

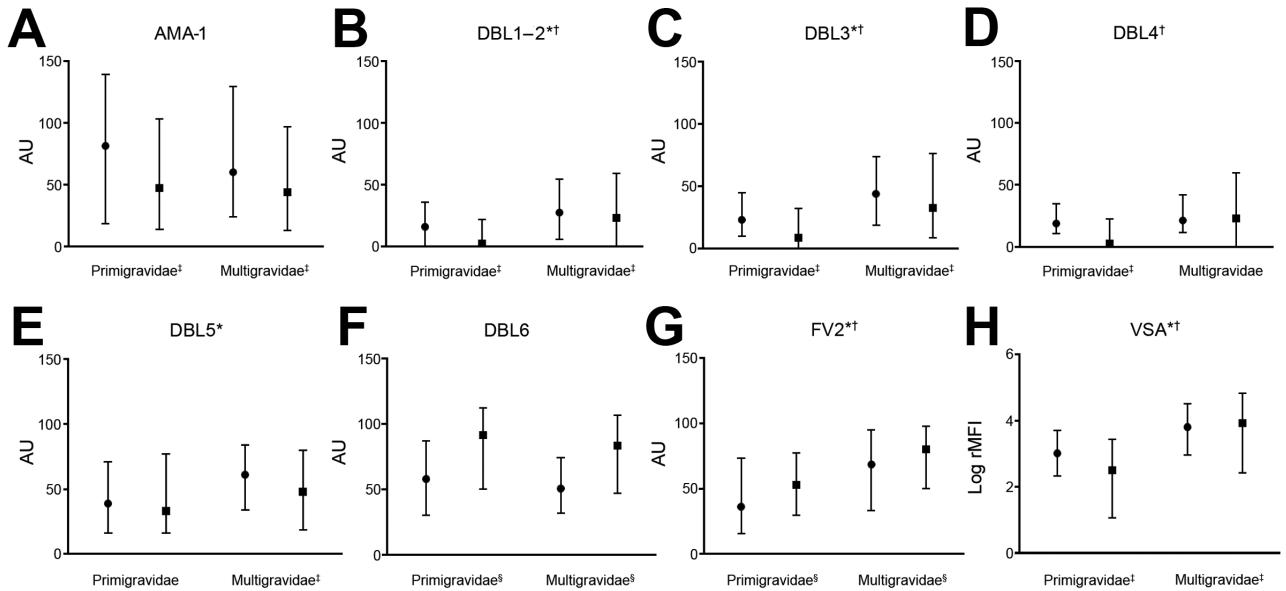


Figure 1. Antibody levels at study inclusion and delivery, by parity, against placental malaria in pregnant women, Benin. A) Apical membrane antigen 1 (AMA-1); B–F) Duffy binding-like (DBL) antigen; G) Full-length ectodomain of variant surface antigen 2 chondroitin sulfate (FV2); H) Variant surface antigen (VSA). Solid circles indicate medians for inclusion, solid squares indicate medians for delivery, and error bars indicate interquartile ranges. AU, absorbance units; rMFI, relative median fluorescence intensity. *Parity dependence at inclusion ($p < 0.05$ by Fisher exact test). †Parity dependence at delivery ($p < 0.05$ by Fisher exact test). ‡Decrease between inclusion and delivery ($p < 0.05$ by paired Wilcoxon test). §Increase between inclusion and delivery ($p < 0.05$ by paired Wilcoxon test).

Effect of Gravidity and Pregnancy-Associated *P. falciparum* Infections on Plasma to Inhibit Binding of Infected Erythrocytes to CSPG

The ability of plasma samples to inhibit infected erythrocyte binding to CSPG was higher for multigravidae than for primigravidae at inclusion ($p < 0.001$) and delivery ($p < 0.008$) (Figure 3). Unlike antibodies measured by ELISA and fluorescent-activated cell sorting, the binding inhibitory capacity increased between inclusion and delivery (Figure 3, panel A) for primigravidae ($p = 0.006$) and multigravidae ($p < 0.001$). When women were divided into subgroups according to infection history, the inhibitory capacity increased between inclusion and delivery only among women who were infected at least once ($p < 0.001$) (Figure 3, panel B).

Binding Inhibitory Capacity of Plasma and Pregnancy Outcomes

The level of IBA at inclusion was not associated with protection from adverse pregnancy outcomes (Table 3). Because IBA levels increased at delivery for women with infection during the follow-up period, we investigated the relationship between IBA levels at delivery and protection from poor outcomes. Among the 309 women infected at least once before delivery, an increase in plasma IBA activity between inclusion and delivery was associated with absence of placental infection at delivery (Figure 4, panel

A), absence of LBW (Figure 4, panel B), and absence of PTB (Figure 4, panel C) ($p < 0.0001$ for all comparisons).

Multivariate logistic regression models confirmed the protection conferred by IBA capacity at delivery against adverse outcomes of pregnancy (Table 4). Women with higher binding inhibitory capacity at delivery were less likely to deliver LBW babies (odds ratio < 1 for all quartiles in comparison to first quartile; $p = 0.028$). There was a stronger relationship with protection from SGA ($p = 0.0084$). Because of a major interaction term, we divided women into subgroups according to the presence or absence of infection at inclusion. For women not infected at inclusion, higher IBA capacity at delivery was associated with a lower risk for placental malaria ($p = 0.008$) and PTB ($p = 0.032$), but these associations were not evident for those infected at inclusion (Figure 3, panel B; Table 4).

Discussion

Primigravidae have the most severe consequences of pregnancy-associated malaria because they lack specific protective immunity. Specific immune responses are usually initiated during the first pregnancy and result in protection in subsequent pregnancies. Although several studies showed that antibodies against infected erythrocytes in the placenta are associated with improved pregnancy outcomes (13,15,32), the precise mechanisms involved remain to be clarified. Use of vaccination as a new approach to prevent

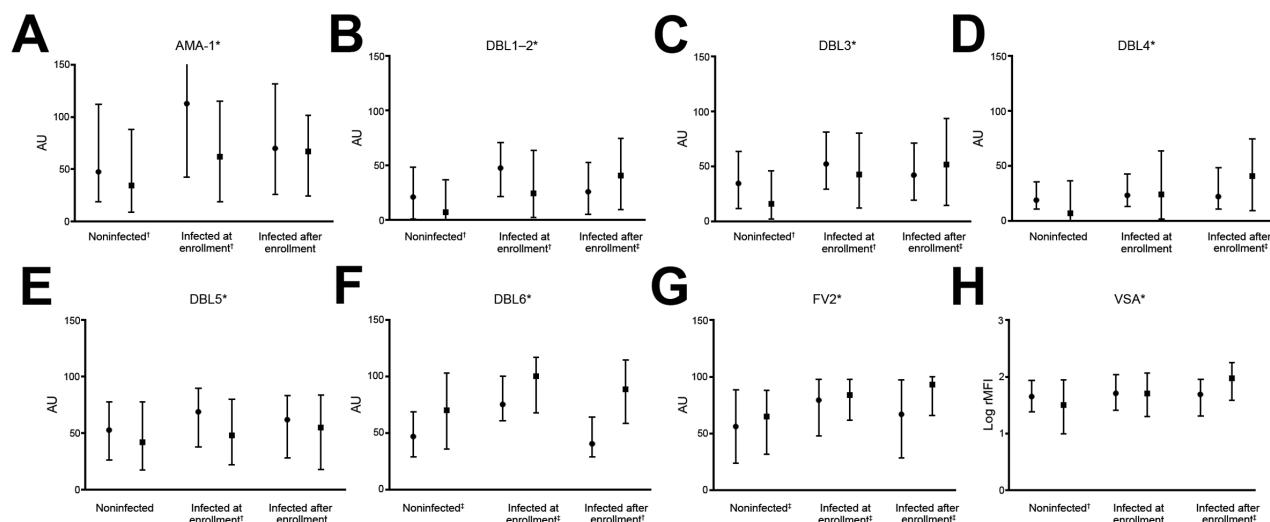


Figure 2. Antibody levels at study inclusion and delivery, by parasitemia during pregnancy, against placental malaria in pregnant women, Benin. A) Apical membrane antigen 1 (AMA-1); B–F) Duffy binding-like (DBL) antigen; G) Full-length ectodomain of variant surface antigen 2 chondroitin sulfate (FV2); H) Variant surface antigen (VSA). Solid circles indicate medians for inclusion, solid squares indicate medians for delivery, and error bars indicate interquartile ranges, and error bars indicate interquartile ranges. AU, absorbance units; rMFI, relative median fluorescence intensity. *Significantly higher in women with parasitemia during pregnancy ($p < 0.05$ by Fisher exact test). †Decrease between inclusion and delivery ($p < 0.05$ by paired Wilcoxon test). ‡Increase between inclusion and delivery ($p < 0.05$ by paired Wilcoxon test).

placental infections requires that specificities of these antibodies be determined. The current consensus is that the VAR2CSA protein specifically expressed by placental parasites is the primary target of such antibodies, but the epitopic target(s) within this large protein are yet to be defined.

One noteworthy observation is early appearance of antibodies against infected erythrocytes in the placenta in primigravidae. These women were recruited mainly at the beginning of their second trimester of pregnancy; such antibodies were present in 40%–70%. Similar prevalences among primigravidae were reported in Senegal (13) and Cameroon (33), which suggests that women are infected with placental-type parasites early in pregnancy, consistent with our previous report of placental-type parasites in pregnant women in the first trimester (34). This finding emphasizes the need to prevent *P. falciparum* infection in early pregnancy.

Parasitemia transiently decreased from 16% to 4% during pregnancy and increased to 12% at delivery. This decrease is the result of IPTp because the decrease was centered at intake periods (G. Cottrell, N. Tuikue Ndam, unpub. data). Despite IPTp, 46% of women had parasitemia during the follow-up period, which highlights the need to improve current approaches to prevent pregnancy-associated malaria.

Overall, levels of antibodies against several antigens tended to decrease during pregnancy, including those specific for placental-type parasites. Although plasma volume expansion associated with pregnancy might contribute to this overall decrease in antibody, the decrease observed also reflects a lack of exposure, as shown by the profiles in women who remained uninfected after inclusion. A previous study showed that levels of IgG against VAR2CSA

Table 2. Percentage of antibody responders, by parity, in study of protective antibodies against placental malaria and poor outcomes during pregnancy, Benin*

Antigen or assay	Enrollment			p value	Delivery			p value
	All, n = 710	Primigravida e, n = 115	Multigravidae, n = 595		All, n = 710	Primigravidae, n = 115	Multigravidae, n = 595	
AMA1	77	73	78	0.265	69	70	69	0.732
VSA	67	46	71	<0.0001	58	32	63	<0.0001
DBL1–DBL2	53	41	56	0.005	46	25	50	<0.0001
DBL3	65	45	69	<0.0001	51	28	55	<0.0001
DBL4	42	40	43	0.569	46	26	50	0.002
DBL5	71	55	74	<0.0001	58	49	60	0.035
DBL6	67	70	66	0.607	81	85	80	0.434
FV2	71	51	75	<0.0001	81	70	83	0.004
IBA	59	42	62	<0.0001	66	61	67	0.168

*Antibody responders were defined as persons having an antibody level beyond the threshold. The threshold was defined for each antigen as mean reactivity + 3 SD for 30 unexposed French pregnant women. AMA1, apical membrane antigen 1; VSA, variant surface antigen; DBL, Duffy binding-like antigen; FV2, full-length ectodomain of variant surface antigen 2 chondroitin sulfate; IBA, inhibition of binding assay.

Table 3. Association of antibody levels at enrollment with pregnancy-associated malaria and pregnancy outcomes in 710 pregnant women, Benin*

Characteristic	No malaria infections/mo		Placental malaria		Low birthweight	
	IRR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
VSA, range log RMFI	n = 709	0.288	n = 607	0.022†	n = 692	0.721
Second quartile, 2.8–3.7	0.85 (0.55–1.31)†		0.58 (0.28–1.18)		1.25 (0.65–2.43)	
Third quartile, 3.7–4.4	0.84 (0.54–1.30)†		0.43 (0.21–0.91)†		0.84 (0.41–1.75)	
Higher quartile, >4.4	1.14 (0.75–1.74)		0.40 (0.19–0.85)†		1.05 (0.51–2.17)	
VAR2CSA DBL1–DBL2, AU	n = 709	0.223†	n = 607	0.61	n = 692	0.003†
Second quartile, 4–26	0.79 (0.51–1.24)†		1.07 (0.49–2.36)		0.71 (0.39–1.30)	
Third quartile, 26–54	0.74 (0.47–1.17)†		1.46 (0.69–3.05)		0.36 (0.17–0.74)†	
Higher quartile, >54	1.01 (0.64–1.60)		0.95 (0.43–2.13)		0.33 (0.16–0.71)†	
VAR2CSA DBL3, AU	n = 706	0.0001†	n = 604	0.011†	n = 689	0.0236
Second quartile, 17–40	0.43 (0.27–0.70)†		0.45 (0.21–0.99)†		0.54 (0.28–1.05)	
Third quartile, 0–70	0.45 (0.27–0.75)†		0.41 (0.19–0.90)†		0.29 (0.13–0.65)	
Higher quartile, >70	0.80 (0.48–1.32)		0.40 (0.18–0.92)†		0.50 (0.24–1.07)	
VAR2CSA DBL4, AU	n = 703	0.122†	n = 601	0.69	n = 686	0.352†
Second quartile, 11–21	0.69 (0.44–1.07)†		0.84 (0.40–1.78)		0.83 (0.44–1.57)	
Third quartile, 21–40	0.71 (0.45–1.08)†		0.70 (0.32–1.53)		0.67 (0.34–1.32)†	
Higher quartile, >40	0.88 (0.57–1.36)		1.07 (0.52–2.22)		0.63 (0.31–1.30)†	
VAR2CSA DBL5, AU	n = 705	0.019	n = 603	0.79	n = 688	0.675
Second quartile, 29–56	0.67 (0.43–1.04)		1.15 (0.54–2.44)		1.39 (0.70–2.75)	
Third quartile, 59–84	0.82 (0.52–1.29)		0.98 (0.45–2.14)		1.25 (0.60–2.62)	
Higher quartile, >84	1.30 (0.83–2.05)		1.31 (0.60–2.84)		1.54 (0.75–3.14)	
VAR2CSA DBL6, AU	n = 352		n = 306		n = 343	0.118
Second quartile, 32–52	0.94 (0.51–1.73)	0.96	0.56 (0.20–1.56)	0.21	0.25 (0.08–0.79)	
Third quartile, 52–74	0.84 (0.44–1.60)		0.29 (0.09–0.93)		0.83 (0.36–1.96)	
Higher quartile, >74	0.95 (0.50–1.83)		0.47 (0.17–1.36)		0.65 (0.26–1.60)	
FV2, AU	n = 698		n = 596		n = 681	
Second quartile, 29–65	0.83 (0.53–1.29)	0.0005	1.39 (0.66–2.93)	0.34	0.80 (0.42–1.54)	0.55
Third quartile, 65–94	0.62 (0.39–0.99)		0.74 (0.32–1.69)		0.58 (0.28–1.21)	
Higher quartile, >94	1.52 (0.97–2.39)		0.84 (0.37–1.93)		0.82 (0.39–1.71)	
IBA, % inhibition	n = 703	0.226†	n = 602	0.65	n = 686	0.21†
Second quartile, 25–40	0.93 (0.59–1.45)		0.83 (0.39–1.76)		1.06 (0.57–1.97)	
Third quartile, 40–60	1.30 (0.84–2.01)†		0.98 (0.46–2.09)		0.68 (0.34–1.37)†	
Higher quartile, >60	1.21 (0.77–1.90)†		1.34 (0.64–2.82)		0.62 (0.30–1.29)†	
AMA1, AU	n = 706	0.463	n = 604	0.58	n = 689	0.155†
Second quartile, 23–61	0.98 (0.62–1.55)		0.78 (0.37–1.65)		0.71 (0.36–1.42)†	
Third quartile, 61–131	0.76 (0.48–1.20)		0.58 (0.27–1.25)		0.68 (0.35–1.33)†	
Higher quartile, >131	1.04 (0.65–1.65)		0.73 (0.35–1.50)		0.64 (0.32–1.27)†	

*Antibodies were tested individually after adjustment for study site, rank of gestation, and malaria at enrollment. p values were obtained by using the Wald test. Bold indicates significant p values after the Holm-Bonferroni correction was used for multiple testing. Analysis of placental malaria was restricted to 607 women who reached delivery, had antibody measurements, and had a placental blood smear. Analysis of low birthweight was restricted to 692 women who reached delivery and had an evaluation of birthweight. IRR, incidence rate ratio; OR, odds ratio; VSA, variant surface antigen; RMFI, relative mean fluorescence intensity; VAR2CSA, variant surface antigen 2 chondroitin sulfate; DBL, Duffy binding-like antigen; AU, absorbance units; FV2, full-length ectodomain of variant surface antigen 2 chondroitin sulfate; IBA, inhibition of binding assay; AMA1, apical membrane antigen 1.

†When ORs for 2 contiguous quartiles were similar, p values were computed after grouping those quartiles.

decreased rapidly over a 3-month period in the absence of antigenic stimulation (13). In women who were infected and treated only at inclusion, all antibody levels decreased between inclusion and delivery. This finding was not accompanied by their functional capacity to inhibit binding of infected erythrocytes that was maintained, which reiterates the need to distinguish between assay results in the context of vaccine development and emphasizing utility of functional assays.

Conversely, in women infected during the follow-up period, levels of antibody against VAR2CSA increased between inclusion and delivery and were higher for multigravidae than for primigravidae. The binding inhibitory capacity of plasma was also higher in multigravidae. Our study analyzed binding inhibition properties for a large number of pregnant women and corroborated results of

earlier studies, which showed parity-dependent acquisition of binding inhibitory capacity of plasma (13,14).

Multivariate analyses indicated that responses to only a restricted number of VAR2CSA domains could be deemed protective against negative outcomes during pregnancy. DBL3-specific antibodies were associated with reduced rates of infections during pregnancy and placental infection at delivery. In women in Cameroon early during pregnancy, high levels of IgG against multiple VAR2CSA domains were associated with a lower risk for placental infection at delivery (33). In our study, high levels of IgG against VAR2CSA early during pregnancy were also related to protection against subsequent infection, which highlights the role of DBL3-specific IgG. In addition, DBL1–DBL2–specific antibodies were associated with a 67% reduction in LBW. This finding emphasizes the key

role of the VAR2CSA N-terminal region that contains the minimal CSA binding site (20,21,35,36). A recent report suggested that IgG responses to the VAR2CSA minimal binding site was not pregnancy specific and that levels of these antibodies at delivery were not associated with protection from placental infection (37), which are in contrast with data from other studies.

Although the design of the study of Babakhanyan et al. (37), differed only slightly from that of our study, antigenic constructs were radically different. We used a larger VAR2CSA N-terminal construct (DBL1–DBL2), whereas Babakhanyan et al. used a minimal internal domain (ID1–ID2) construct. The other major difference was the method used to measure antibody. We used an ELISA and optimized conditions for each antigen. However, Babakhanyan et al. used a Luminex (Austin, TX, USA) assay that measures multiple analytes simultaneously in 1 reaction well and fixed default background values, which resulted in difficulties with interpretation of results. Our data highlight the need to clarify the contribution of antibodies to ID1–ID2, one of the current vaccine candidates, in protection against placental malaria. Our data indicate that antibody responses to DBL3X and DBL1–DBL2 represent surrogates of protection against placental malaria.

The functional capacity of antibodies to mediate inhibition of infected erythrocyte adherence to CSA is enhanced after infection and is sustained despite the decrease in levels of antibodies against VAR2CSA. We divided women into various subgroups by history of infection, which showed the pivotal role of infections in increasing quality of antibodies. The rationale for vaccination to prevent placental malaria is that it should induce immune memory against infected erythrocytes in the placenta, lead to an accelerated response at exposure, and limit deleterious effects of infections on pregnancy outcomes. We previously demonstrated that pregnant women might be exposed to placental-type parasites in early pregnancy, exposure increases with gestational age, and that women are more often infected with placental-type parasites later in pregnancy (38). These findings might explain why the relative increase in IBA levels was greater for infections after inclusion than in those at inclusion (Figure 3, panel B). This capacity at delivery protects against placental infection, LBW, and PTB. Antibody maturation after natural boosting leads to acquisition of a binding inhibitory property that contributes to clearing or preventing placental infection. The rationale for the association of delivery with LBW, SGA, and PTB, which has complex etiologies, is not as clear as that for placental infection. Previous studies showed that *P. falciparum* infections at delivery are strongly associated with LBW and PTB. We showed that functional antibody response at delivery is

associated with the absence of placental infection. Such functional response might prevent placental infections during pregnancy.

Our data support the idea that inducing protective immunity against placental parasites by vaccination requires induction of antibodies that inhibit binding of infected erythrocytes

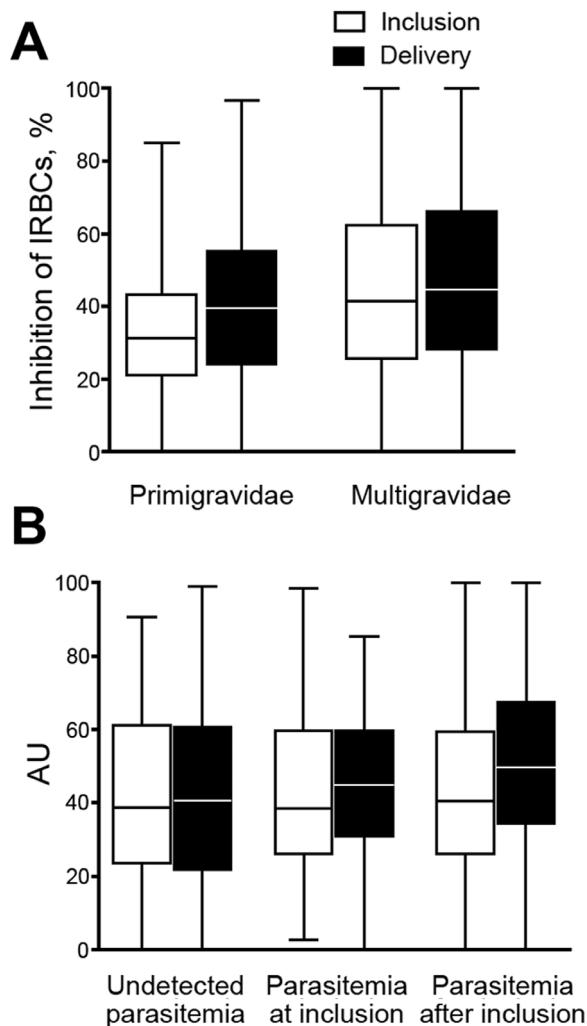


Figure 3. Binding inhibition profile of plasma from pregnant women against placental malaria, Benin. Plasma binding inhibitory capacity according to parity ($n = 109$ primigravidae and 573 multigravidae) (A) and to parasitemia during follow-up (B) ($n = 384$ women with undetected parasitemia, 115 with parasitemia detected at study inclusion, and 183 with parasitemia detected after inclusion). A) Binding inhibitory capacity was significantly higher at inclusion in multigravidae than in primigravidae and increased at delivery compared with that at inclusion in both groups (all $p < 0.05$). B) Significant increase between inclusion and delivery and a higher level at delivery in women with documented parasitemia during pregnancy ($p < 0.05$, by Fisher exact test). Horizontal lines indicate medians, boxes indicate interquartile ranges, and error bars indicate ranges. IRBCs, infected red blood cells; AU, absorbance units.

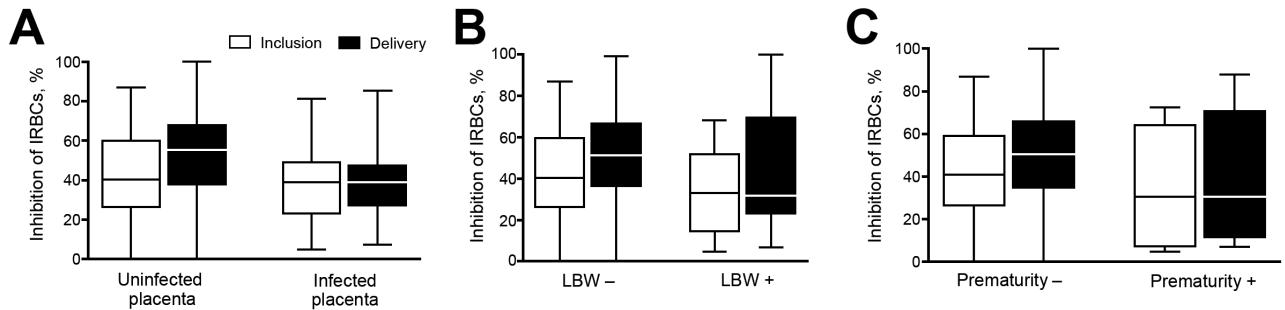


Figure 4. Binding inhibitory capacity of plasma, by adverse outcomes, in pregnant women with documented *Plasmodium falciparum* infection during follow-up, Benin. Binding inhibition was assessed according to adverse outcomes in the subgroup of women who had ≥ 1 parasitemia documented between study inclusion and delivery. A) Placental infection (52 infected placentas and 214 uninfected placentas). B) Low birthweight (LBW) (36 with LBW and 254 without LBW). C) Preterm birth (29 preterm and 269 not preterm). Horizontal lines indicate medians, boxes indicate interquartile ranges, and error bars indicate ranges. Plasma binding inhibitory capacity was significantly higher at delivery in women without adverse outcomes ($p < 0.05$, by Fisher exact test), and the increase between inclusion and delivery was also significant ($p < 0.05$, by paired-Wilcoxon test). No associations were observed at inclusion. IRBCs, infected red blood cells.

to CSA. We measured the quantity and quality of antibodies in a cohort of pregnant women for whom detailed clinical histories were available. Results provide evidence that support current efforts to develop a subunit vaccine based on VAR2CSA constructs derived from its N-terminal portion.

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Table 4. Association between inhibition capacity at delivery and 4 outcomes in 309 women with documented *Plasmodium falciparum* infection during follow-up, Benin*

IBA levels at delivery, % inhibition	Placental malaria†		Low birthweight‡		SGA		Preterm birth†	
	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value	OR (95% CI)	p value
Overall sample	NA	NA	n = 290	0.028	n = 264	0.0084	NA	NA
Second quartile, 28–43	NA	NA	0.39	NA	0.22	NA	NA	NA
Third quartile, 43–63	NA	NA	0.27	NA	0.34	NA	NA	NA
Higher quartile, >63	NA	NA	0.22	NA	0.35	NA	NA	NA
			(0.15–1.01)		(0.08–0.59)			
			(0.10–0.76)		(0.13–0.88)§			
			(0.07–0.72)		(0.13–0.94)§			
Women not infected at inclusion (first infection after inclusion)	n = 163	0.0085	NA	NA	NA	NA	n = 173	0.0324
Second quartile, 28–43	0.66	NA	NA	NA	NA	NA	0.10	NA
Third quartile, 43–63	0.19	NA	NA	NA	NA	NA	0.09	NA
Higher quartile, >63	0.21	NA	NA	NA	NA	NA	0.18	NA
	(0.22–2.04)						(0.01–0.93)§	
	(0.05–0.72)§						(0.01–0.89)§	
	(0.06–0.76)§						(0.03–1.18)	
Women infected at inclusion	n = 103	0.604	NA	NA	NA	NA	n = 87	0.969
Second quartile, 28–43	1.29	NA	NA	NA	NA	NA	0.68	NA
Third quartile, 43–63	2.02	NA	NA	NA	NA	NA	0.87	NA
Higher quartile, >63	0.90	NA	NA	NA	NA	NA	NA	NA
	(0.30–5.50)						(0.14–3.20)	
	(0.52–7.91)						(1.96–3.83)	
	(0.19–4.35)							

*SGA, small for gestational age; OR, odds ratio; IBA, inhibition of binding assay; NA, not applicable. Analysis was conducted by using multivariate logistic regression adjusting for center, gravidity, infection at inclusion, and testing the interaction between IBA level and infection at inclusion.

†In the absence of a major interaction, results of the model without interaction are shown.

‡In the instance of a major interaction, results were stratified by the presence or absence of infection at inclusion.

§When ORs for 2 contiguous quartiles were similar, p values were computed after grouping those quartiles.

N.T.N., P.D., and A.J.F.L. conceived and designed the study; J.D., F.V., and N.T.N. conducted laboratory experiments; L.D.-N. and N.T.N. analyzed data; N.F., M.A.N., A.S., and A.M. provided reagents, materials, and analysis tools; J.D., L.D., A.J.F.L., P.D., and N.T.N. drafted and finalized the manuscript.

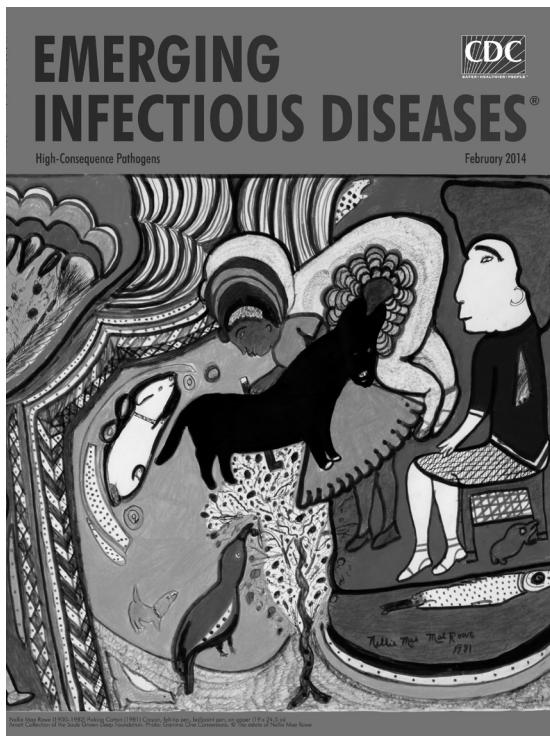
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Canine Distemper in Endangered Ethiopian Wolves

Christopher H. Gordon, Ashley C. Banyard, Alo Hussein, M. Karen Laurenson, James R. Malcolm, Jorgelina Marino, Fekede Regassa, Anne-Marie E. Stewart, Anthony R. Fooks, Claudio Sillero-Zubiri

The Ethiopian wolf (*Canis simensis*) is the world's rarest canid; ≈ 500 wolves remain. The largest population is found within the Bale Mountains National Park (BMNP) in south-eastern Ethiopia, where conservation efforts have demonstrated the negative effect of rabies virus on wolf populations. We describe previously unreported infections with canine distemper virus (CDV) among these wolves during 2005–2006 and 2010. Death rates ranged from 43% to 68% in affected subpopulations and were higher for subadult than adult wolves (83%–87% vs. 34%–39%). The 2010 CDV outbreak started 20 months after a rabies outbreak, before the population had fully recovered, and led to the eradication of several focal packs in BMNP's Web Valley. The combined effect of rabies and CDV increases the chance of pack extinction, exacerbating the typically slow recovery of wolf populations, and represents a key extinction threat to populations of this highly endangered carnivore.

Infectious diseases are a major cause of population declines in wildlife (1). Canine distemper virus (CDV; family *Paramyxoviridae*, genus *Morbillivirus*) constitutes one such threat and has caused outbreaks in a diverse range of wild mammals: black-backed jackals (*Canis mesomelas*) (2); lions (*Panthera leo*) (3); spotted hyenas (*Crocuta crocuta*) (4); fennecs (*Vulpes zerda*); rhesus monkeys (*Macaca mulatta*) (5); and aquatic species, including Lake Baikal seals (*Phoca sibirica*) and Caspian seals (*Phoca caspia*) (6). CDV has also affected several threatened carnivores, including the world's most endangered felid, the Iberian lynx (*Lynx pardinus*) (7); the Santa Catalina Island

fox (*Urocyon littoralis catalinae*) (8); and the Amur tiger (*Panthera tigris altaica*) (9). Rapidly expanding human populations increase domestic dog contact with wild canids (10,11), exacerbating the risk for disease transmission (12,13). CDV infections in different species are serologically indistinguishable due to the existence of a single serotype of the virus.

The Ethiopian wolf (*Canis simensis*) is recognized as the rarest canid species in the world and as the most threatened carnivore in Africa. Fewer than 500 adult and subadult wolves remain in half a dozen suitable Afroalpine habitat ranges (14). The largest population is in the Bale Mountains National Park (BMNP) in southeastern Ethiopia, where wolf populations reach densities of up to 1.4 adults and subadults/km² (15). On average, family packs contain 6 adult and subadults (range 2–20) and protect a home range of ≈ 6 km² (16). Such high wolf densities, large packs, and intense social behaviors increase the risks for disease transmission (17). As a result of rabies outbreaks during 1991–1992 (18), 2003 (19), and 2008–2009 (20), wolf subpopulations in BMNP were dramatically reduced by 45%–75%.

Serologic evidence for CDV within wolf populations has been reported (21); of 30 samples tested during 1989–1992, a total of 9 (30%) were seropositive for CDV. This finding among wild mammal populations shows that survival rates among animals with canine distemper (CD) infection can be high, as most clearly evidenced in populations of rare or threatened species that are likely to be closely monitored by field conservation efforts. Furthermore, it is well established that the virulence of CDV can vary greatly depending on the infecting virus strain, the immunologic competence of the infected host, and the presence of preexisting infections that can be exacerbated by the immunosuppressive effect of infection with a morbillivirus (22).

Population viability analyses have been used to predict the effect of epizootics on wolf populations, and the findings suggest that periodic CD epizootics would play a relatively minor role in population persistence, even when modeled together with rabies (23,24). However, estimated CD-associated death rates in these models were low (15%–20%), and a caveat of the study findings was that the effect on wolf populations should be reassessed if death rates

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were $\geq 40\%$ (23). These models predicted that populations could recover from outbreaks of rabies or CDV, but if the interval between disease outbreaks was < 30 months, the likelihood of local extinction would be high in the absence of low-coverage parenteral vaccination campaigns (25).

To confirm whether CDV poses an extinction threat to Ethiopian wolves, we examined the effect of CDV infection on pack and population dynamics during 2 CD epizootics in BMNP and quantified their effect on the wolf populations. We investigated the source of CD epizootics in village dogs close to the geographic onset of the outbreaks and compared CD-associated deaths between domestic dogs and wild canids.

Animal care and use protocols for the ethical handling of domestic dogs in this study were approved by the Oxford University Zoology Ethical Review Committee (case no. ZERC040905). Animal care and use protocols adhere to the Animals (Scientific Procedures) Act regulations (1986) in the United Kingdom. Furthermore, all animal handling protocols were approved by the Ethiopian Wildlife Conservation Authority.

Materials and Methods

The BMNP, in south-central Ethiopia ($6^{\circ}54'N$, $39^{\circ}42'E$), contains the largest remaining continuous range of Afroalpine habitat (26), upon which Ethiopian wolves are dependent. Wolves in BMNP are found in 3 major subpopulations, all linked by narrow geographic corridors: Morebawa, the Web Valley, and Sanetti Plateau (Figure 1). Wolves are present throughout the Afroalpine range but occur in high densities in these subpopulations.

Since 2001, the Ethiopian Wolf Conservation Programme has closely monitored focal wolf packs in all 3 subpopulations. A total of 18 focal wolf packs were intensively monitored in the 3 areas; 41 packs in other areas, including the Worgona Valley and Chafadalacha, were monitored less intensively (Figure 1) (27). Time series of wolf pack densities of the focal packs in Sanetti and the Web Valley were calculated at 6-month intervals; intensive monitoring data and disease information were incorporated in the time series. Wolves up to 1 and 1–2 years of age are classified as juveniles and subadults, respectively; full adult physical appearance and sexual maturity are attained at 2 years of age. We calculated long-term population trends annually and presented them densities of adult and subadult wolves around the time of breeding. To calculate densities, we considered the area occupied by each population as the 95% kernel of all wolf sightings during the breeding season (October–March).

Intensive monitoring enabled the timely recovery of wolf carcasses and the detection of disease epizootics, and detailed pack composition data enabled the recognition of missing wolves, even when their carcasses were

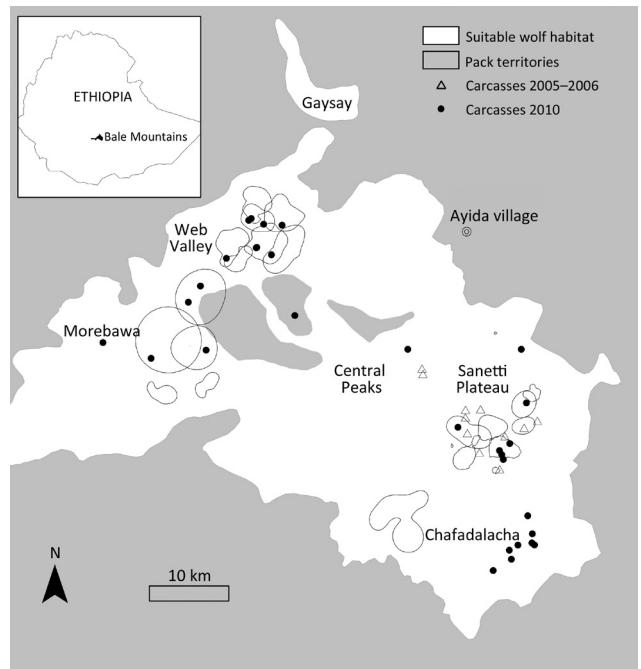


Figure 1. Bale Mountains National Park in Ethiopia, showing location of wolves that died during the 2005–2006 and 2010 canine distemper virus outbreaks in Worgona Valley and Sanetti Plateau and the location of Ayida village, the source of the outbreaks.

not found. Wherever possible, wolf carcasses were subjected to detailed postmortem examination and sampling, following established protocols (28). Samples, including lymph node, lungs, spleen, and brain tissue, were collected when possible.

Interviews were conducted in 62 households in Ayida village, 2 km from a wolf pack in Worgona Valley, after reports of a disease outbreak in the area. Domestic dogs that were suspected to have recovered from the virus were captured, and blood samples were obtained for testing.

We analyzed tissue samples for the presence of CDV antibody by using a semiquantitative solid-phase ELISA (ImmunoComb; Biogal, Galed Labs, Galed, Israel). Where possible, we macerated tissues and extracted total cellular RNA by using Trizol (Invitrogen, Carlsbad, CA, USA). Reverse transcription PCR was performed, and a segment of the phosphoprotein (P) gene was generated as described previously (29). We sequenced positive reaction products of the correct size (429 bp) in their entirety with primer sequences removed from the consensus. We amplified a section of the hemagglutinin (H) gene in the same manner, using H-specific primers (CDVF1 5'TTAGGGCTCAGG-TAGTCCAACA 3' to CDVR1 5' GACAAGGCCGACTC-CAGACAA 3') to yield a 1,122-bp product. P gene and H gene data were aligned with available data by using MEGA6 (30). In all cases, assessing statistical significance using χ^2 values was done with degrees of freedom = 1.

Results

CDV Outbreak 2005–2006

In July 2005, a total of 65 domestic dog deaths were reported in Ayida village (Figure 1), which was just 2 km from the nearest wolf pack in Worgona Valley. In 62 households surveyed, 49% (65/132) dogs owned by villagers had died. Owners commonly reported that infected dogs showed symptoms consistent with a CDV infection, including ocular discharge, convulsive head nodding, loss of appetite, and death. An additional 28% (37/132) of the dogs had been sick but recovered, implying CDV infection in 77% (102/132) of the village dogs. Of 16 serum samples collected from dogs that had recovered, 9 (56%) were positive for CDV antibodies by ELISA.

On September 15, 2005, a wolf with hind leg ataxia, hunching of the back, hair loss, and lethargy was observed in Worgona Valley. On September 21, a wolf carcass was discovered, and in December, a juvenile carcass found. In addition, 7 known wolves disappeared from 4 study packs in the Worgona Valley during September–December. In total, 9 of 19 wolves died or disappeared, resulting in a presumed 47% death rate among adult and subadult wolves across 4 packs.

In November 2005, a known female wolf emigrated from Shiya pack in Worgona to Garba Guracha pack in Sanetti, ≈5 km to the east, and in January 2006, a wolf carcass was discovered in the Garba Guracha pack. During January–April, 13 additional carcasses were recovered in Sanetti (Figure 1), and 10 wolves were observed with clinical symptoms consistent with CDV infection; 4 of the wolves recovered and survived the outbreak. In addition to the 14 wolves that were confirmed dead, 17 other known wolves disappeared from Sanetti during the same period (Table 1), bringing the suspected death rate to 54% (31/58 known wolves) among the 9 packs. Death rates were higher among subadults (83%) than adults (34%). Samples were collected from 3 of 14 carcasses; 2 had positive test results.

CDV Outbreak 2010

In April 2010, 3 wolf carcasses were discovered in Web Valley and Morebawa (Figure 1), and during July–August,

9 more were discovered. In September, 5 carcasses were found in Chafadalacha (30 km from Morebawa), and in November, 5 were detected in Sanetti (25 km from Web Valley). In total, 31 carcasses were recovered, and 7 samples were collected for analysis. In addition, 3 domestic dog carcasses were recovered.

In Web Valley, during April–August 2010, a total of 8 wolves were found dead, and another 13 disappeared and were presumed dead, indicating an estimated death rate of 68% (21/31 known wolves from 7 packs) (Table 2). In Sanetti, 8 carcasses were recovered, and 19 more wolves went missing during October–December 2010, indicating a death rate of 43% (27/63 known wolves from 7 packs) (Table 2). In Morebawa, 5 wolf carcasses were found, and 11 wolves went missing during May–August, indicating a death rate of 47% (16/34 from 6 packs). In focal areas, the death rate among subadult wolves (87%, 20/23) was higher than that among adults (39%, 28/71); the death rate among juvenile wolves was 93% (27/29). Ten additional carcasses were recovered in nonfocal wolf areas, such as Chafadalacha and Central Peaks.

For the 2 CDV outbreaks combined, the death rates among subadult (85%) and adult (38%) wolves were significantly higher than the expected annual natural death rate of 15% (19) ($\chi^2_1 = 42.98$, $N = 106$, $p < 0.001$) and ($\chi^2_1 = 175.69$, $N = 46$, $p < 0.001$) respectively, but significantly more subadults than adults died or disappeared ($\chi^2_1 = 28.45$, $N = 152$, $p < 0.001$). The death rate among juvenile wolves (93%) during the 2010 CDV outbreak was significantly higher than the expected annual natural death of 37% (15) ($\chi^2_1 = 39.16$, $N = 29$, $p < 0.001$).

CD Diagnosis

An amplicon for the P gene could be amplified from only 1 sample among those analyzed from the 2005–2006 outbreak. This sequence grouped phylogenetically with sequences reported for isolates from domestic dogs in the United States and Germany (Figure 2, panel A). Three postmortem samples from the 2010 outbreak were positive for the CDV P gene or the H gene, and the sequences aligned most closely with isolates from domestic dogs in Japan (Figure 2).

Table 1. Age and sex distribution of Ethiopian wolves in focal packs monitored in Sanetti Plateau, Ethiopia, before, during, and after a 2005–2006 outbreak of canine distemper virus

Focal pack	November 2005, before the outbreak				April 2006, during the outbreak				November 2006, after the outbreak			
	Adult, M	Adult, F	Subadult	Total	Adult, M	Adult, F	Subadult	Total	Adult, M	Adult, F	Subadult	Total
Badagassa	4	3	3	10	2	2	0	4	2	2	0	4
Batu	3	2	4	9	2	2	0	4	2	2	0	4
BBC	4	1	7	12	4	1	3	8	2	1	0	3
Garba Guracha	2	2	4	8	2	1	1	4	2	1	1	4
Nyala	3	3	2	8	1	1	1	3	1	1	1	3
Quarry	3	2	3	8	3	1	2	6	3	1	2	6
Billisa	2	1	0	3	2	1	0	3	2	1	0	3
Total	21	14	23	58	16	9	7	32	14	9	4	27

Table 2. Age and sex distribution of Ethiopian wolves in focal packs monitored in Sanetti Plateau and Web Valley, Ethiopia, before, during, and after a 2010 outbreaks of canine distemper virus

Focal pack	April 2010, before outbreaks				November 2010, during outbreaks				April 2011, after outbreaks			
	Adult,		Subadult	Total	Adult,		Subadult	Total	Adult,		Subadult	Total
	M	F			M	F			M	F		
Sanetti Plateau												
Badagassa	3	1	3	7	5	2	1	8	3	1	0	4
Batu	4	2	0	6	3	1	0	4	4	1	0	5
BBC	11	3	3	17	7	2	1	10	4	2	1	7
Garba Guracha	4	1	3	8	4	1	2	7	4	1	1	6
Nyala	2	3	2	7	2	1	0	3	1	1	0	2
Quarry	5	3	3	11	4	3	0	7	4	3	0	7
Bilisa	3	2	2	7	3	2	2	7	4	1	0	5
Total	32	15	16	63	28	12	6	46	24	10	2	36
Web Valley												
Darkeena	1	1	0	2	0	0	0	0	0	0	0	0
Mulamu	3	2	1	6	0	0	0	0	0	0	0	0
Meggity	3	1	0	4	2	1	0	3	1	1	0	2
Kotera	1	0	0	1	0	0	0	0	0	0	0	0
Sodota	2	2	6	10	1	1	0	2	0	0	0	0
Alandu	3	2	0	5	3	1	0	4	3	1	1	5
Tarura	2	1	0	3	2	1	0	3	2	1	0	3
Total	15	9	7	31	8	4	0	12	6	3	1	10

CDV Effects on Population and Pack Dynamics

Between 2002 and 2013, focal packs in the Sanetti subpopulation were affected by 2 CDV epizootics (2005–2006 and 2010), but no rabies epizootics were observed. Wolf numbers fluctuated in Sanetti in response to CDV infection; the interepizootic interval was 4 years (Figure 3). An immediate lull in population growth followed both epizootics. In 2006, two Sanetti packs (BBC and Lencha) coalesced to form 1 pack, meaning, in essence, that 1 pack became extinct. Breeding success during or immediately after the epizootics was also affected. During 2005–2006, only 4 (44%) focal packs in Sanetti bred; during 2006–2007, only 3 (38%) bred; and during 2010–2011, only 4 (57%) bred. In 2005–2006 in Sanetti, only 4 pups in total survived from 3 packs, but in February during the epizootic, all 4 pups in a fourth pack, Badagassa, died. The remaining 5 packs in Sanetti did not breed that season. Breeding remained suppressed in the 2006–2007 breeding season: only 3 of 8 packs produced pups, of which 10 survived to independence at 6 months of age. During the 2010–2011 breeding season, 3 of 7 focal packs did not breed; another 2 packs bred but lost their pups before emergence at 3 weeks of age. Four of 9 pups from the other 2 packs died before they reached independence.

Subsequent to this initial 2-year lull in reproduction, wolf numbers recovered strongly: by the second outbreak in 2010, wolf numbers and wolf density in Sanetti had surpassed pre-CDV outbreak levels. The combined wolf density for the 7 focal packs in Sanetti more than doubled during 2007–2010 (Figure 3).

During 2002–2013, Web Valley wolf packs were affected by rabies epizootics in 2003 and 2008–2009 and by an CDV epizootic in 2010 (Figure 4). Death rates were 62% (19) and 59% (39/66), respectively, for the 2 rabies

epizootics and 68% for the CDV epizootic. Four of 7 Web Valley packs, including 86% of the subadult wolves that were born during the 2008–2009 breeding season, were eradicated by the CDV epizootic. Two new packs formed in the Web Valley in 2012.

Discussion

CDV Diagnosis

The detection of CDV-associated deaths among populations of rare Ethiopian wolves is of paramount significance for their effective protection and survival. Alongside the ongoing threat from rabies, deaths from CDV highlight the real and present threat that emerging viral diseases pose to these endangered carnivores. Molecular typing of viral pathogens is of great utility in identifying and managing threats to susceptible populations. The phylogenetic clustering of wolf-derived CDV isolates with domestic dog-derived isolates from geographically distinct areas is not surprising. CDV isolates were originally reported to cluster geographically, however, increased reporting and genetic analysis of CDV isolates has shown that translocation of animals, often internationally, can spread the virus globally. Thus, geographically distinct viruses are often found to cluster (22). Furthermore, data for CDV isolates are scarce, and our epidemiologic understanding of this virus remains unclear in the absence of genetic data.

CDV Effects on Population and Pack Dynamics

The detailed death information gathered from both epizootics contradicts the predictions of previous population viability analysis models, which were determined on the basis of lower estimated CDV death rates (15%–20%). Those

models indicated that CDV would have little effect on wolf population persistence or pack size (23,24). However, the observed death rates in our study were 2–4 times higher than those in the earlier studies (23). When compared with the effect of natural death rates of $\approx 15\%$ per year for subadult and adult wolves (19), the high death rates observed

in our study can rapidly alter population and pack dynamics. The missing subadult and adult wolves in Sanetti in 2005–2006 (29%) and 2010 (35%) and in Web Valley in 2010 (42%) represented more than the 15% natural death rate, providing further confirmation that these missing wolves had died from CD.

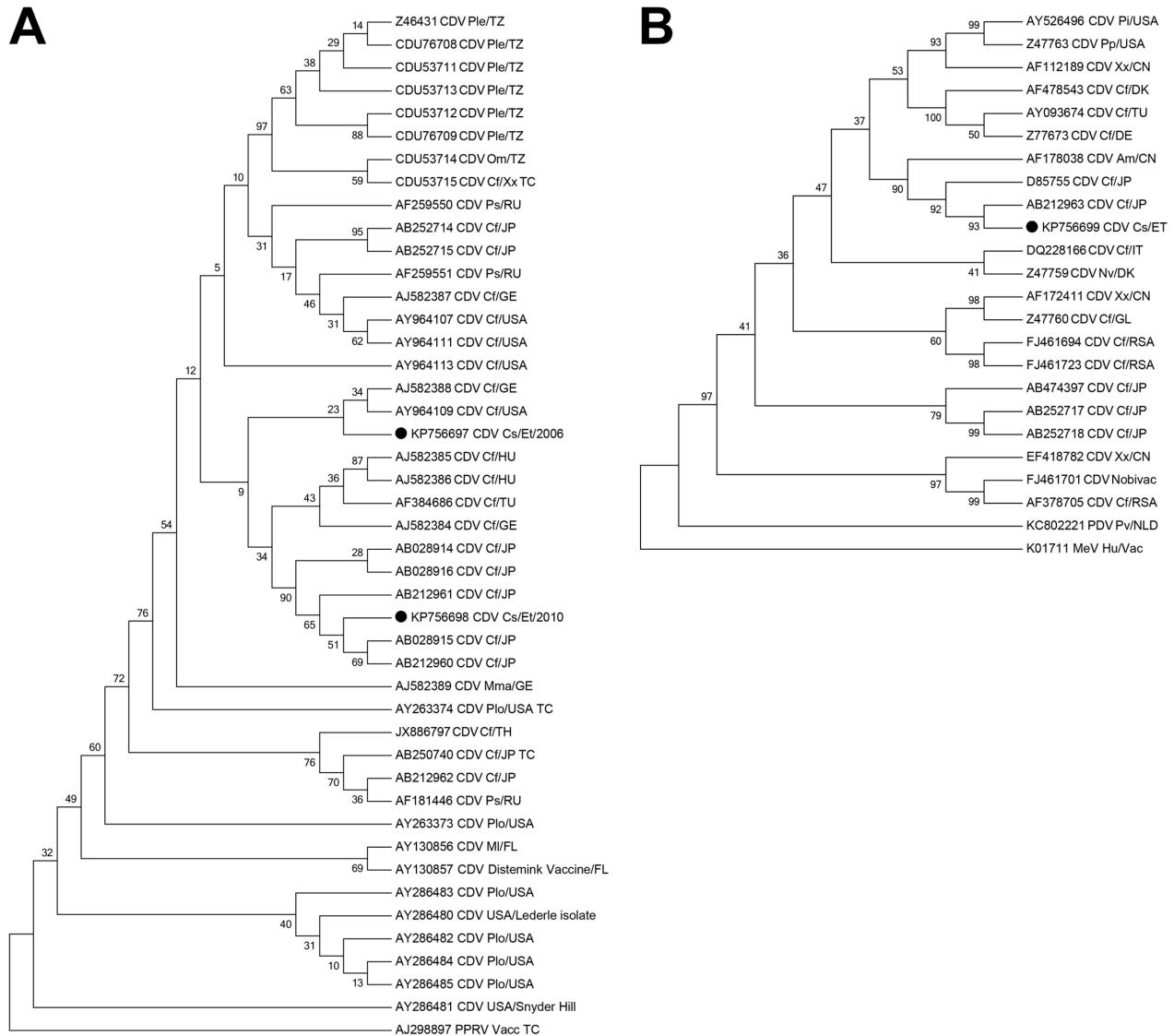


Figure 2. Phylogenetic neighbor-joining trees of canine distemper virus (CDV) isolates from samples collected during outbreaks in 2006 and 2011 (A) and 2010 (B). Evolutionary analyses were conducted in MEGA6 (30). A) Tree constructed using the phosphoprotein gene (331 nt). Evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The analysis involved 45 nt sequences and a total of 331 positions in the final dataset. B) Tree constructed by using the hemagglutinin gene (1,334 nt). Bootstrap values (10,000 replicates) are indicated at relevant nodes. Black dot indicates Ethiopian wolf samples. Species from which the viruses were isolated are indicated by the following abbreviations: Am, *Ailuropoda melanoleuca* (giant panda); Cf, *Canis familiaris* (dog); Cs, *Canis simensis* (Ethiopian wolf); Hu, human; Mma, *Martes martes* (European marten); MI, *Mustela lutreola* (European mink); Mm, *Meles meles* (badger); Nv, *Neovison vison* (American mink); Om, *Otocyon megalotis* (bat-eared fox); Ple, *Panthera leo* (lion); Plo, *Procyon lotor* (raccoon); Pp, *Panthera pardus* (black leopard); Ps, *Phoca sibirica* (Baikal seal); Pv, *Phoca vitulina* (harbor seal); Xx, species unidentified. Country of sample origin are indicated as follows: CN, China; DK, Denmark; ET, Ethiopia; FL, Finland; GE, Germany; GL, Greenland; HU, Hungary; IT, Italy; JP, Japan; NLD, the Netherlands; RU, Russia; RSA, South Africa; TU, Turkey; TZ, Tanzania. TC denotes where isolates have undergone extensive tissue culture passage. Phylogenetic outgroups are indicated as follows: PPRV, peste des petits ruminants virus; PDV, phocine distemper virus; and MeV, measles virus.

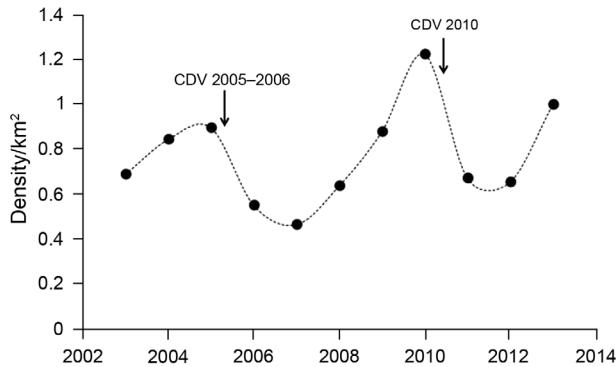


Figure 3. Adult and subadult Ethiopian wolf population in Sanetti Plateau, Ethiopia, 2002–2013. Dots indicate wolf population estimates at different time points; arrows indicate canine distemper virus outbreaks in this study.

Death rates for wolves with CD were comparable to those for dogs in Ayida village. The genetic identity of the virus and the close proximity between the village and wolf habitat makes it almost certain that the village dogs were the source of the 2005–2006 epizootic among wolves; this finding supports the assertion that CDV is transmitted to wild canids by domestic dogs (12). Some dogs in Ayida recovered from CD, and the number of surviving dogs combined with the number of dead dogs suggests that the prevalence of CDV infection during the epizootic was much higher than the reported number of deaths. Wolves can also survive exposure to CDV, as evidenced by the detection of a CDV seropositive wolf in 2011 and the detection of seropositive wolves in 1989–1992 (21).

Once disease is transmitted from a domestic dog to an individual wolf, the intense social behavior of wolf packs enables pathogens to spread almost instantly within the pack. Adjacent packs interact at territorial boundaries, permitting further transmission through the population. However, the 2 epizootics in this study showed different transmission patterns across subpopulations (Figure 1). The 2005–2006 epizootic spread a relatively short distance from the lower density wolf habitats in Worgona Valley to the adjacent high-density habitats in Sanetti, where the disease died out. In contrast, the 2010 epizootic moved temporally and geographically from Morebawa to Web Valley and Sanetti, crossing through geographic bottlenecks and areas of lower wolf density between these subpopulations. Interpack contact rates are reduced by low pack connectivity within geographic bottlenecks, reducing the probability of disease transmission. This fact reinforces the severity of the 2010 epizootic, which left 64 wolves dead or missing from the 3 focal subpopulations. Monitoring efforts were less intense in the nonfocal, lower density wolf areas, so it was difficult to gauge death in these areas. The reported losses from focal packs, combined with unknown deaths

from lower density areas, suggest that this was the single most catastrophic disease event for Ethiopian wolves reported to date; the spread of CDV to all areas of BMNP caused losses that outnumber reports from all previous rabies epizootics (18–20).

CDV had a considerable effect on younger wolves: death rates among subadults were >2 times higher than those among adults. Lower death rates in adult wolves will aid recovery of packs by keeping breeding units (packs) intact, assuming survival of at least 1 adult female. Although juvenile wolves usually have natural death rates of 37% during high wolf density periods and 29% during periods of population recovery (15), juvenile death rates were 3 times these levels after the 2010 epizootic. Lower death rates among adult wolves may reflect previous low-level exposure, and thus immunity.

Although the mechanism by which CDV affects reproduction is uncertain, both CDV outbreaks clearly affected breeding success and pup survival in Sanetti. In periods of high wolf densities, 75% of packs typically breed successfully, and during periods of population recovery, 83% of packs typically breed successfully (15); however, <50% of Sanetti packs bred successfully immediately after both CDV epizootics. After this lull, breeding was not impaired, and once the juveniles were recruited into the population, growth rates were rapid in Sanetti: wolf densities doubled over a 3-year period. With the exception of the 2 packs that coalesced, all breeding packs in Sanetti were maintained, and at least 1 adult female survived in each pack. Four years after the 2005–2006 outbreak, wolf densities had recovered above pre-outbreak levels, and signs suggest a similar outcome following the 2010 outbreak.

Ethiopian wolf populations can recover from CDV epizootics, but the capacity to recover will be impaired when intervals between epizootics are short, as was seen in Web Valley. The brevity of the second interepizootic interval (20 months) meant that wolf numbers had only just started to recover following the 2008–2009 rabies epizootic before the CDV epizootic began. After the 2010 CDV outbreak, death rates were high: 4 of 7 packs were eradicated. These pack extinctions confirmed modeling predictions that the probability of pack extinctions greatly increases as the length of the interepizootic period decreases (23). Although concurrent rabies and CDV infections likely caused these extreme death scenarios in Web Valley wolves, there is evidence of high death rate CDV epidemics in lions coinciding with high levels of *Babesia* spp. infection resulting from climatic extremes (31); thus, other factors should be fully explored.

The loss of breeding units can slow population growth because it is rare for packs of Ethiopian wolves to split (32), even though large litter sizes and high juvenile survival may occur following a decrease in population

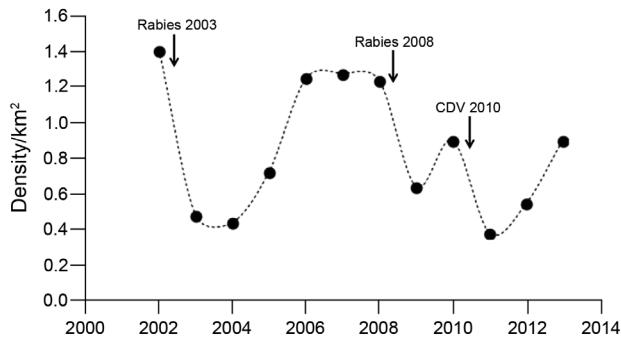


Figure 4. Density of adult and subadult Ethiopian wolf population in Web Valley, Ethiopia, 2002–2013. Dots indicate wolf population estimates at different time points; arrows indicate known rabies epizootics and canine distemper virus outbreaks in this study. Of 7 wolf packs in Web Valley, 4 went extinct after the 2010 canine distemper virus outbreak; in 2011, two new packs formed in the area.

density (15,33). Dispersal movements are constrained by the scarcity of suitable, unoccupied habitat, although some subordinate females disperse once recruited to adult status (32). Packs will expand their territory if opportunities arise, usually following the disappearance of a neighboring pack (34). After the 2010 CDV epizootic, available habitat in optimal Afroalpine areas was abundant in Web Valley, and in 2012, two new packs formed (Figure 4), hinting at the resilience of this species. Several of the founder members of these new packs came through the corridor from Morebawa and joined surviving solitary wolves from extinct Web Valley packs. This finding confirms that corridors facilitate migration and recovery after epizootics (35), and such migration is critical for minimizing genetic drift from bottlenecks (36). Such mechanisms indicate that individual wolves take advantage of breeding opportunities and low population density, filling available habitat to form new packs. The size and structure of the BMNP wolf population lends robustness to wolf numbers and provides greater resilience against disease catastrophes (23).

Conservation Implications

CDV is a major threat to the persistence of some threatened carnivore populations, including the Ethiopian wolves. Long-term disease management plans are vital for conservation of susceptible species, and vaccination of host and target populations remains a key strategy for disease management (37,38). Even with incomplete CDV control in domestic dogs, any reduction in disease incidence should have a beneficial effect on the persistence of a wild endangered species.

Population viability models indicate that disease-induced population fluctuations and extinction risks can be markedly reduced by the vaccination of a small proportion of wolves (23,25). However, CDV vaccines for wild species

are not currently at the same stage of development as rabies vaccines. In particular, although licensed for domestic species, live attenuated CDV vaccines can cause adverse reactions in wildlife species. Monovalent canarypox-vectored CDV recombinant vaccines hold the greatest promise for protection of wild canids against CDV (39), and trials of the Nobivac D and P antigens have also been conducted on wildlife (40), with no adverse reactions. The high prevalence of CDV in the surrounding domestic dog population and the apparent frequent incursion of CDV into the BMNP wolves makes finding new disease control strategies all the more urgent, particularly for smaller wolf populations, among which extinction probabilities are even higher with any reduction in interepizootic periods (23). The extent of knowledge regarding CDV and its effects is clearly demonstrated in the well-monitored Ethiopian wolf populations, resulting in suggested conservation solutions that are far reaching in their potential application to other susceptible threatened carnivore species.

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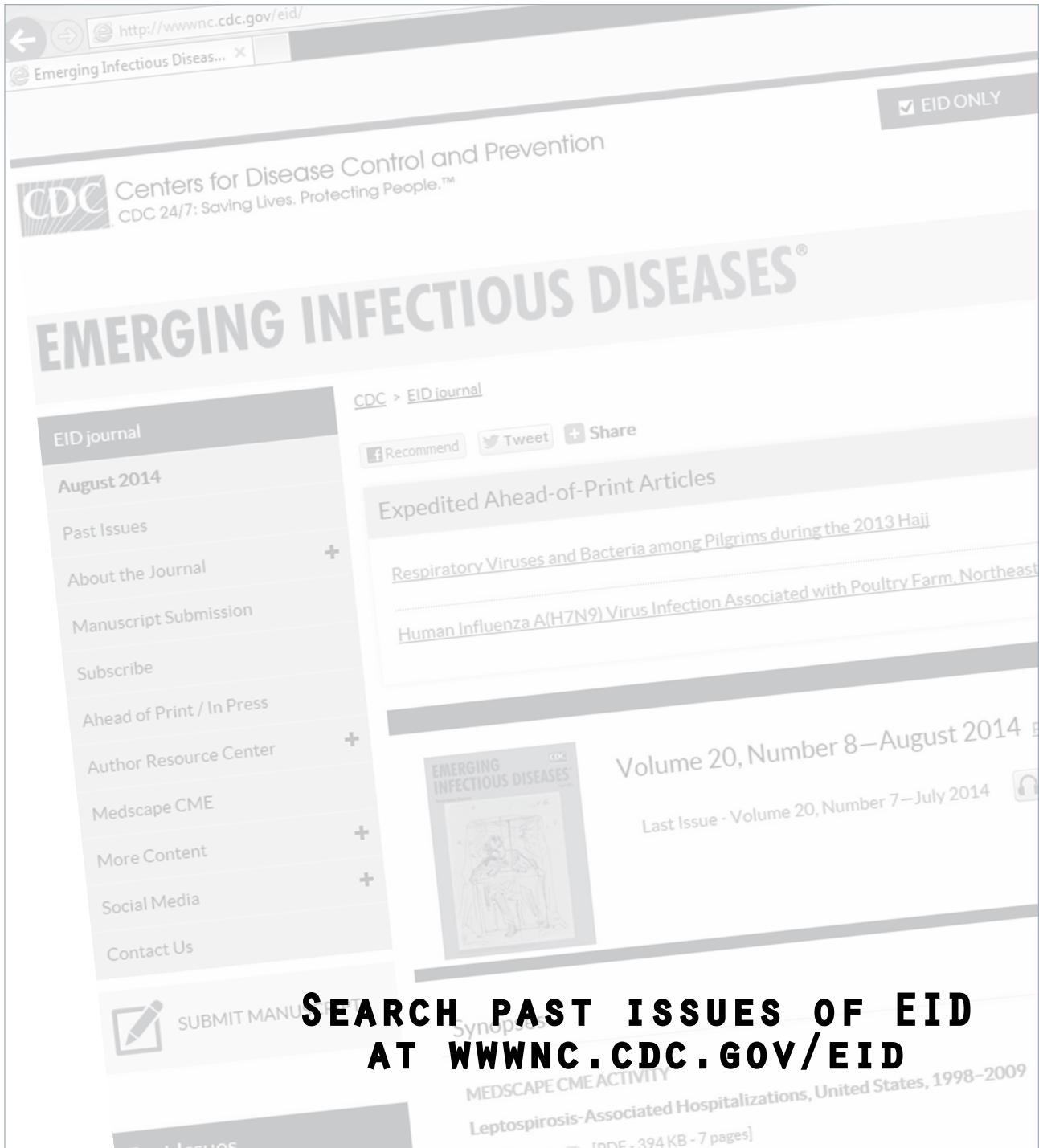
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Comparative Sequence Analyses of La Crosse Virus Strain Isolated from Patient with Fatal Encephalitis, Tennessee, USA

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We characterized a La Crosse virus (LACV) isolate from the brain of a child who died of encephalitis-associated complications in eastern Tennessee, USA, during summer 2012. We compared the isolate with LACV sequences from mosquitoes collected near the child's home just after his postmortem diagnosis. In addition, we conducted phylogenetic analyses of these and other sequences derived from LACV strains representing varied temporal, geographic, and ecologic origins. Consistent with historical findings, results of these analyses indicate that a limited range of LACV lineage I genotypes is associated with severe clinical outcomes.

La Crosse virus (LACV) (family *Bunyaviridae*, genus *Orthobunyavirus*) is the primary cause of arthropod-borne viral (arboviral) encephalitis in children in the United States. LACV has a genome of 3 negative-stranded RNA segments (small, medium, and large) and is endemic to forested regions along the Mississippi and Ohio River basins, east of the Rocky Mountains (1–5). These forests provide a habitat for the known principal vector of LACV, *Aedes triseriatus* mosquitoes. Amplifying hosts include small mammals that develop levels of viremia sufficient to transmit LACV to mosquitoes during the summer months. Human infections occur during the summer and early fall, when humans are at greatest risk for mosquito bites.

In recent years, LACV reportedly has increased above endemic levels in regions of the southeastern United States, including areas of eastern Tennessee (6). The reason for this apparent increase remains unknown; possible causes include a change in transmission dynamics contributed to by invasive vector species or emergence of a relatively virulent strain of LACV in those regions. Confounding a better understanding of the latter, LACV has been historically

difficult to isolate from humans. In fact, just 3 human isolates, derived over >50 years, were described in GenBank (accession nos. EF485033–35, EF485030–32, GU206139) before our study began.

We report the multisegment genomic characterization of an LACV strain isolated from the brain of a child who died of encephalitis-associated complications in eastern Tennessee, USA, in July 2012. To the best of our knowledge, this represents only the fourth human isolate of LACV that has been described at the nucleotide sequence level. In addition, we have determined the coding sequences of LACV strains derived from mosquitoes reared from eggs collected within 16 radial kilometers (10 miles) of the child's home during summer 2012, after the postmortem diagnosis.

Case Description

In July 2012, a 6-year-old boy from Union County, Tennessee, was seen at an emergency department after 2 focal seizures and symptoms consistent with viral encephalitis. Because of the geographic location, seasonal timing of illness, and clinical presentation, LACV was suspected as the cause of illness. A serum sample was determined to be immunofluorescence assay–positive for LACV IgM and IgG. On day 2 after admission, the boy displayed altered mental status, decreased mobility, hallucinations, and vomiting. By day 3, he was responsive only to painful stimuli and showed decreased arousal. His condition progressively deteriorated, and he was pronounced brain dead and died on day 5 after admission. At autopsy, a section of the temporal lobe was taken, immediately frozen, and sent to the Centers for Disease Control and Prevention (CDC), Division of Vector-Borne Diseases (Fort Collins, CO, USA), for analysis.

Materials and Methods

At CDC, the sample was determined to be LACV-positive by real-time reverse transcription PCR (RT-PCR) by using previously described methods (7). This result was confirmed by next-generation sequencing by using the Ion

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Torrent PGM system (Life Technologies, Grand Island, NY, USA) and methods described elsewhere (8). After nucleic acid sequence detection, the brain sample was subjected to a standard isolation method in an attempt to derive an LACV isolate. Briefly, 200 μ L of 10^0 and 10^{-1} dilutions of supernatant taken from a homogenized preparation of the temporal lobe sample were inoculated onto confluent Vero cells in T25 flasks. The flasks were then incubated at 37°C and reviewed daily for cytopathic effect. Cytopathic effect was identified in flasks inoculated with both the 10^0 and 10^{-1} dilutions on day 4 postinoculation. LACV was confirmed in the supernatants collected from these flasks by real-time RT-PCR.

To detect LACV in the geographic area, oviposition, BG sentinel traps, (Bioagents AG, Regensburg, Germany; and CDC light and gravid traps, (Clarke, St. Charles, IL, USA), were placed at 49 sites consisting of cemeteries and houses within 10 miles of the patient's home (the presumed site of infection). Traps were set during September 5–October 3, 2012. A total of 816 egg papers were collected, and on arrival at the Tennessee Department of Health, eggs were counted and each paper flooded 3 times with deionized water. Egg papers were dried, and eggs were allowed to embryonate for 1 week in a humidified container. Each egg paper was placed in a plastic tray (28 × 11 × 12 cm) containing 0.5–1 L of deionized water, and emerging larvae were fed bovine liver powder ad libitum (#02900396 MP Biomedicals, Solon, OH, USA). Each tray was placed into an incubator at 26 ± 2°C, 60%–80% relative humidity, and a 12-h light–12-h dark photoperiod. Pupae were collected by using plastic pipettes and transferred into small bowls containing 0.1 L of deionized water that were housed in 60-mL screened cages for adult emergence. Adults were removed by using hand-held insect vacuums and placed in another chamber, frozen, and then morphologically identified to species and sex (9). Adults identified from the field or reared from egg papers were pooled in cohorts of ≤ 25 specimens of the same species, sex, trap, date, and site.

Each pool of mosquitoes was combined with 3 copper BBs and 1 mL of Eagle's minimum essential medium with 2% fetal bovine serum, 0.5% NaHCO₃, and 1% antimicrobial solution (10,000 IU/mL penicillin, 10,000 μ g/mL streptomycin, 25 μ g/mL amphotericin B; Sigma-Aldrich Co., St. Louis, MO, USA). The samples were homogenized on a Retsch MM300 shaker (Retsch GmbH & Co. KG, Haan, Germany) for 90 sec, centrifuged at 5,000 rpm for 5 min, and stored at –80°C. RNA was extracted from 200 μ L of each sample by using the QIAamp Viral Isolation 96-well protocol on the BioRobot 9604 or the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA), following the manufacturer's protocol.

All samples were tested for orthobunyavirus small segment RNA by RT-PCR by using published primers

from Kuno et al (10) (forward BCS82C 5'-ATGACT-GAGTTGGAGTTTCATGATGTGCGC-3', reverse BC-S332V 5'-TGTTTCCTGTTGCCAGGAAAAT-3'). Each 25- μ L reaction contained 11.25 μ L of nuclease-free water, 2.5 μ L of 10× RT-PCR buffer, 200 μ M dNTPs, 2 U/ μ L of RT, 5 U/ μ L of Easy-A High Fidelity DNA polymerase (Agilent Technologies, Santa Clara, CA, USA), 50 pmol of each primer, and 5 μ L of extracted RNA. We conducted RT-PCR in the GeneAmp PCR system 9700 (Applied Biosystems) with the following cycle times and temperatures: 1 cycle at 45°C for 30 min; 1 cycle at 95°C for 10 min; 45 cycles of 95°C for 30 s, 55°C for 60 s, 68°C for 120 s; and 1 cycle at 68°C for 5 min. RT-PCR products were visualized by using the E-Gel electrophoresis system (Life Technologies). The positive control originated from a pool of 10 *Ae. triseriatus* mosquitoes collected from Fulton County, Georgia, USA, in 2002. RNAase-free deionized water was used as a negative RT-PCR control.

Products of positive RT-PCR reactions were gel purified by using the QIAquick Gel Extraction Kit protocol (QIAGEN) and then sequenced at the Tennessee Department of Health Laboratory Services (Nashville, TN, USA) with a 3130 × 1 genetic sequencer (Applied Biosystems) by using the BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). We then analyzed the resulting sequences using Sequencer 4.6 software (Gene Codes Corp., Ann Arbor, MI, USA) and compared them with sequences in GenBank by BLAST analyses (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) for identification. Samples with a $\geq 96\%$ identity with other published LACV sequences were considered preliminarily identified as LACV positive.

To confirm results from multiple genomic segments, we reextracted RNA from all LACV-positive pools and retested them using real-time RT-PCR with a second published primer set targeting the medium segment of the viral genome (7). Using these methods, we determined that 4 pools of *Ae. triseriatus* mosquitoes were LACV positive. The positive pools came from egg papers collected at a cemetery on October 3, 2012.

We generated multisegment genomic sequence data from the newly derived LACV human isolate, along with the 4 LACV-positive *Ae. triseriatus* mosquitoes (2 male and 2 female), by next-generation sequencing methods conducted as previously described (8). Results were confirmed by spot sequencing using traditional Sanger methods, as described elsewhere (8).

After nucleotide sequence level characterization of the Tennessee 2012 LACV strains of both human and mosquito origin, we conducted comparative sequence and phylogenetic analyses using MEGA5 software and methods described previously (11). Phylogenetic methods were applied to 2 representative Tennessee 2012 *Ae. triseriatus* mosquito strains, along with the Tennessee 2012 human

sequence and a diverse set of data from GenBank (Figure). GenBank accession numbers for sequences described in our study are KP271104–KP271118.

Results and Discussion

The 4 LACV strains derived from *Ae. triseriatus* mosquito eggs were highly similar to the patient’s isolate, with $\geq 99\%$, $\geq 97\%$, and $\geq 98\%$ nt sequence identities shared among compared small, medium, and large segment sequences, respectively. The Tennessee 2012 LACV strains grouped with strong support in lineage I, along with all described human isolates of LACV from varied geographic and temporal origins (Figure). This finding is consistent with results from analyses of more limited historical datasets (12,13), including data generated from direct amplification of LACV cDNA from central nervous system tissues of persons who died (14) and indicates that a restricted range of LACV genotypes is associated with severe clinical outcomes. In addition, a sequence from GenBank that was generated from *Ae. triseriatus* mosquitoes collected in North Carolina during summer 2000 grouped with strong support along with the Tennessee 2012 human and mosquito strains (Figure). Coincidentally, we analyzed samples

from a child from North Carolina who, during summer 2000, had fatal LACV-associated encephalitis (A.J. Lambert, unpub. data). At that time, we were unable to derive an isolate or generate sequence data because of the low levels of LACV, as inferred by real-time RT-PCR, in those samples (A.J. Lambert, unpub. data). The presence of a strain highly similar to the Tennessee isolates in a vector mosquito collected at the same general time and location of the fatal case in North Carolina during 2000 suggests that a Tennessee 2012–like lineage I genotype also might have been responsible for the North Carolina case.

Taken together, our results confirm the epidemiologic importance of LACV lineage I strains in the emergence of fatal LACV disease throughout broad temporal and geographic ranges, including the southeastern United States. Furthermore, identification of LACV lineage I strains in male *Ae. triseriatus* mosquitoes collected near the presumed site of infection in this case verifies transovarial maintenance and the ecologic importance of the endemic vector in Tennessee. Our findings justify further studies, designed to discriminate possible differences in the ecology, epidemiology, and biology of LACV strains from varied phylogenetic lineages.

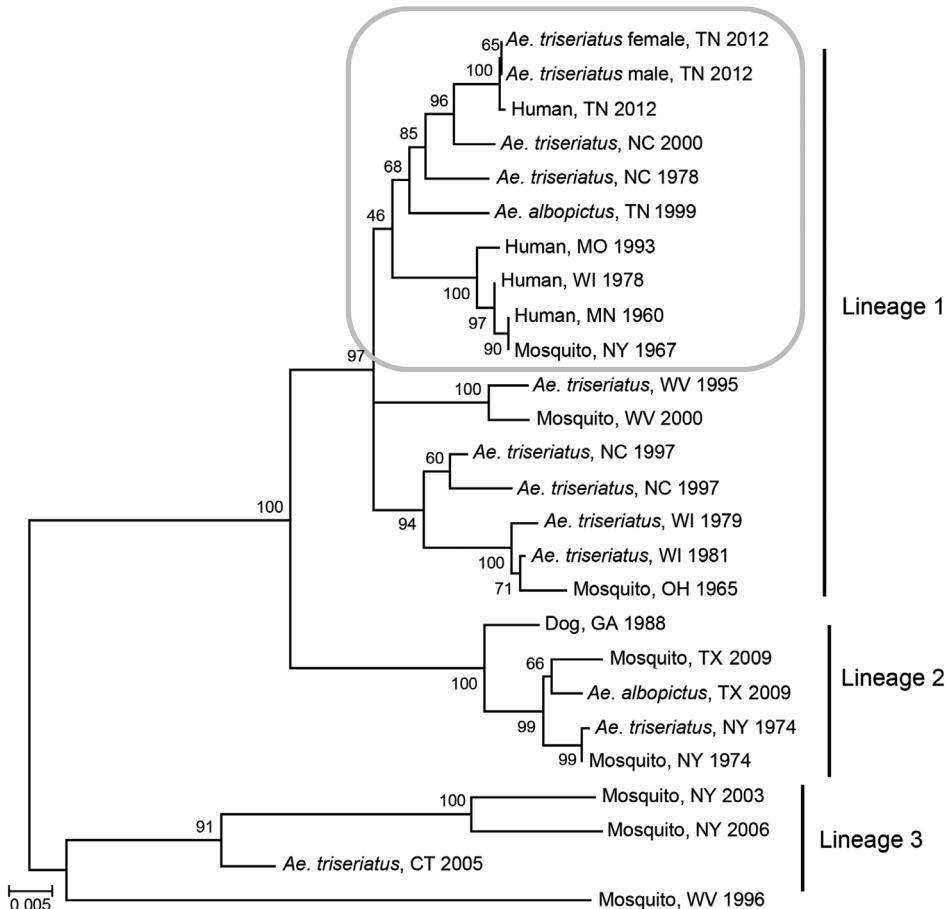


Figure. Phylogeny of medium segment sequences of selected La Crosse virus strains of varied temporal, geographic, and ecological origin. Taxon descriptions are restricted in some cases according to a limited amount of information in GenBank. A neighbor-joining method was used with 2,000 replicates for bootstrap testing. Scale bar represents 0.005 nt substitutions per site. Box indicates the area of phylogenetic interest.

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Dr. Lambert is a research microbiologist in the Division of Vector-Borne Infectious Diseases, CDC. Her research interests include the evolution and global emergence of arthropod-borne bunyaviruses.

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Low-level Circulation of Enterovirus D68—Associated Acute Respiratory Infections, Germany, 2014

Janine Reiche, Sindy Böttcher, Sabine Diedrich,
Udo Buchholz, Silke Buda, Walter Haas,
Brunhilde Schweiger, Thorsten Wolff

We used physician sentinel surveillance to identify 25 (7.7%) mild to severe infections with enterovirus D68 (EV-D68) in children and adults among 325 outpatients with acute respiratory infections in Germany during August–October 2014. Results suggested low-level circulation of enterovirus D68 in Germany. Viruses were characterized by sequencing viral protein (VP) 1 and VP4/VP2 genomic regions.

Enterovirus D68 (EV-D68) belongs to the family *Picornaviridae*, genus *Enterovirus*, species *Enterovirus D*. Since its initial discovery in 1962 (1), EV-D68 infections in humans have been reported rarely. However, since 2008, clusters of acute respiratory infections (ARIs) associated with EV-D68 have been reported worldwide, including Europe (2–5).

During mid-August 2014–January 2015, the United States and Canada had nationwide outbreaks of EV-D68 infections associated with severe respiratory disease (6,7). The US Centers for Disease Control and Prevention and state public health laboratories confirmed 1,153 persons in 49 states and the District of Columbia infected by EV-D68 (8). Over the same period, >200 specimens tested were positive for EV-D68 throughout Canada (7). Clinical symptoms ranged from mild to severe disease requiring intensive care and mechanical ventilation. Children were predominantly affected, in particular if they had asthma or wheezing (6,9). After ARIs, symptoms of neurologic illness or acute flaccid myelitis developed in an increasing number of children (10,11).

To describe EV-D68 circulation in a large country in Europe in the fall of 2014, we investigated specimens from patients with respiratory illness for EV-D68. This investigation was conducted within the national outpatient ARI sentinel surveillance in Germany.

The Study

Nasal swab specimens from outpatients with influenza-like illness (ILI), ARI, or both were collected by sentinel physicians participating in sentinel surveillance in Germany

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during weeks 32–44 in 2014 and sent to the Robert Koch Institute (Berlin, Germany). All specimens were tested in parallel for respiratory viruses, including influenza viruses A and B, rhinovirus/enterovirus, respiratory syncytial virus, adenovirus, and metapneumovirus by specific real-time reverse transcription PCRs (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-1900-Techapp1.pdf>). The rhinovirus/enterovirus real-time PCR detected rhinovirus at a limit of detection of 26 copies/reaction. EV-D68 was identified at a slightly lower sensitivity of 118 copies/reaction.

Rhinovirus/enterovirus–positive specimens were screened for EV-D68 by sequencing the viral protein (VP) 4/VP2 gene regions. Rhinovirus/enterovirus–negative specimens and samples without sequencing results were additionally analyzed by using a specific EV-D68 PCR (12). VP4/VP2 and VP1 regions were sequenced (GenBank accession nos. KP189380–KP189403 and KP657730–KP657747) for EV-D68–positive specimens.

Rhinovirus/enterovirus was detected in 44% (143/325) of the specimens; 98 were identified as rhinovirus types A–C and 25 as EV-D68. The remaining 20 specimens could not be subtyped, but were negative for EV-D68 by using the specific PCR. The proportion of EV-D68 corresponded to 7.7% of the study collective. EV-D68 was initially detected from week 36 (August) through 38 (September) and continuously from week 41 through week 44 in October (Figure 1).

In addition to the other viruses tested, EV-D68–positive specimens were screened for parainfluenza virus 1–4, coronaviruses (NL63, OC43, HKU1, 229E), and bocavirus. None of these viruses was detected in EV-D68–positive patients, which suggested that EV-D68 played a major role in causing respiratory disease.

Major symptoms associated with EV-D68 infection included sudden onset, fever/shivers, cough, and sore throat (Table 1). Pneumonia was diagnosed in a 7-year-old boy and a 10-year-old girl, and a 2-year-old girl was hospitalized because of obstructive bronchitis. For 11 (44%) of 25 case-patients, a concurrent chronic condition was reported: 5 with a respiratory condition, 3 with a cardiac condition, 2 with diabetes, and 1 with a neurologic disorder. EV-D68 was detected in 10 children and 15 adults; 56% of these patients were male.

Patients infected with EV-D68 came from different federal states in Germany; no epidemic cluster or outbreak

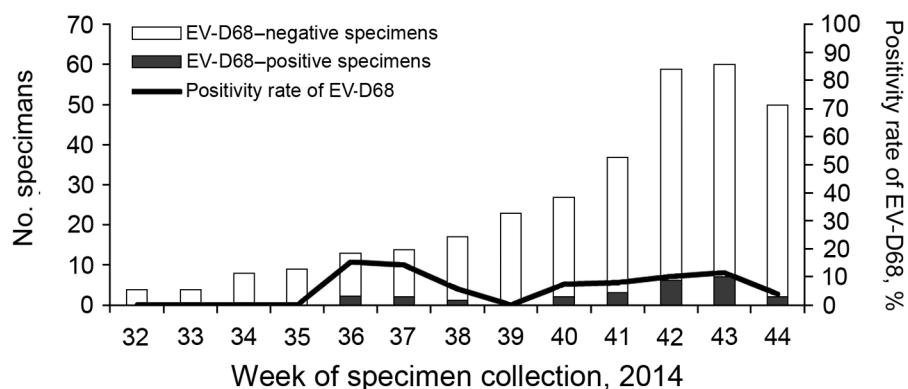


Figure 1. Detection of enterovirus D68 (EV-D68), Germany, week 32–44, 2014.

was detected in the context of these patients. Syndromic surveillance data of corresponding sentinel practices showed only a partial coincidence of EV-D68–positive pa-

tients and an increase of ARI activity in the practice. However, the increase in ARI activity was probably caused by unrelated RV activity.

Table 1. Demographic and clinical characteristics of 25 patients infected with enterovirus D68, Germany, weeks 32–44, 2014*

Patient	Week†	Age, y/sex	Federal state	Sudden onset of disease	Fever/shivers	Cough	Sore throat	Clinical follow-up	Admission to hospital	Underlying condition
1	36	6/F	Thuringia	+	+	+	+	ILI	No	None
2	36	42/M	North Rhine-Westphalia	+	+	+	+	ILI	No	Respiratory system
3	37	61/M	North Rhine-Westphalia	+	+	+	+	ILI	No	Cardiac system
4	37	42/F	North Rhine-Westphalia	–	+	+	+	ARI	No	Respiratory system
5	38	11/M	Thuringia	+	+	+	+	ILI	No	None
6	40	35/F	Lower Saxony	+	+	+	–	ILI	No	Respiratory system, diabetes
7	40	53/M	North Rhine-Westphalia	+	+	+	+	ILI	No	Cardiac system
8	41	2/M	Thuringia	+	+	+	–	ILI	No	None
9	41	62/M	Hesse	+	–	+	+	ARI	No	None
10	41	7/M	Rheinland-Palatinate	+	+	+	+	Pneumonia	No	Respiratory system
11	42	8/F	Bavaria	NA	NA	+	+	ARI	No	None
12	42	25/M	Hesse	+	–	+	–	ARI	No	None
13	42	22/M	Lower Saxony	+	+	+	+	ILI	No	None
14	42	14/F	Baden-Württemberg	–	–	+	+	ARI	No	None
15	42	43/M	Bavaria	+	–	+	+	ARI	No	None
16	42	10/F	Berlin	–	+	+	+	Broncheal pneumonia	No	Respiratory system
17	43	3/F	North Rhine-Westphalia	+	+	+	–	ILI	No	None
18	43	12/F	Bavaria	+	–	+	+	ARI	No	None
19	43	52/M	Lower Saxony	+	+	+	+	ILI	No	Diabetes
20	43	26/M	North Rhine-Westphalia	+	+	+	–	ILI	No	None
21	43	44/M	North Rhine-Westphalia	+	–	+	+	ARI	No	Cardiac system
22	43	2/F	Hesse	+	+	+	–	Obstructive bronchitis	Yes	None
23	43	41/M	Saarland	+	+	+	+	ILI	No	None
24	44	2/F	Schleswig-Holstein	+	–	+	–	ARI	No	None
25	44	73/F	North Rhine-Westphalia	–	–	+	+	ARI	No	Neurologic disorder

*+, positive; ILI, influenza-like illness; –, negative; ARI, acute respiratory infection; NA, not available.

†Data are listed by week of symptom onset.

Sequence analysis is not regularly performed for rhinovirus/enterovirus–positive specimens within sentinel surveillance in Germany. However, comparative data can be provided for week 36 through week 20 for the 2009–10 and 2010–11 seasons (Table 2). During those seasons, patients with ILI in 5 age groups (<1–4, 5–15, 16–34, 35–60, and >60 years) were investigated by using the rhinovirus/enterovirus real-time reverse transcription PCR. Within the seasons, an average of 21% (198/952 for 2009–10) and 13% (128/1002 for 2010–11) of specimens were positive for rhinovirus/enterovirus (Table 2). At least 20% of the rhinovirus/enterovirus–positive specimens were arbitrarily chosen for sequencing (mainly RV A, B, or C; 1 echovirus), but no EV-D68 was identified.

Phylogenetic analysis of EV-D68 strains detected in Germany was conducted for the VP1 and the VP4/VP2 genomic regions (Figure 2). Analysis placed EV-D68 isolates from Germany into major groups 1 or 3 and clustered with strains from the United States and the Netherlands from 2014, which indicated circulation of similar strains in the United States and Europe.

Conclusions

In the 2014 outbreak in the United States, ≈36% (2,600) of specimens were positive for EV-D68; children were predominantly affected. Because testing was prioritized for children with severe respiratory illness, there were probably more cases of mild infections (8). Information on EV-D68 circulation during this period for Europe is rare. This finding might be caused by insufficient sampling of patients with ARI or limited detection of EV-D68 by laboratory diagnostics (9). Sporadic cases of neurologic disease after EV-D68 infection were reported from France and the United Kingdom (9,10).

Investigation for EV-D68 has been continuously performed in the Netherlands since the increase in infections in

2010 (13). The ILI/ARI sentinel system in the Netherlands identified 24 EV-D68 infections in 2010, none in 2011, 7 in 2012, 3 in 2013, and 5 in 2014 (by week 40) (13,14), which probably increased toward the end of that year (9). For the 2014 season, a hospital in the Netherlands reported an increase of EV-D68; 16 patients were infected (12). Such an increase in EV-D68 infections was already seen in 2010 at the same hospital along with an increased number of cases throughout the country (13). This finding increased the possibility that an increase in EV-D68 infections in primary care will also extend to increased numbers of infections in patients in secondary care. So far, we report low EV-D68 circulation in Germany: 25 sporadic cases in 2014.

Clinical patterns in patients in Germany were largely determined by the ILI/ARI case definition for collecting specimens. Most (88%) patients had mild disease. Severe disease was observed in 3 children who had obstructive bronchitis and pneumonia, and 1 child required hospital care. Similarly, mild respiratory disease was predominantly observed for patients in the Netherlands (14). However, more severe cases were detected among hospitalized children who had life-threatening respiratory illness, as described in the United States (6,12,14). More than half of patients with severe respiratory illness in Germany and the Netherlands had concurrent conditions (12,14), which seem to be a major factor for development of severe disease after EV-D68 infection (6).

Phylogenetic analysis of EV-D68 from Germany showed high similarity with current strains from the United States and the Netherlands (12,14). These new genetic clusters reflect the evolution of EV-D68 and might be associated with an increasing activity of this virus. For an improved understanding of the factors determining local and temporal differences in respiratory disease outbreaks, continuous collection of global data by representative surveillance systems is needed.

Table 2. Detection of rhinovirus and enterovirus by national outpatient ARI sentinel surveillance, Germany, weeks 36–20, 2009–2010 and 2010–2011*

Age group, y	No. specimens tested	No. rhinovirus/enterovirus–positive specimens (%)	No. rhinoviruses/enteroviruses sequenced	No. rhinoviruses detected	No. enteroviruses detected
2009–2010					
<1–4	156	45 (29)	11	11	0
5–15	386	71 (18)	18	17	1†
16–34	225	40 (18)	12	12	0
35–60	157	34 (22)	19	19	0
>60	28	8 (29)	4	4	0
Total	952	198 (21)	64	63	1
2010–2011					
<1–4	271	56 (21)	21	21	0
5–15	363	40 (11)	20	20	0
16–34	189	20 (11)	17	17	0
35–60	153	12 (9)	11	11	0
>60	26	0 (0)	0	0	0
Total	1,002	128 (13)	69	69	0

*ARI acute respiratory infection.

†Echovirus 9.

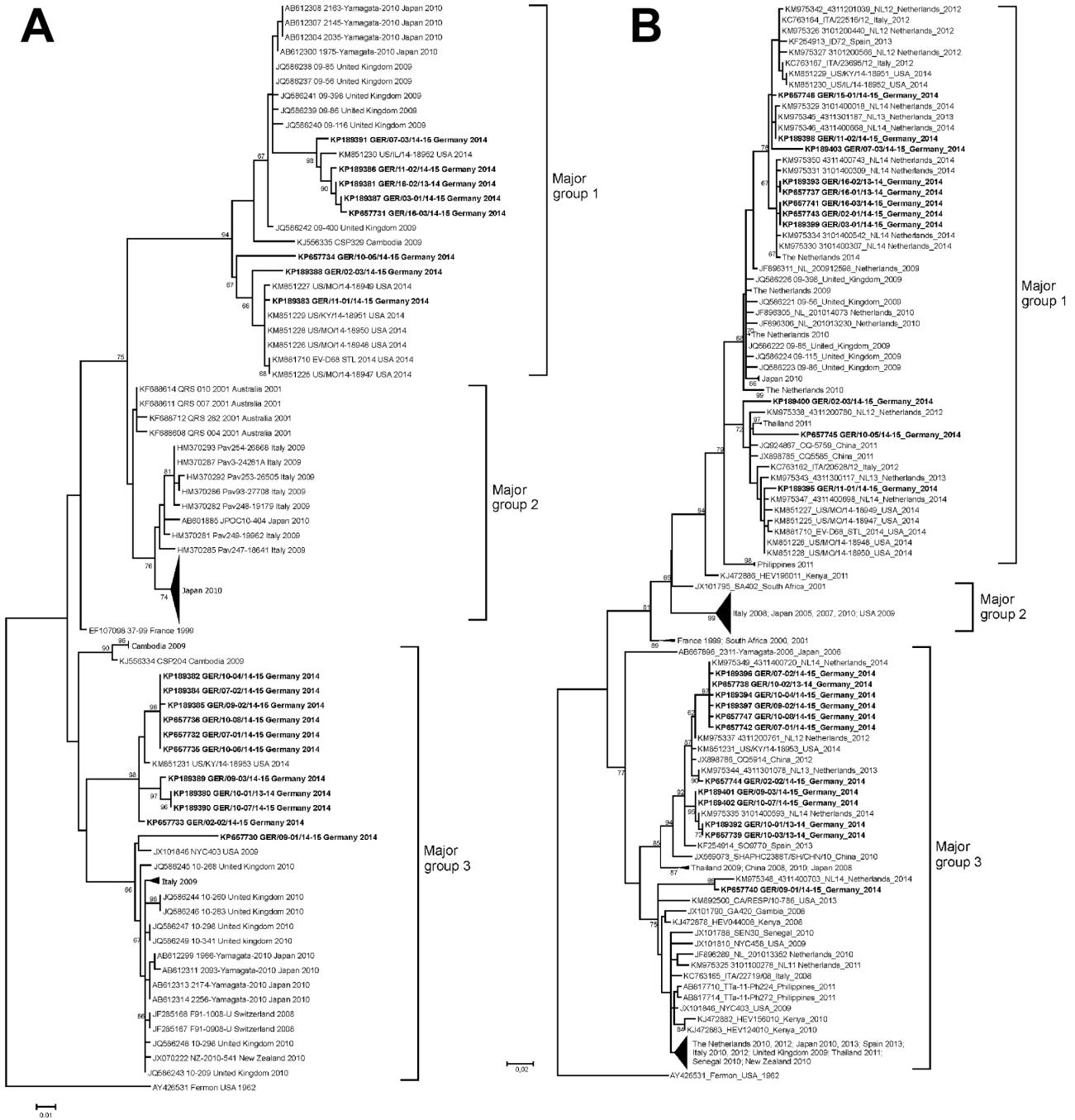


Figure 2. Phylogenetic analysis of selected enterovirus D68 (EV-D68) strains based on nucleotide sequences of A) partial viral protein (VP) 4/VP2 region (357 nt) corresponding to nt 733–1089 in the Fermon strain (GenBank accession no. AY426531); and B) partial VP1 region (339 nt) corresponding to nt 2521–2859 in the Fermon strain. Trees were constructed by using maximum-likelihood estimation (Tamura 3-parameter with 5 gamma distributed rates among sites) with 1,000 replicates through MEGA 5.2 (<http://www.megasoftware.net/>). The Fermon strain was used as an outgroup. Reference sequences were selected from the United States, countries in Europe, and other regions, mainly during 2005–2014. Selected reference sequences are not identical in both trees because complementary VP1 and VP4/VP2 sequence data are not available for all reference viruses. Major groups 1, 2, and 3 are shown as described by Meijer et al. (13,14). Only bootstrap values >65% are shown at branch nodes. EV-D68 strains from Germany are indicated in bold. Scale bars indicate nucleotide substitutions per site. Some parts of the trees are collapsed. For an expanded version of Figure 2, see the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/5/14-1900-Techapp1.pdf>).

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Rates and Risk Factors for Coccidioidomycosis among Prison Inmates, California, USA, 2011

Dr. Charlotte Wheeler
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Rapid Emergence of Highly Pathogenic Avian Influenza Subtypes from a Subtype H5N1 Hemagglutinin Variant

Erik de Vries, Hongbo Guo,¹ Meiling Dai,¹
Peter J.M. Rottier, Frank J.M. van Kuppeveld,
Cornelis A.M. de Haan

In 2014, novel highly pathogenic avian influenza A H5N2, H5N5, H5N6, and H5N8 viruses caused outbreaks in Asia, Europe, and North America. The H5 genes of these viruses form a monophyletic group that evolved from a clade 2.3.4 H5N1 variant. This rapid emergence of new H5Nx combinations is unprecedented in the H5N1 evolutionary history.

A highly pathogenic avian influenza (HPAI) A(H5N1) virus (A/goose/Guangdong/1/1996) was first detected in China in 1996. Multiple clades, defined by phylogenetic characterization of the H5 hemagglutinin (HA) (1), have evolved and spread across Asia, Africa, and Europe, causing enormous losses to the poultry industry. A total of 694 human infections (death rate 58%) were recorded during 2003–2014 (2).

During the evolution of HPAI H5N1 viruses, reassortment events involving the 6 internal gene segments have often been detected (reviewed in [3]), but novel subtypes (i.e., combinations of HPAI H5 with other N subtypes) have rarely been isolated. In 2014, a novel highly virulent reassortant HPAI H5N6 virus (4) caused multiple outbreaks in Southeast Asia and 1 lethal human infection, which led the Food and Agricultural Organization of the United Nations to issue a warning (5). Outbreaks of novel HPAI H5N8 virus in South Korea (6,7), China (8), and Japan raised further concern, and in November 2014, this subtype emerged outside Eastern Asia, causing outbreaks in poultry farms in Germany, the Netherlands, the United Kingdom, Canada, and the United States.

The Study

To determine the evolutionary history of the HA proteins of these novel HPAI subtypes, we collected all HPAI H5 coding region sequences for all subtypes, except H5N1, and then aligned them with 850 H5N1 HA sequences representing all HPAI H5N1 clades (selected from ≈5,000 total sequences) and constructed a phylogenetic tree (Figure 1). Reassortment events leading to the generation of novel

H5Nx subtypes are almost uniquely restricted to a single branch of the tree; the branch contains all isolates of the recent HPAI H5N2, H5N5, H5N6, and H5N8 outbreaks. The only other H5Nx reassortants that have been identified are a limited number of H5N2 subtype isolates that are present in 5 other branches of the tree.

A more detailed analysis (Figure 2) revealed that the monophyletic H5 clade harboring all the recent novel H5Nx reassortants evolved from early members of H5N1 clade 2.3.4 (a group of highly similar H5N1 viruses isolated in China in 2005). On January 12, 2015, the World Health Organization recommended designation of the novel H5 clade as 2.3.4.4 in anticipation of a revised H5 nomenclature (11). A previously described (12) H5N5 virus (A/duck/Guangdong/wy24/2008) is the first detected reassortant subtype within this clade; the donor of the NA segment of this virus could not clearly be identified (13). Subsequent reassortment events between viruses harboring an HA segment originally derived from the novel H5N5 viruses and a range of other avian influenza viruses have generated the H5N2, H5N6, and H5N8 subtypes.

The HA protein of A/wild duck/Hunan/211/2005, a member of H5N1 clade 2.3.4, is highly similar to that of other clade 2.3.4 members, differing from the HA of the earliest known H5N5 descendant (A/duck/Guangdong/wy24/2008, clade 2.3.4.4) at only 12 aa positions. Seven amino acid substitutions are subsequently maintained in all descending viruses: K86R, T160A, N187D, K222Q, S227R, N244H, and A267T. Substitutions K222Q and S227R are unique to clade 2.3.4.4 and have not been observed previously in any HPAI H5N1 viruses.

Within the subtree shown in Figure 2, the H5N2 viruses are present in 2 branches. N2 of A/duck/Jiangsu/m234/2012 was derived from an H11N2 virus (14); the N2 of the other viruses in this branch were derived from an avian H3N2 virus (15). In addition, 12 H5N1 reassortants were found to be spread over different branches of the subtree (Figure 2). The N1 proteins of these reassortants are derived from different H5N1 viruses that descended from H5 clade 2.3.2. Whereas the N1 of 8 identical isolates from Vietnam (A/Muscovy duck/Vietnam/LBM631/2014) is highly similar to the N1 of clade 2.3.2.1b virus A/barn swallow/Hong Kong/1161/2010 (1), the N1 of A/duck/eastern China/108/2008 is highly similar to the N1 of clade 2.3.2.1c virus A/duck/Hunan/8/2008

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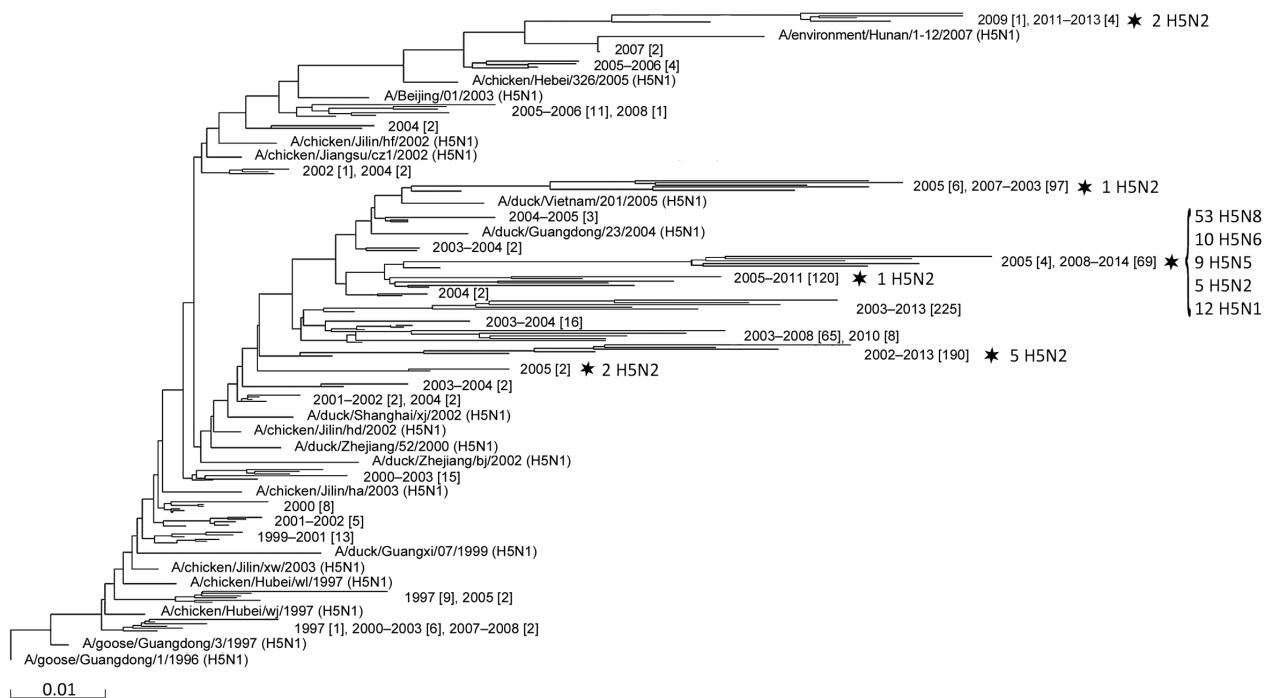


Figure 1. Phylogenetic tree showing the evolutionary history of the hemagglutinin (HA) proteins of novel highly pathogenic avian influenza (HPAI) H5 HA subtype viruses. By using MUSCLE (9), we aligned the coding region sequences for 89 HPAI H5 HA subtype viruses, excluding H5N1, with those for 850 H5N1 HA viruses representing all HPAI H5N1 clades (1); the 89 H5 HA sequences were identified in the NCBI Influenza Virus Resource (10) and the GISAID EpiFlu Database (<http://www.gisaid.org>). A phylogenetic tree was constructed by using the PHYLIP Neighbor Joining algorithm using the F84 distance matrix (<http://www.ncbi.nlm.nih.gov/genomes/FLU/DatasetExplorer/fluPage.cgi?pageInclude=References.inc#PHYLIP>). The number of sequences present in a branch is indicated between brackets. Stars indicate the branches that contain subtypes other than H5N1. The genotypes (H5N2, H5N5, H5N6, and H5N8) and their numbers of occurrence in a particular branch are indicated at right. Scale bar indicates evolutionary distance (nucleotide substitutions per site). Details for GISAID-derived sequences are shown in the Table.

(1), suggesting that independent reassortment events have taken place.

The H5N8 and the H5N6 viruses have segregated into 2 branches. Analysis of the N6 proteins (data not shown) indicates that the H5N6 viruses (all from southern China) are the result of 2 independent reassortment events with avian H6N6 strains. Unfortunately, sequences from the recent H5N6 outbreaks in Vietnam (5) are not yet present in the databases. The 2 different H5N8 virus clusters most likely evolved from a single H5N8 reassortant virus (A/duck/Jiangsu/k1203/2010) that was isolated in China in 2010 (13). Both clusters were identified in Korea in 2014, whereas members of the most evolved cluster were detected later in 2014 in Japan, Germany, the Netherlands, and the United Kingdom.

Conclusion

Since 1996, reassortment events involving H5N1 HPAI viruses have, as far as detected, only rarely led to the generation of new H5Nx subtypes. The 2008 generation of an

H5N5 reassortant virus (prototype A/duck/Guangdong/wy/24/2008) represents the creation of a new HPAI virus that has led to the generation of a range of novel H5Nx reassortants that acquired novel NA proteins (H5N2, H5N6, and H5N8). The H5N6 reassortant became of particular concern after spreading over a wide geographic area in Southeast Asia and causing a fatal human infection in China (5). Meanwhile, the H5N8 subtype spread to Europe in November 2014, resulting in large economic losses in the poultry industry. On the basis of reports from the World Organisation for Animal Health, H5N8 and H5N2 viruses were detected in Canada and the United States in December 2014.

In this study, we exclusively focused on the unique occurrence of new HA–NA combinations. Recent publications have already described the reassortment events of the internal gene segments of several of the viruses mentioned above (6–8, 11–14). In contrast to novel HA–NA combinations, novel constellations of internal gene segments are far from unique and have frequently been

Table. Details for GISAID-derived sequences of the hemagglutinin genome segment of various influenza A(H5) subtype viruses descended from a highly pathogenic avian A(H5N1) virus hemagglutinin variant*

Segment ID	Country	Collection date	Isolate name	Originating/submitting laboratory
EPI544756	Germany	2014 Nov 04	A/turkey/Germany-MV/R2472/2014	Friedrich-Loeffler-Institut
EPI530063	China	2013 Dec 02	A/environment/Shenzhen/25–24/2013	BGI-Shenzhen
EPI533583	China	2014 Apr 21	A/Sichuan/26221/2014	WHO Chinese National Influenza Center
EPI548623	Netherlands	2014 Nov 15	A/chicken/Netherlands/14015531/2014	Central Veterinary Institute
EPI547678	Netherlands	2014 Nov 14	A/Chicken/Netherlands/14015526/2014	Central Veterinary Institute
EPI530054	China	2014 Jan 10	A/duck/Jiangxi/95/2014	BGI-Shenzhen
EPI548493	Japan	2014 Nov 18	A/duck/Chiba/26–372–61/2014	National Institute of Animal Health
EPI548485	Japan	2014 Nov 18	A/duck/Chiba/26–372–48/2014	National Institute of Animal Health
EPI547673	UK	2014 Nov 14	A/duck/England/36254/14	Animal and Plant Health Agency
EPI543002	China	2014 Jan 20	A/duck/Beijing/FS01/2014	Institute of Microbiology, Chinese Academy of Sciences
EPI542617	China	2013 Nov 10	A/duck/Beijing/FS01/2013	Institute of Microbiology, Chinese Academy of Sciences
EPI431456	China	2011 Dec 07	A/duck/Hebei/3/2011	Institute of Microbiology, Chinese Academy of Sciences
EPI431448	China	2011 Dec 01	A/duck/Hebei/2/2011	Institute of Microbiology, Chinese Academy of Sciences

*The GISAID EpiFlu Database is available at <http://www.gisaid.org>. BGI, Beijing Genomics Institute; ID, identification; UK, United Kingdom; WHO, World Health Organization.

observed for HPAI H5N1 viruses (3). Our analysis indicates that new HPAI viruses have emerged that carry H5 proteins capable of matching with multiple NA subtypes. Whether the formation of new HA–NA combinations confers a selective advantage that contributed to the emergence of these novel subtypes is not known and requires elaborate research. However, the balance between HA (receptor binding) and NA (receptor cleavage) protein activities is known to be critical to cell entry and host tropism and may be an important factor that lead to the emergence of new HA–NA combinations. In contrast to HPAI H5N1, the novel clade 2.3.4.4 viruses, excluding H5N6 viruses, have not caused human infections. However, it is unknown to what extent the repeated acquisition of a new NA proteins could enhance the rate of evolution of the HA protein. Obviously such changes could further affect host and tissue specificity, potentially having serious consequences. Therefore, surveillance is required to monitor further spread, evolution, and potential changes in host range.

Acknowledgments

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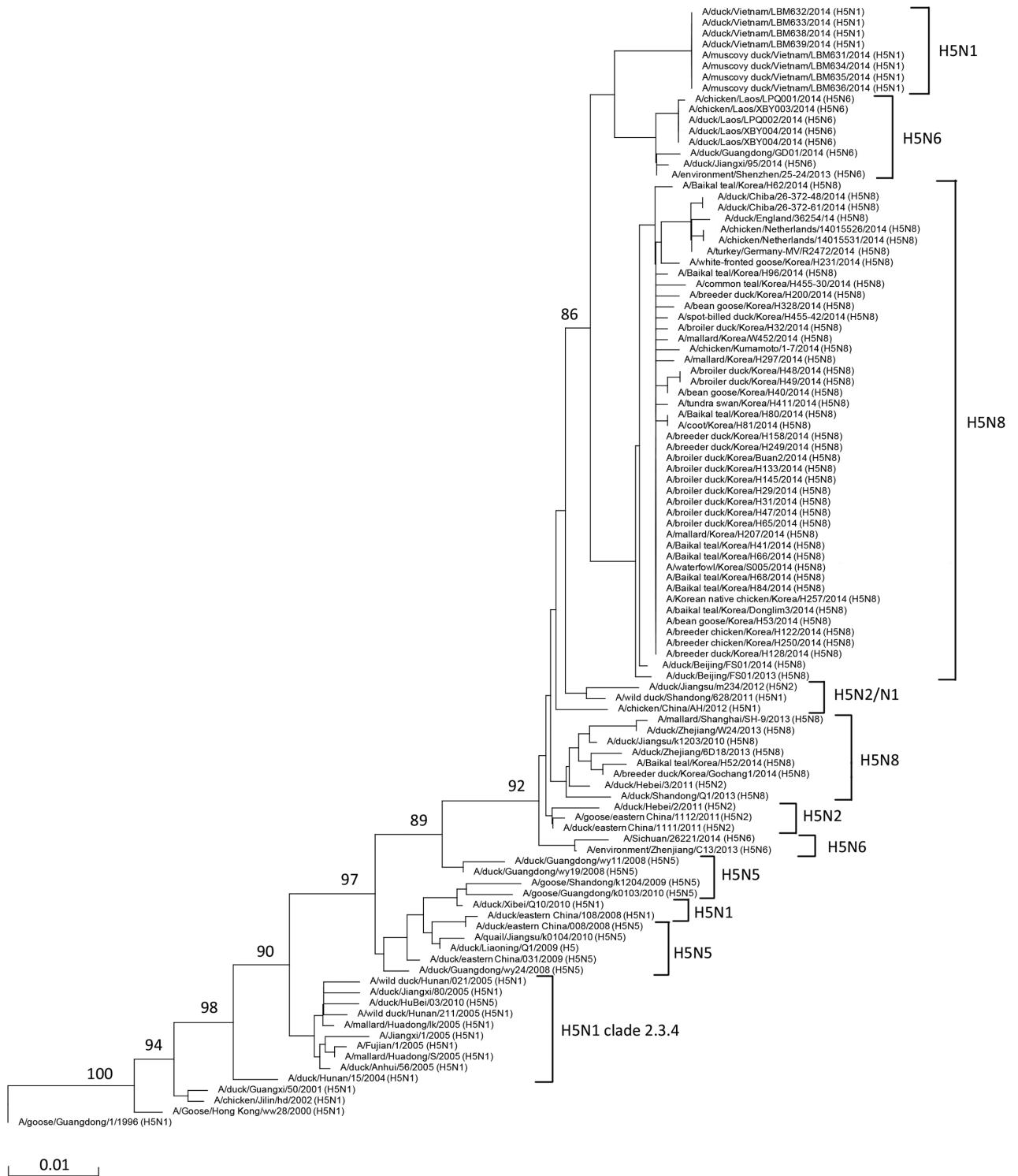


Figure 2. Hemagglutinin protein tree (neighbor-joining, point accepted mutation distance matrix model) of subtypes present in branch descending from highly pathogenic avian influenza A(H5N1) cluster 2.3.4 (see Figure 1). MUSCLE (9) was used to align protein sequences. Subtype group positions are indicated at right. Bootstrap values (n = 1,000) at key nodes are indicated. Scale bar indicates evolutionary distance (nucleotide substitutions per site).

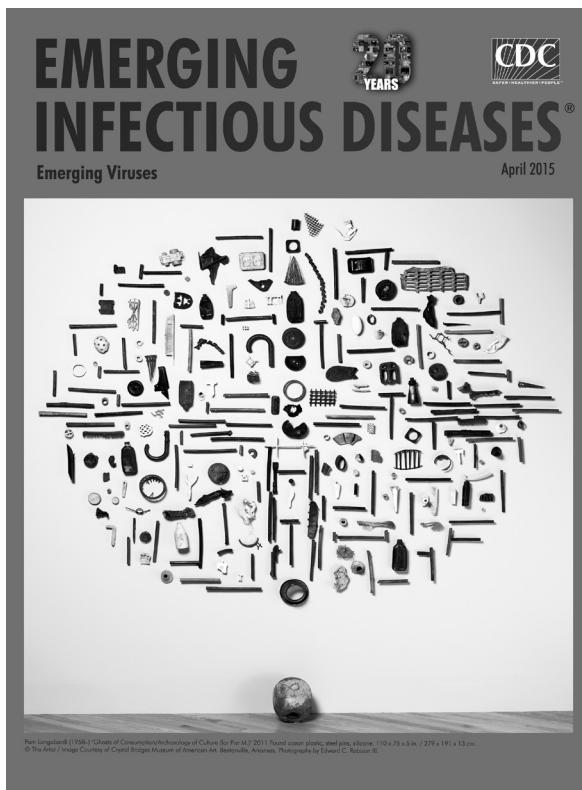
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Antimicrobial Drug Resistance of *Vibrio cholerae*, Democratic Republic of the Congo

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Georges Nguefack-Tsague,
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We analyzed 1,093 *Vibrio cholerae* isolates from the Democratic Republic of the Congo during 1997–2012 and found increasing antimicrobial drug resistance over time. Our study also demonstrated that the 2011–2012 epidemic was caused by an El Tor variant clonal complex with a single antimicrobial drug susceptibility profile.

Cholera is an acute intestinal infection caused by *Vibrio cholerae* (1). Although hydration remains the primary treatment for cholera, antimicrobial drug therapy is recommended for severely ill patients (2). However, multidrug-resistant *V. cholerae* strains have long been observed in Africa (3), and strains exhibiting new resistance phenotypes have emerged during recent epidemics (4). It is therefore critical to carefully monitor changes in strains' susceptibility to antimicrobial drugs in each African country and adapt treatment recommendations accordingly.

Few longitudinal studies assessing shifts in the resistance of *V. cholerae* to antimicrobial drugs in Africa have been established. The available studies are limited either to a restricted area (5) or a short time period (6). We describe

the long-term evolution of antimicrobial drug susceptibility of an extensive set of *V. cholerae* isolates collected in the Democratic Republic of the Congo (DRC). We applied whole-genome sequencing and multiple locus variable-number tandem-repeat analysis (MLVA) to clarify the mechanisms behind the aggressive epidemic of 2011–2012 that spread throughout the country, affecting regions to which cholera was not endemic (7).

The Study

Sample collection included all available isolates from major outbreaks in the DRC during 1997–2012, which were stored at the National Institute of Biomedical Research. The Table shows the locations where the 1,093 tested isolates were collected.

V. cholerae O1 strains stored in nutrient agar during 1997–2012 were cultured on thiosulfate citrate bile salts agar and nutrient agar at the National Laboratory, Kinshasa, DRC. The strains were enriched in alkaline peptone water liquid medium and incubated at 37°C for 18–24 h. Biochemical and serogroup characterization was subsequently performed according to standard protocols (8).

Antimicrobial drug susceptibility testing of 1,093 confirmed *V. cholerae* isolates was performed by using the Kirby-Bauer disk diffusion method (9) with Mueller agar (bioMérieux, Marcy l'Etoile, France). *V. cholerae* O1 Ogawa and Inaba reference strains (ATCC) served as controls. Isolates were tested against 9 antimicrobial drugs as follows: ampicillin (10 µg), chloramphenicol (30 µg), sulfamethoxazole/trimethoprim (1.25 + 23.75 µg), tetracycline (30 µg), doxycycline (30 µg), norfloxacin (5 µg), ciprofloxacin (5 µg), nalidixic acid (30 µg), and erythromycin (30 µg) (bioMérieux). Interpretation of inhibition diameters (sensitive, intermediate, and resistant) was performed according to Clinical and Laboratory Standards Institute guidelines (10). When no interpretive criteria for *V. cholerae* were available from these guidelines, breakpoints for *Enterobacteriaceae* were applied by using *Escherichia coli* ATCC 25922 for quality control. The few intermediate results were categorized as resistant for this study. The strains were then regrouped into 21 resistance profiles.

Seventy-four clinical isolates from the 2011–2012 epidemic, spatiotemporally representative of outbreak diffusion, were subcultured and transported to Marseille, France. In Marseille, the strains were recultivated and identified as previously described (11). For DNA extraction, a 50-colony aliquot of cultured cells was suspended in 500

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Table. Distribution of 1,093 *Vibrio cholerae* isolates, by year and province, Democratic Republic of the Congo, 1997–2012

Year	Province, no. isolates										Total
	Bandundu	Bas-Congo	Equateur	Kasai-Oriental	Katanga	Kinshasa	Maniema	North Kivu	Oriental	South Kivu	
1997	0	4	0	0	0	2	0	0	0	0	6
1998	0	2	0	0	15	14	0	4	7	0	42
1999	8	0	0	0	1	22	0	0	0	0	31
2000	3	0	10	0	0	13	0	0	0	0	26
2001	0	0	0	0	16	0	0	0	0	0	16
2002	0	0	0	0	44	0	0	0	0	0	44
2003	0	0	0	19	21	4	0	0	0	0	44
2004	0	0	0	0	1	2	0	3	0	0	6
2005	0	0	0	0	1	0	0	2	0	0	3
2006	2	3	0	0	9	0	0	0	0	0	14
2007	0	0	0	0	15	1	0	1	5	0	22
2008	0	0	0	0	30	0	5	4	17	0	56
2009	0	0	0	0	112	0	0	2	0	11	125
2010	0	0	0	0	10	0	0	0	0	5	15
2011	46	0	17	0	0	73	4	151	45	0	336
2012	11	25	16	0	1	27	0	196	31	0	307
Total	70	34	43	19	276	158	9	363	105	16	1,093

μL NucliSENS easyMAG lysis buffer (bioMérieux). DNA was extracted by using a NucliSENS easyMAG platform (bioMérieux) according to the manufacturer's instructions. MLVA-based genotyping of the *V. cholerae* isolates and eBURST analysis (<http://eBURST.mlst.net>) were performed as previously described (11). To perform a phylogenetic assessment of the core *V. cholerae* genome on the basis of genome-wide single nucleotide polymorphisms, whole-genome sequencing was performed on an isolate (L286) collected at epidemic onset by using a HiSeq Illumina System (Illumina, San Diego, CA, USA) as previously described (12).

A spatiotemporal analysis was performed on the basis of the antibiogram profiles of *V. cholerae* isolates collected in the DRC during 1997–2012. The strain profiles were plotted by year (Figure 1) and then mapped by year and province. Using these data, we regrouped them into 5 representative periods (Figure 2). The *V. cholerae* strains displayed an increasingly complex resistance phenotype to various antimicrobial drugs. Sulfamethoxazole/trimethoprim resistance was observed initially, followed by resistance to nalidixic acid, erythromycin, and chloramphenicol during the early 2000s. Although sensitivity to fluoroquinolones seemed to be preserved, strain resistance patterns continued to evolve with the circulation of isolates resistant to tetracyclines and ampicillin from 2007–2010. Finally, isolates collected during 2011–2012, which was marked by the westward spread of a major epidemic (7), displayed a single antimicrobial drug susceptibility profile: resistance to most antimicrobial drugs except cyclines and fluoroquinolones.

Serotype analysis of the 1,093 *V. cholerae* isolates showed that Inaba strains were restricted to the western region of the country, Ogawa strains were isolated in the east and south, and Hikojima strains were restricted to Oriental

Province, in the northeastern region of the country. During 2001–2010, Inaba and Ogawa serotypes were observed, but Ogawa predominated; during 2011–2012, these serotypes switched, and the Ogawa serotype was almost completely replaced by Inaba.

To examine the particular 2011–2012 epidemic that spread throughout the DRC (7), 74 *V. cholerae* isolates were assessed by using MLVA and eBURST analysis. Overall, the isolates displayed 19 different MLVA genotypes, of which 18 grouped into 1 clonal complex. eBURST analysis indicated that the clonal complex likely arose from a founder strain identified at the beginning of the epidemic. Furthermore, whole-genome sequence analysis of an isolate identified in March 2011 in Lubunga, Oriental Province (L286), revealed that the strain was an El Tor variant with CTX-3 type phage and a RS1 satellite phage. Phylogeny analysis situated this DRC strain close to the major Kenyan clade in the most recent wave of the seventh pandemic (data not shown).

Conclusions

Analysis of a panel of *V. cholerae* clinical isolates from the DRC from 1997–2012 highlighted a loss of sensitivity to leading antimicrobial drugs, although strains remain susceptible to fluoroquinolones. However, a risk for emergence and spread of fluoroquinolone-resistant strains exists, as has been shown elsewhere in Africa (13). Because resistance to nalidixic acid is frequently associated with decreased susceptibility to fluoroquinolones, nalidixic acid resistance must be detected to monitor the emergence of highly resistant strains (14).

Our findings also provide new insight regarding the cholera epidemic of 2011–2012. This epidemic appears to have been caused by the expansion of a specific *V. cholerae* subpopulation, which rapidly diffused country-

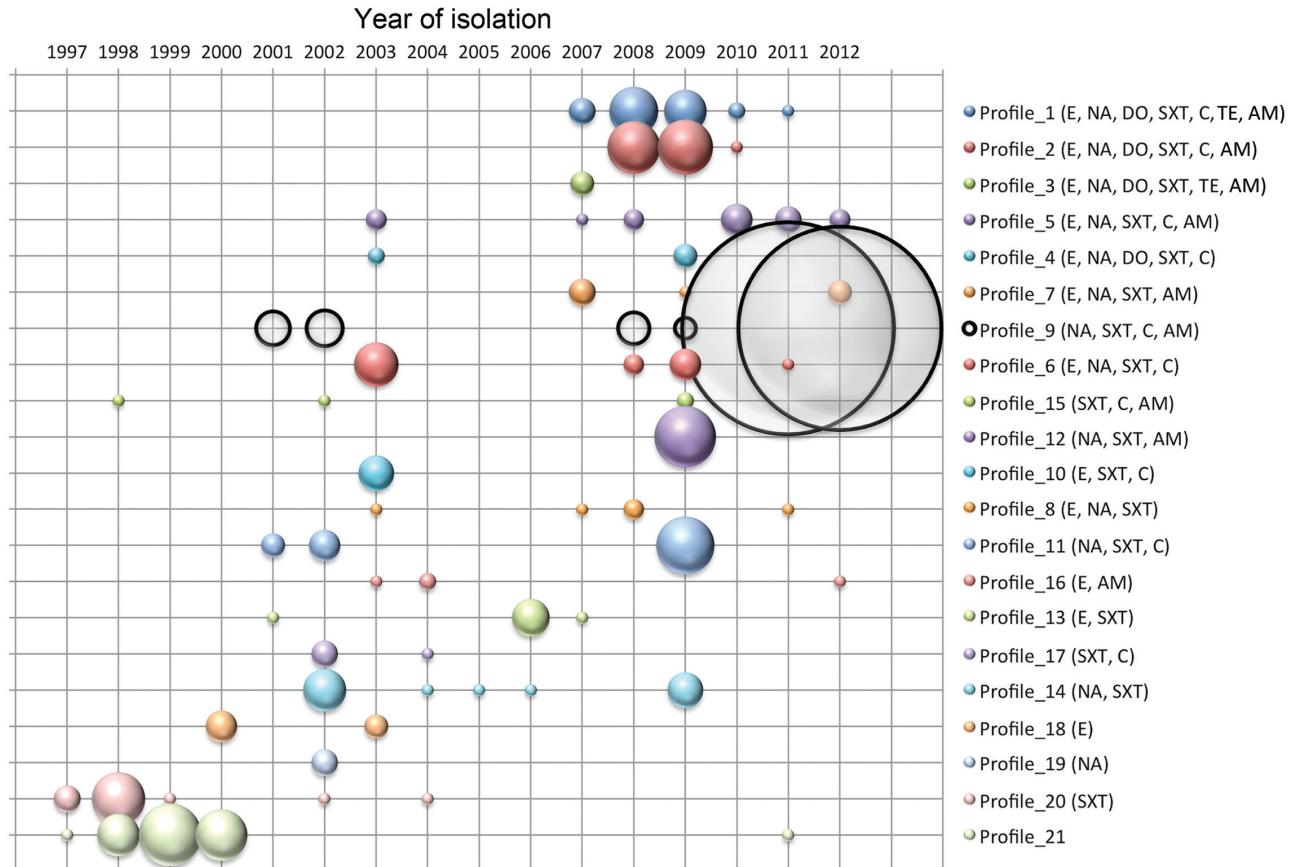


Figure 1. *Vibrio cholerae* strain antimicrobial drug resistance profiles plotted by year, Democratic Republic of the Congo, 1997–2012. On the basis of the antibiogram results, strains were grouped into 21 antimicrobial drug resistance profiles. The antimicrobial drugs to which the strains displayed resistance are indicated on the right. Circle circumference represents the relative number of isolates per profile. AM, ampicillin; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; DO, doxycycline; NOR, norfloxacin; CI, ciprofloxacin; NA, nalidixic acid; E, erythromycin.

wide. Furthermore, sequence analysis showed that the clone responsible for this epidemic, an El Tor variant with CTX-3 type phage, falls close to the major Kenyan clade in wave 3 of the seventh pandemic. This observation correlates with a 2011 study demonstrating that the seventh cholera pandemic had been caused by specific strains originating from a unique ancestral clone that have spread globally in successive waves (12). The 2011–2012 isolates displayed a specific antimicrobial drug resistance pattern, characterized by the return of tetracycline and doxycycline sensitivity. The outbreak strain also represented a serotype switch from Ogawa to Inaba. However, further MLVA genotyping of preoutbreak isolates is required to determine whether these strains were already present in the region or if they represent a new *V. cholerae* population.

This study demonstrates that molecular and microbiological analyses of *V. cholerae* isolates provide extensive insight into the mechanisms of cholera epidemics.

MLVA and whole-genome sequencing are powerful tools for elucidating epidemic dynamics because these methods have been used to link distinct outbreaks and identify the origin of certain epidemic *V. cholerae* strains (15). Improved sampling of clinical isolates is essential to monitor changes in pathogen antimicrobial drug resistance and elucidate the dissemination pathways of toxigenic strains to ensure proper management of patients requiring antimicrobial drug treatment and to appropriately direct the public health response.

Acknowledgments

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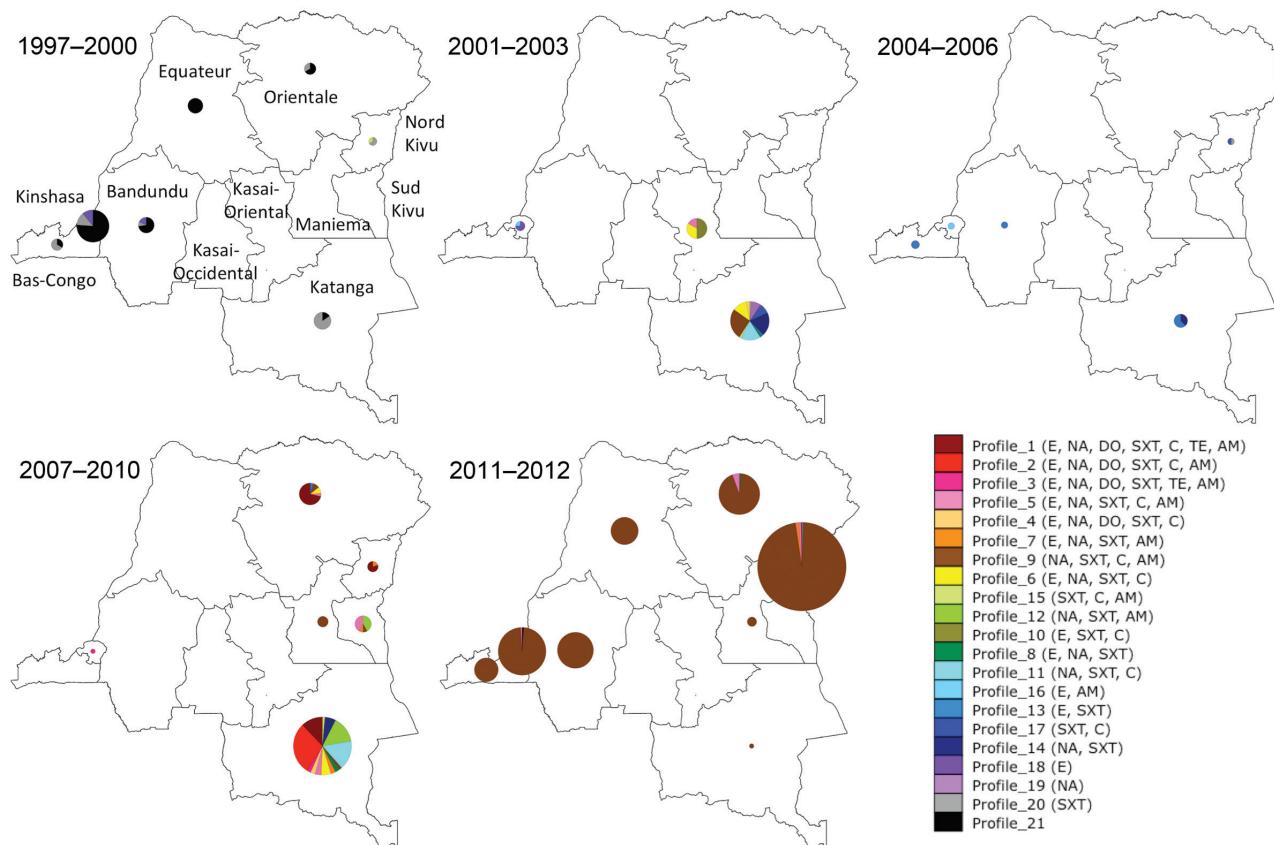


Figure 2. Spatiotemporal localization of isolate antimicrobial drug resistance profiles by time period and province, Democratic Republic of the Congo, 1997–2012. Strains were grouped into 21 antimicrobial drug resistance profiles. The antimicrobial drugs for which the strains displayed resistance are indicated in the lower right panel. Patterns of antimicrobial drug resistance were further grouped into 5 periods. Circle circumference represents the relative number of strains, while the colors correspond to the different antimicrobial drug resistance profiles. Provinces are indicated in the 1997–2000 map. The maps were generated by using QGIS version 2.4.0-Chugiak (<http://qgis.org/api/2.4/>). AM, ampicillin; C, chloramphenicol; SXT, sulfamethoxazole/trimethoprim; TE, tetracycline; DO, doxycycline; NOR, norfloxacin; CI, ciprofloxacin; NA, nalidixic acid; E, erythromycin.

The collection of strains and biological analyses were supported by many partners, including the World Health Organization, the Veolia Foundation, and the African Cholera Surveillance Network. Epidemiologic surveillance was financially supported by the World Health Organization, Epicentre, and the Belgian Development Cooperation.

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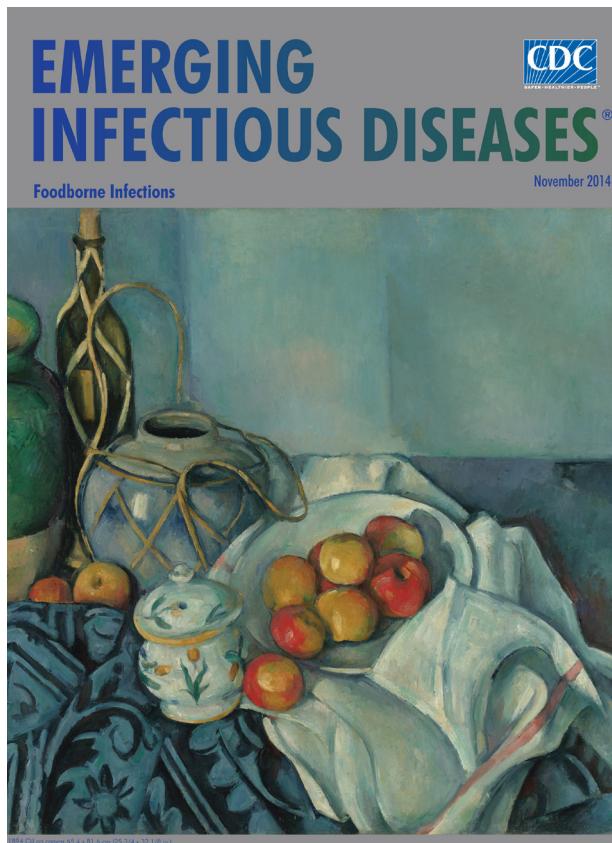
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Transmission Potential of Influenza A(H7N9) Virus, China, 2013–2014

Adam J. Kucharski,¹ Harriet L. Mills,¹
Christl A. Donnelly, Steven Riley

To determine transmission potential of influenza A(H7N9) virus, we used symptom onset data to compare 2 waves of infection in China during 2013–2014. We found evidence of increased transmission potential in the second wave and showed that live bird market closure was significantly less effective in Guangdong than in other regions.

From February 19, 2013, through April 22, 2014, a total of 429 cases of influenza A(H7N9) virus infection in humans in China were reported and occurred in 2 outbreak waves. During the first wave in spring 2013, live bird markets were closed in several parts of China (1,2); these market closures substantially reduced the risk for infection in affected regions (3). During a second wave in autumn 2013 (4), markets were again closed in some provinces (5–7). Analysis of the largest clusters of subtype H7N9 virus infection in 2013 suggested that the basic reproduction number (R_0 , the average number of secondary cases generated by a typical infectious host in a fully susceptible population) was higher in some clusters than in others (8,9), although the absence of sustained transmission implied that R_0 was less than the critical value of 1. To determine the transmission potential of influenza A(H7N9) virus in the first and second waves in 2013, we compared symptom onset data. We also measured the extent to which market closures in 2014 reduced spillover hazard (i.e., risk for animal-to-human infection).

The Study

We focused on the locations of the 6 largest outbreaks: Shanghai, Zhejiang, and Jiangsu (first wave) and Guangdong, Zhejiang, and Jiangsu (second wave). To infer market hazard and human-to-human transmission potential, we used a statistical model of infection spillover (9). We assumed that cases could be generated in 1 of 2 ways: on each day, the expected number of reported cases was equal to the sum of animal exposure and secondary cases generated by earlier infectious hosts (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-1137-Techapp1.pdf>).

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DOI: <http://dx.doi.org/10.3201/eid2105.141137>

Use of such a framework enables estimation of the degree of human-to-human transmission from symptom onset data and of exposure hazard from markets; the accuracy of these estimates is greatly improved when the timing of a sudden change in hazard, such as a market closure, is known (9). We therefore constrained the timing of the drop in exposure hazard to reported market closure dates (online Technical Appendix Table 1). We also estimated R_0 for each of the 6 outbreaks. For patients with known exposure, cluster reports suggest that the serial interval (time delay between symptom onset in primary and secondary case-patients) could be 7–8 days (online Technical Appendix Table 2). We therefore assumed a serial interval of 7 days for our main analysis and tested a range of values from 3 to 9 days during sensitivity analysis. We adjusted for potential delays between symptom onset and case report on the basis of the distribution of delays to date (online Technical Appendix Figure 1).

During the first wave, cases were initially concentrated around Shanghai; reports centered on the city and neighboring Zhejiang and Jiangsu (Figure 1, panel A). A wave-like relationship between location and onset timing was apparent; distance between the location of the first case-patient in Shanghai and subsequent case-patients increased over time (Figure 1, panel B). The pattern of cases at the start of the second wave suggests that infection did not spread outward from a single source; in October 2013, initial cases occurred in Guangdong and Zhejiang.

We used our statistical model to estimate the relative contributions of animal-to-human and human-to-human transmission. In Zhejiang, Shanghai, and Guangdong, market hazard clearly increased and decreased at the start and end of the outbreak, respectively (Figure 2). We also estimated R_0 for different regions over the 2 outbreak waves (Table). Although our estimates for Jiangsu did not change significantly between the 2 waves, for Zhejiang, R_0 was significantly higher for the second wave than for the first wave in spring 2013 ($p = 0.045$). We estimated R_0 to be 0.06 (95% credible interval [CrI] 0.00–0.25) in the first wave and 0.35 (95% CrI 0.15–0.65) in the second.

Using our estimates for R_0 and market hazard, we estimated the number of cases in each outbreak that resulted from human-to-human rather than animal-to-human transmission. We found evidence of a small but significant amount of transmission between humans in the first and second waves (Table). Our findings agree with reports of possible human clusters in the first wave (1,10–12) and corroborate media reports of possible human clusters in Zhejiang and Guangdong

¹These authors contributed equally to this article.

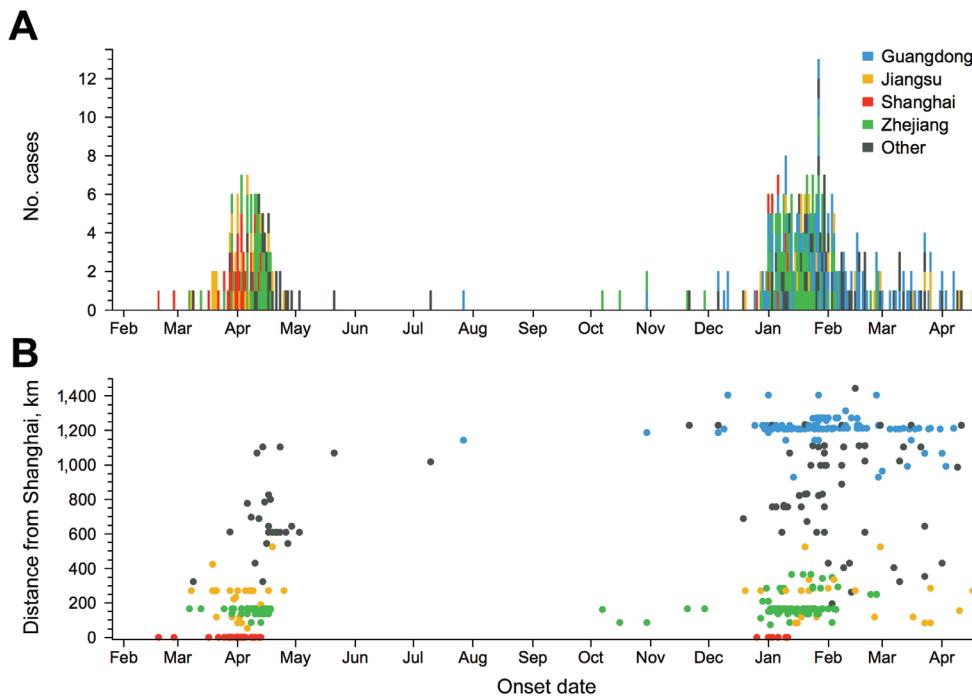


Figure 1. Spatial and temporal distribution of reported cases of influenza A(H7N9) virus infection among humans, China, 2013–2014. Onset of the first case in wave 1 was February 19, 2013 (although the case was not reported until the end of March 2013); onset of the last case in wave 1 was July 27, 2013; only 4 cases occurred in May–July 2013. Onset of the first case in wave 2 was October 7; onset of the last case in our time series was April 17, 2014. A) Case onset reports across all regions. Colors indicate the 4 largest geographic clusters; black indicates all other cases. B) Spatial pattern of reported cases. Points show geodesic distance between the first reported case of influenza A(H7N9) virus infection (in Shanghai) and location of each subsequent reported case. Cases are colored by region as in panel A.

during 2013–2014. We identified 5 clusters during the first wave (February–April 2013) and 8 clusters during the second wave (November 2013–May 2014); the clusters in both waves had median size of 2 cases per cluster (online Technical Appendix Table 2). These conclusions were robust under different assumptions about the duration of serial interval (online Technical Appendix Figures 2, 3).

During the second wave, market closures in Zhejiang began on January 22, 2014, and ended on January 26, 2014 (Table). The reduction in spillover hazard after these closures was significant. We estimated that closures for a serial interval of 7 days reduced hazard by 97% (95% CrI 92%–99%). During 2013, estimated effectiveness was similar in Zhejiang (99%; 95% CrI 97%–100%) and Shanghai (99%; 95% CrI 95%–100%). These estimates are in agreement with those from other analyses for the first wave (3). The 95% CrI was broader for Jiangsu, however, where estimated effectiveness was 97% (95% CrI 80%–100%). In Guangdong, Guangzhou markets closed on February 16, 2014, and reopened on February 28; markets in other cities in Guangdong closed around the same time for 2 weeks. Our results suggest that these closures reduced hazard by 73% (95% CrI 53%–89%). This reduction was significantly smaller than that for Shanghai and Zhejiang ($p < 0.01$). Our result was robust at different serial intervals of infection (online Technical Appendix Figure 4).

Despite the effectiveness of closures during the first wave, interventions in most regions were delayed until

after the Chinese New Year (January 31, 2014). Some regions are investigating alternative market practices: Guangzhou has implemented a trial of a permanent ban on live poultry sales in certain markets, potentially to extend over the entire city by 2024 (5). Our results support recommendations made after the first wave of outbreaks in 2013 (3), which suggest that prompt closure of markets could lead to substantially fewer infections. However, our finding that the relative effectiveness of the shorter closure in Guangdong was lower suggests that such interventions are needed for a sufficiently long time to prevent recurrence.

Our study has limitations. First, case data were insufficient for us to jointly infer serial interval and transmissibility. We therefore tested our results against a wide range of plausible assumptions about the serial interval of infection (online Technical Appendix). We also assumed that the market hazard increased and decreased in a simple stepwise manner (Figure 2). Local market density could also influence the size of spillover hazard and, hence, effectiveness of interventions (13). If the market hazard could be better characterized (e.g., by longitudinal serologic surveillance [14]), the accuracy of our estimates would probably be improved (9). When estimating R_0 , we did not incorporate individual-level variability in transmission and potential superspreading events. However, the framework that we used can still produce reliable estimates of R_0 when a population contains superspreaders (9).

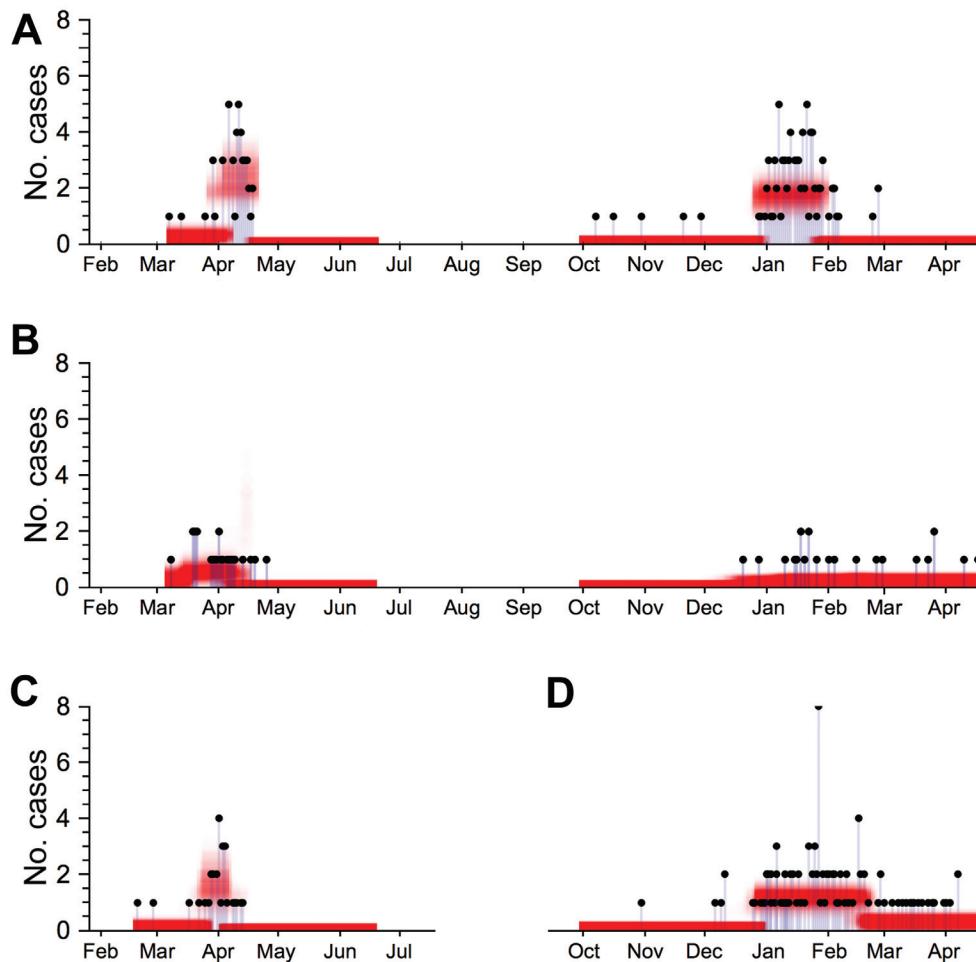


Figure 2. A posteriori probability estimates of spillover hazard for influenza A(H7N9) virus infection in China, by region. Black dots show total number of reported influenza A(H7N9) virus cases for which symptom onset occurred on a given date. Red shading shows a posteriori probability estimate of spillover hazard (i.e., the expected number of cases resulting from animal-to-human transmission on each day). A serial interval of 7 days was assumed. A) Zhejiang, 2013–2014; B) Jiangsu, 2013–2014; C) Shanghai, first outbreak wave, 2013; D) Guangdong, second outbreak wave, 2013–2014.

Conclusions

We found no evidence of reduced human-to-human transmission between the 2 waves. For a serial interval of 7 days, we estimated that R_0 increased in Zhejiang. Furthermore, the effectiveness of live bird market closures varied between regions; short-term closures were substantially less effective than interventions in other regions. These results emphasize the value of prompt and sustainable control measures during outbreaks of influenza A(H7N9) virus infection.

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Table. Estimates of human-to-human transmission and effectiveness of live bird market closures, China, 2013–2014*

Region, outbreak wave	Total no. cases	R_0 (95% CrI)	Human-to-human transmission, no. cases (95% CrI)	Hazard reduction, % (95% CrI)
Shanghai, first	29	0.32 (0.06–0.60)	11.0 (2.3–14.8)	99 (95–100)
Jiangsu				
First	23	0.24 (0.03–0.69)	6.7 (2.0–12.2)	97 (80–100)
Second	26	0.13 (0.01–0.41)	2.9 (0.1–8.7)	NC
Zhejiang				
First	46	0.06 (0.00–0.25)	3.8 (0.8–12.4)	99 (97–100)
Second	92	0.35 (0.15–0.65)	32.5 (17.3–48.9)	97 (92–99)
Guangdong, second	103	0.16 (0.01–0.54)	16.7 (1.0–48.6)	73 (53–89)

*A serial interval of 7 days was assumed. For sensitivity analysis, see online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/5/14-1137-Techapp1.pdf>). CrI, credible interval; NC, not calculated; R_0 , reproduction number (average number of secondary cases generated by a typical infectious host in a fully susceptible population).

Fogarty International Center with the Science and Technology Directorate, Department of Homeland Security.

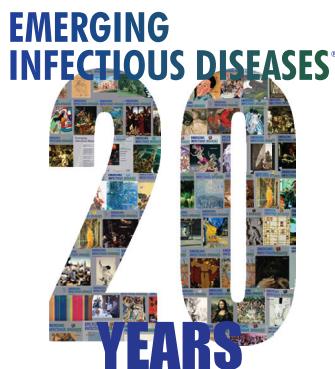
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A History of the *Emerging Infectious Diseases* Journal



Dr. James Hughes and Dr. D. Peter Drotman discuss the history of the *Emerging Infectious Diseases* journal



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Postmortem Stability of Ebola Virus

Joseph Prescott, Trenton Bushmaker,
Robert Fischer, Kerri Miazgowicz,
Seth Judson, Vincent J. Munster

The ongoing Ebola virus outbreak in West Africa has highlighted questions regarding stability of the virus and detection of RNA from corpses. We used Ebola virus–infected macaques to model humans who died of Ebola virus disease. Viable virus was isolated ≤ 7 days post euthanasia; viral RNA was detectable for 10 weeks.

The ongoing outbreak of Ebola virus (EBOV) infection in West Africa highlights several questions, including fundamental questions surrounding human-to-human transmission and stability of the virus. More than 20,000 cases of EBOV disease (EVD) have been reported, and $>8,000$ deaths have been documented (1). Human-to-human transmission is the principal feature in EBOV outbreaks; virus is transmitted from symptomatic persons or contaminated corpses or by contact with objects acting as fomites (2). Contact with corpses during mourning and funeral practices, which can include bathing the body and rinsing family members with the water, or during the removal and transportation of bodies by burial teams has resulted in numerous infections (3).

Assessing the stability of corpse-associated virus and determining the most efficient sampling methods for diagnostics will clarify the safest practices for handling bodies and the best methods for determining whether a person has died of EVD and presents a risk for transmission. To facilitate diagnostic efforts, we studied nonhuman primates who died of EVD to examine stability of the virus within tissues and on body surfaces to determine the potential for transmission, and the presence of viral RNA associated with corpses.

The Study

We studied 5 cynomolgus macaques previously included in EBOV pathogenesis studies and euthanized because of signs of EVD and viremia. Two animals were infected with EBOV-Mayinga and 3 with a current outbreak isolate (Makona-WPGC07) (4).

Immediately after euthanasia, multiple samples were collected: oral, nasal, ocular, urogenital, rectal, skin, and blood (pooled in the body cavity) swab samples and tissue biopsy specimens from the liver, spleen, lung, and muscle.

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Swabs were placed in 1 mL of culture medium and tissue samples were placed in 500 μ L of RNAlater (QIAGEN, Valencia, CA, USA), or an empty vial for titration, before freezing at -80°C . Carcasses were placed in vented plastic containers in an environmental chamber at 27°C and 80% relative humidity throughout the study to mimic conditions in West Africa (5). At the indicated time points (≤ 9 days for 2 animals and 10 weeks for 3 animals), swab and tissue samples were obtained and used for EBOV titration on Vero E6 cells to quantify virus or for quantitative reverse transcription PCR (qRT-PCR) (40 cycles) to measure viral RNA, as reported (6,7).

Viral RNA was detectable in all swab samples and tissue biopsy specimens at multiple time points (Figure 1). For swab samples (Figure 1, panel A), the highest amount of viral RNA was in oral, nasal, and blood samples; oral and blood swab specimens consistently showed positive results for all animals until week 4 for oral specimens and week 3 for blood, when 1 animal was negative for each specimen type. Furthermore, oral swab specimens had the highest amount of viral RNA after the first 2 weeks of sampling, although after the 4-week sampling time point, some samples from individual animals were negative.

In all samples, RNA was detectable sporadically for the entire 10-week period, except for blood, which had positive results for ≤ 9 weeks. Tissue samples were more consistently positive within the first few weeks after euthanasia (Figure 1, panel B). All samples from the liver and lung were positive for the first 3 weeks, and spleen samples were positive for the first 4 weeks, at which time lung and spleen samples were no longer tested because of decay and scarcity of tissue. Muscle sample results were sporadic: a sample from 1 animal was negative at the 1-day time point and at several times throughout sampling.

Viable EBOV was variably isolated from swab from all sampling sites. Among blood samples, those from the body cavity had the highest virus titer (2×10^5 50% tissue culture infectious doses/mL) and longest-lasting isolatable virus (7 days post euthanasia) (Figure 2, panel A). Consistent with the qRT-PCR results, for swab samples, oral and nasal sample titers were highest, followed by those for blood samples, and relatively high titers were observed ≤ 4 days post euthanasia (Figure 2, panel B). Similar to the qRT-PCR experiments, virus titers were higher in tissue samples than in swab samples but were not as sustained; all tissue samples were positive at day 3 post euthanasia but negative by day 4.

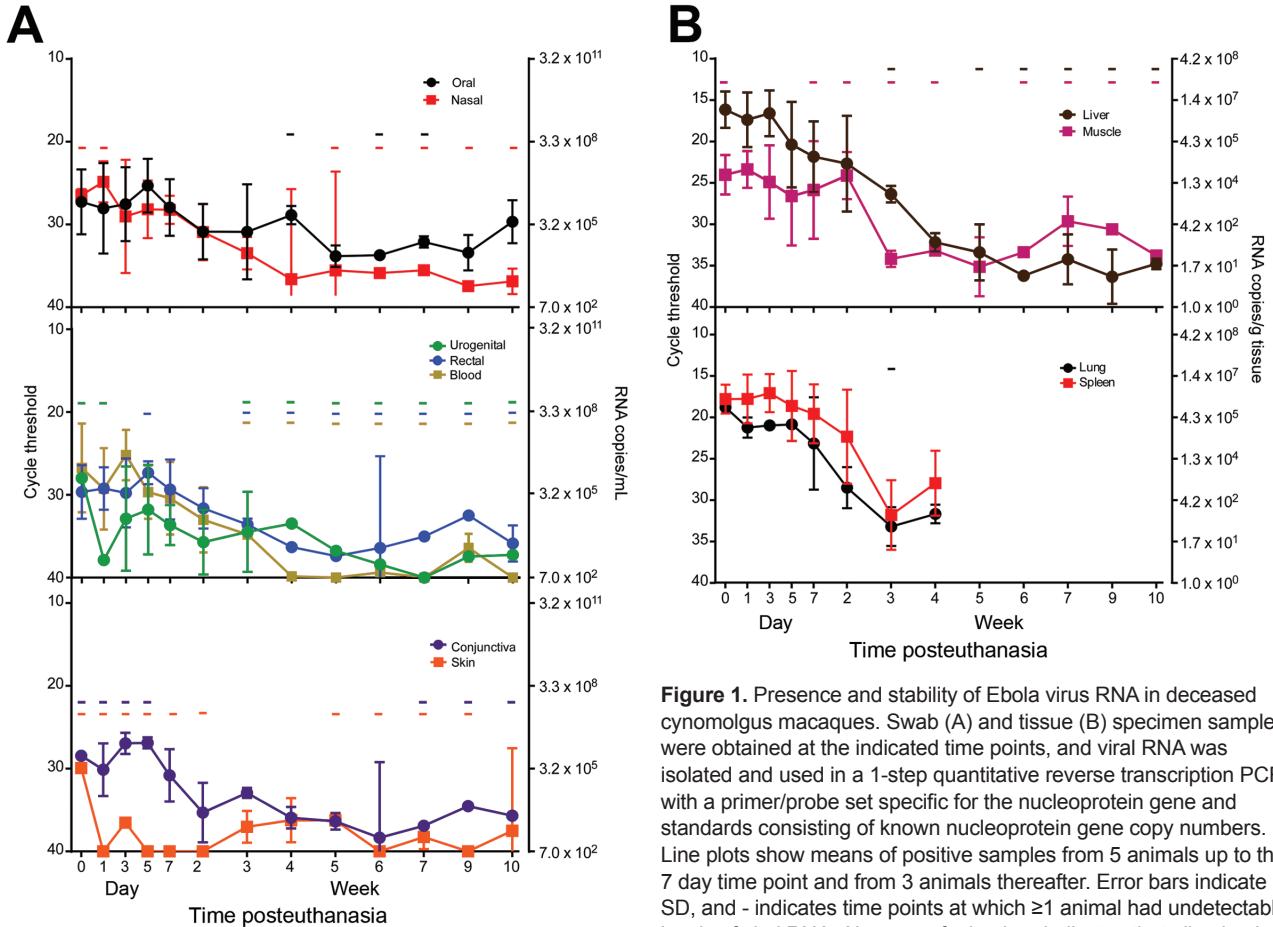


Figure 1. Presence and stability of Ebola virus RNA in deceased cynomolgus macaques. Swab (A) and tissue (B) specimen samples were obtained at the indicated time points, and viral RNA was isolated and used in a 1-step quantitative reverse transcription PCR with a primer/probe set specific for the nucleoprotein gene and standards consisting of known nucleoprotein gene copy numbers. Line plots show means of positive samples from 5 animals up to the 7 day time point and from 3 animals thereafter. Error bars indicate SD, and - indicates time points at which ≥ 1 animal had undetectable levels of viral RNA. Absence of a hyphen indicates that all animals had detectable levels of viral RNA.

Conclusions

The efficiency of detecting EBOV from corpse samples has not been systematically studied; this information is needed for interpreting results for diagnostic samples for epidemiologic efforts during outbreaks. We showed that viral RNA is readily detectable from oral and blood swab specimens for ≤ 3 weeks postmortem from a monkey carcass that was viremic at the time of death, in environmental conditions similar to those during current outbreak (5).

The stability of the target RNA used for RT-PCR is more robust than that of viable virus because degradation of any part of the genome (or proteins and lipids) would compromise the ability of the virus to replicate. Thus, the ability to isolate replicating virus in cell culture from postmortem materials was much less sensitive than detection of viral RNA by qRT-PCR. The sensitivity for quantitating infectious virus is probably lowered because of limitations in isolation efficiency on cell culture and necessary dilutions of tissues for homogenization for titration. Nonetheless, we detected viable virus ≤ 7 days post euthanasia in swab specimens and 3 days in tissues, and showed that infectious virus is present

at least until these times. Because virus titers decreased relatively sharply, despite sensitivity issues, it is unlikely that viable virus persists for times longer than we measured.

Humans who die of EVD typically have high levels of viremia, suggesting that most fresh corpses contain high levels of infectious virus, similar to the macaques in this study (9). Furthermore, family members exposed to EVD patients during late stages of disease or who had contact with deceased patients have a high risk for infection (2). The presence of viable EBOV and viral RNA in body fluids of EVD patients has been studied, and oral swabbing has been shown to be effective for diagnosis of EVD by RT-PCR compared with testing of serum samples from the same persons (10,11). However, detection limits for diagnostic swab samples are unknown for early phases of EVD, and blood sampling is probably more sensitive and reliable for antemortem diagnostics and should be used whenever possible, which has also been shown with closely related Marburg virus (12).

Although these studies included data from outbreak situations, they are limited in their sampling numbers,

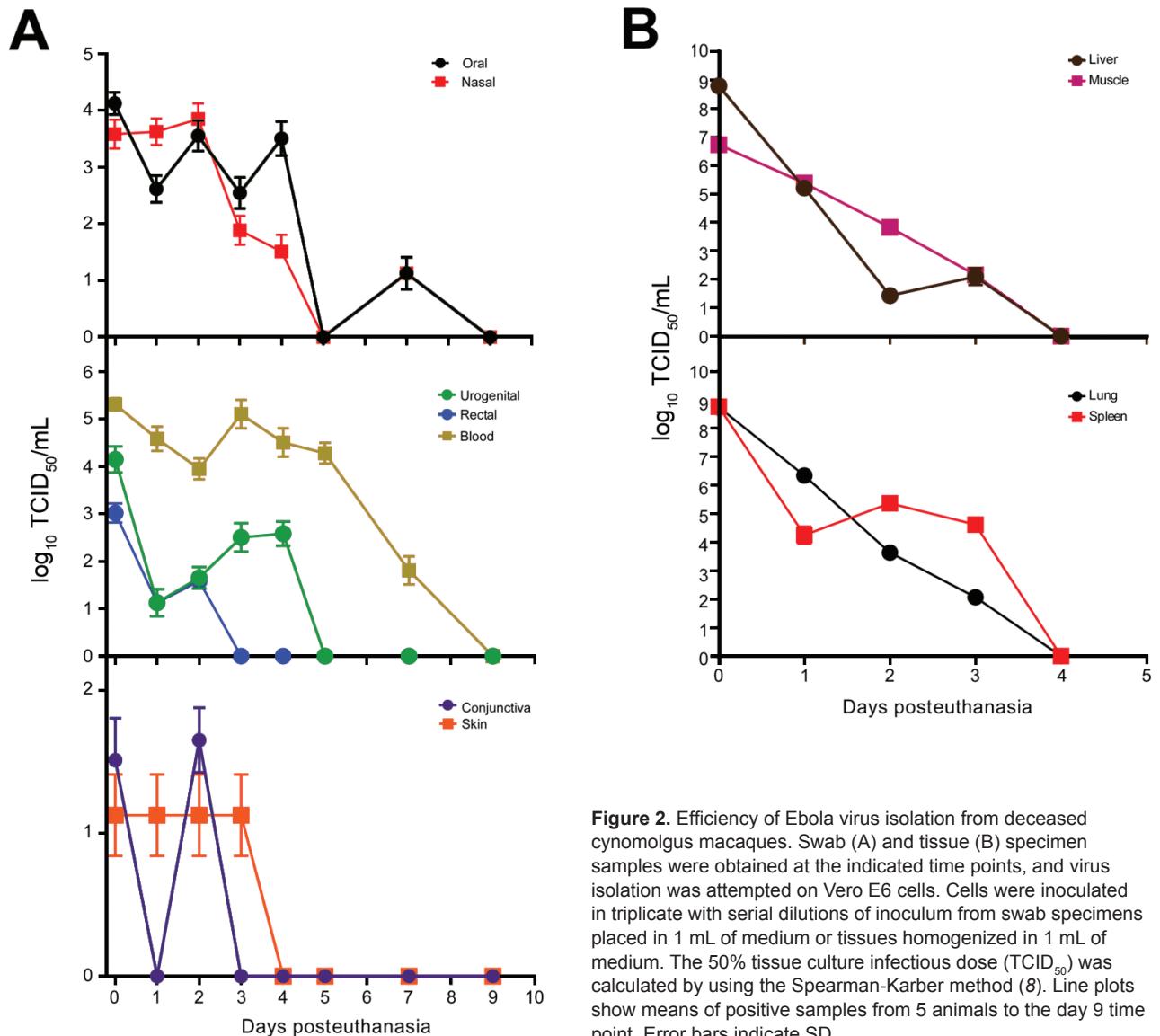


Figure 2. Efficiency of Ebola virus isolation from deceased cynomolgus macaques. Swab (A) and tissue (B) specimen samples were obtained at the indicated time points, and virus isolation was attempted on Vero E6 cells. Cells were inoculated in triplicate with serial dilutions of inoculum from swab specimens placed in 1 mL of medium or tissues homogenized in 1 mL of medium. The 50% tissue culture infectious dose ($TCID_{50}$) was calculated by using the Spearman-Kärber method (8). Line plots show means of positive samples from 5 animals to the day 9 time point. Error bars indicate SD.

swabbing surfaces, and time course, and it is unknown how predictive they are for samples collected postmortem. It is essential to stress that swab samples should be obtained by vigorous sampling to acquire sufficient biologic material for testing, and development of a quality-control PCR target (housekeeping gene target) would be beneficial for sample integrity assessment, which is a limitation of this study.

In summary, we present postmortem serial sampling data for EBOV-infected animals in a controlled environment. Our results show that the EBOV RT-PCR RNA target is highly stable, swabbing upper respiratory mucosa is efficient for obtaining samples for diagnostics, and tissue biopsies are no more effective than simple swabbing for virus detection. These results will directly aid interpretation

of epidemiologic data collected for human corpses by determining whether a person had EVD at the time of death and whether contact tracing should be initiated. Furthermore, viable virus can persist for ≥ 7 days on surfaces of bodies, confirming that transmission from deceased persons is possible for an extended period after death. These data are also applicable for interpreting samples collected from remains of wildlife infected with EBOV, especially nonhuman primates, and to assess risks for handling these carcasses.

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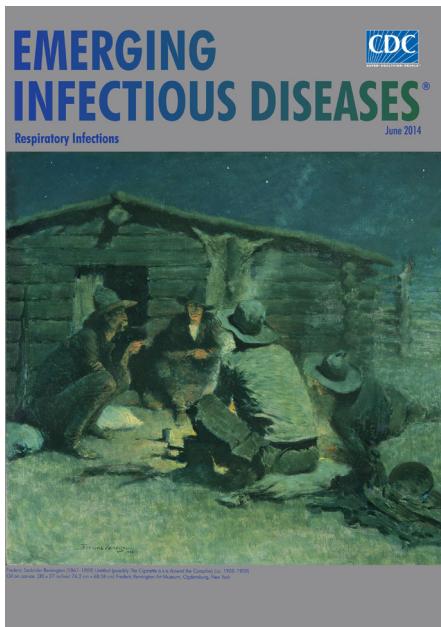
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Influenza A(H5N8) Virus Similar to Strain in Korea Causing Highly Pathogenic Avian Influenza in Germany

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Highly pathogenic avian influenza (H5N8) virus, like the recently described H5N8 strain from Korea, was detected in November 2014 in farmed turkeys and in a healthy common teal (*Anas crecca*) in northeastern Germany. Infected wild birds possibly introduced this virus.

Reassortant highly pathogenic avian influenza (HPAI) viruses of subtype H5N8 were introduced into South Korea in early 2014, possibly by virus-infected wild birds. The virus, which was spread widely by wild birds and within farming networks, caused major outbreaks of HPAI in poultry, was associated with deaths in aquatic wild birds, and spread to Japan (1–4). Related viruses were detected in China (5–7), but before early November 2014, the viruses had been confined to eastern Asia. This study sought to confirm the etiology of a major outbreak of HPAI (H5N8) on a turkey farm in northeastern Germany and to determine the virus's possible origin.

The Study

On November 3, 2014, a sudden increase in deaths among 16-week-old turkeys was noticed at an indoor turkey-

fattening facility in northeastern Germany (8). The affected farm kept 31,000 turkeys and is situated in an area with low density of poultry ≈ 1.3 km east of Lake Galenbeck, a protected, internationally recognized nature reserve frequented by wild birds. The completely fenced farm is surrounded by fields and forest and has restricted access. The turkeys were kept in stables A and B, each with 3 units (A1–3 and B1–3), all connected by a corridor. On November 1 and 2, 2014, 0.5% of turkeys were found dead (expected number of deaths = 0) in unit A3, which is near the stable complex entrance. On November 3 and 4, the number of dead turkeys increased sharply (731 and 899, respectively; 18.4% and 22.6% daily mortality rates). When turkeys were culled on November 6, 2014, ≈ 300 turkeys were alive in A3 (93.4% cumulative mortality rate). In the adjacent unit, A2, onset of disease followed the course occurring in A3 with an increased number of deaths delayed by 1–2 days, but deaths never reached levels found in A3. Units A1, B1, and B2 had been little affected when all turkeys were culled (unit B3 was not in use).

RNA extracted from swab samples of viscous mucus in the oropharynx of dead turkeys and from an organ mixture was positive for influenza A(H5N8) virus by using reverse transcription quantitative PCR, conventional reverse transcription PCR, and sequencing (9). The polybasic hemagglutinin (HA) cleavage site sequence RNSPLRERRRKR*GLF indicated a highly pathogenic phenotype.

Pathomorphologic examination of 2 turkeys revealed herds of pancreatic necrosis associated with fibrinous exudates, necrosis of ileocecal tonsils, and discrete petechiae in peri- and subepicardial locations. Heavily injected subserosal mesenteric vessels dominated the situs. Immunohistologic analysis confirmed systemic infection and revealed influenza virus nucleocapsid protein in 1) ganglions of the adrenal medulla, 2) ependymal cells of the central nervous system (associated with marked lymphocytic meningitis and perivascular cuffing), 3) thymus epithelia, and 4) epithelia of the exocrine pancreas (Figure 1).

A virus isolate (A/turkey/Germany-MV/AR2472/2014; AR2472/14) was obtained in embryonated chicken eggs and in a chicken hepatocyte culture (LMH, ATCC CRL-2117). Full-genome sequencing (using Sanger technology) and nontargeted next-generation sequencing, followed by phylogenetic analyses of the sequences (EPI_ISL_167140), confirmed a close relationship of all 8 segments of

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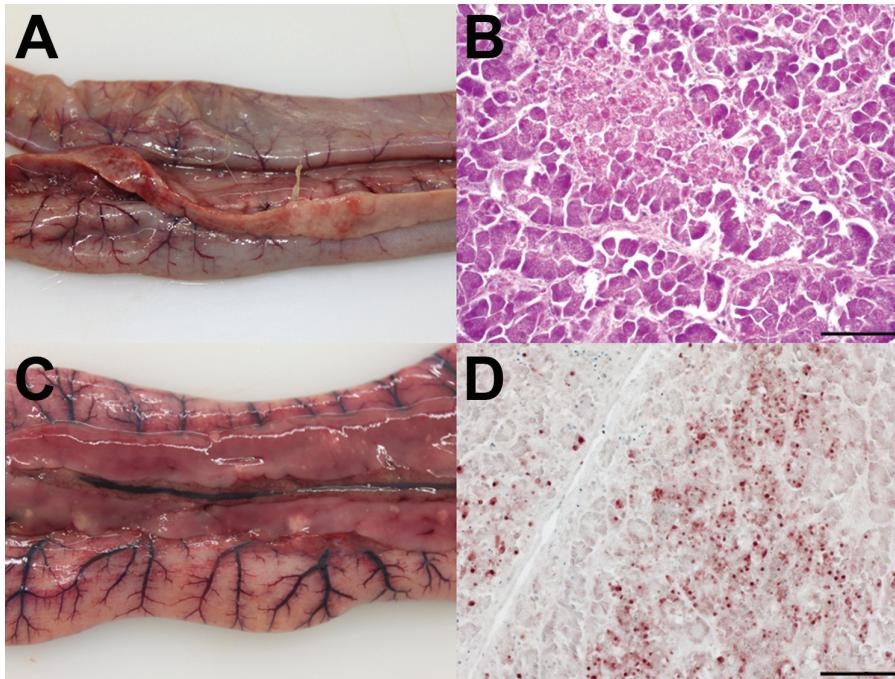


Figure 1. Pathomorphologic results for 2 dead turkeys infected with influenza A(H5N8) virus, Germany.

A, C) Gross pathology showing acute, focally extensive to diffuse pancreatic necrosis with fibrinous serositis.

B, D) Hematoxylin and eosin staining showing acute coagulative necrosis of the pancreas and multifocal staining within the exocrine pancreatic acini for influenza A virus nucleocapsid protein. Scale bars indicate 50 μm (B) and 100 μm (D).

AR2472/14 to HPAI virus subtype H5N8 of clade 2.3.4.4 from South Korea (Figure 2, panel A). Within this clade, 2 sister lineages (A/breeder duck/Korea/Gochang1/2014 and A/Baikal teal/Korea/Donglim3/2014 [Donglim3/14]) can be distinguished. The AR2472/14 isolate forms a cluster with the Donglim3/14 lineage (10), but the derived coding sequences of all segments of AR2472/14 show 14 unique amino acids differing from those of Donglim3/14 (Table).

Three unique amino acid substitutions were found in HA (S197P, A201E, and I390V, numbered according to influenza A virus subtype H5N8) and modeled in the HA structure (11) (Figure 2, panel B). Although S197P is near the receptor-binding site, no HA mutations involve residues previously associated with host specificity, and key residues are in the typical avian configuration: E(190)202, G(225)237, Q(226)238, G(228)240. A201E and S197P are close to antigenic site Sb and could have limited influence on the antigenic profile of AR2472/14. Substitution I390V is in the binding interface of some of the universally neutralizing HA stalk antibodies. Compared with the most geographically widespread H5 clade in birds in East Asia (2.3.2.1), several alterations occur in key antigenic regions, possibly promoting spread of the new clade variant 2.3.4.4. Genetically, A/Sichuan/26221/2014 (H5N6) appears to be the closest human vaccine candidate in preparation (13). Although this influenza A virus subtype differs from AR2472/14 by 19 HA mutations, the mutations are scattered over different regions of the structure (Figure 2, panel B), and putative cross-protection requires confirmation.

In the neuraminidase protein, unique substitutions A190T and M470T were detected in AR2472/14, but on the basis of the deduced protein sequence, AR2472/14 is expected to be sensitive to current neuraminidase inhibitors. In the internal genes, which are shared with the Korean H5N8 subtype, matrix 2 (M2) N31 may confer amantadine resistance, and a C-terminal extension in nonstructural protein 1 masks the PDZ binding motif otherwise involved in host interactions. The sequences of the polymerase basic (PB) 1, nucleoprotein (NP), matrix 1 and 2, and nonstructural (NS) 1 proteins were similar to the prototype sequence of A Donglim3/14; however, the matrix protein had no changes, and the PB1, NP, and NS1 genes had 1 aa change each. Three unique changes were identified in each of the PB2 and polymerase acidic proteins (Table).

Applying restriction measures according to European Union directive 94/2005 (14), such as culling affected flocks, has been effective in stopping further spread of this virus to other poultry farms. Epidemiologic investigations revealed no definite route of introduction of the virus but have excluded incursion by infected turkey eggs or poults; contaminated water, feed, or litter; and vehicles or persons having contact with infected premises in South Korea or East Asia. Introduction by infected wild birds, perhaps facilitated by contaminated litter, feed, water, fomites, or other substance cannot be excluded because an internationally recognized site frequented by wild birds is near the affected turkey farm.

Shortly before the start of the outbreak, large numbers of migratory birds were observed on harvested fields near the

premise. Fecal wild bird samples collected from the environment around the farm were negative for influenza A(H5N8) virus RNA. However, a swab specimen obtained from a healthy common teal (*Anas crecca*) shot from a flock of wild ducks on the island of Ruegen, Germany, on November 17, 2014, showed positive results for HPAI (H5N8) virus.

Since this study began, HPAI H5N8 subtype outbreaks in poultry and infections in wild birds have been reported

in Europe (the Netherlands, England, Italy, Hungary, Sweden, and Germany), Asia (Russia and Japan), and North America (Canada and the United States). On the basis of available sequences, strains from Japan differ only slightly from influenza A(H5N8) viruses from Europe, suggesting that the common ancestor of this new H5N8 subtype variant likely emerged in Asia before recently spreading to Europe (Figure 2, panel A).

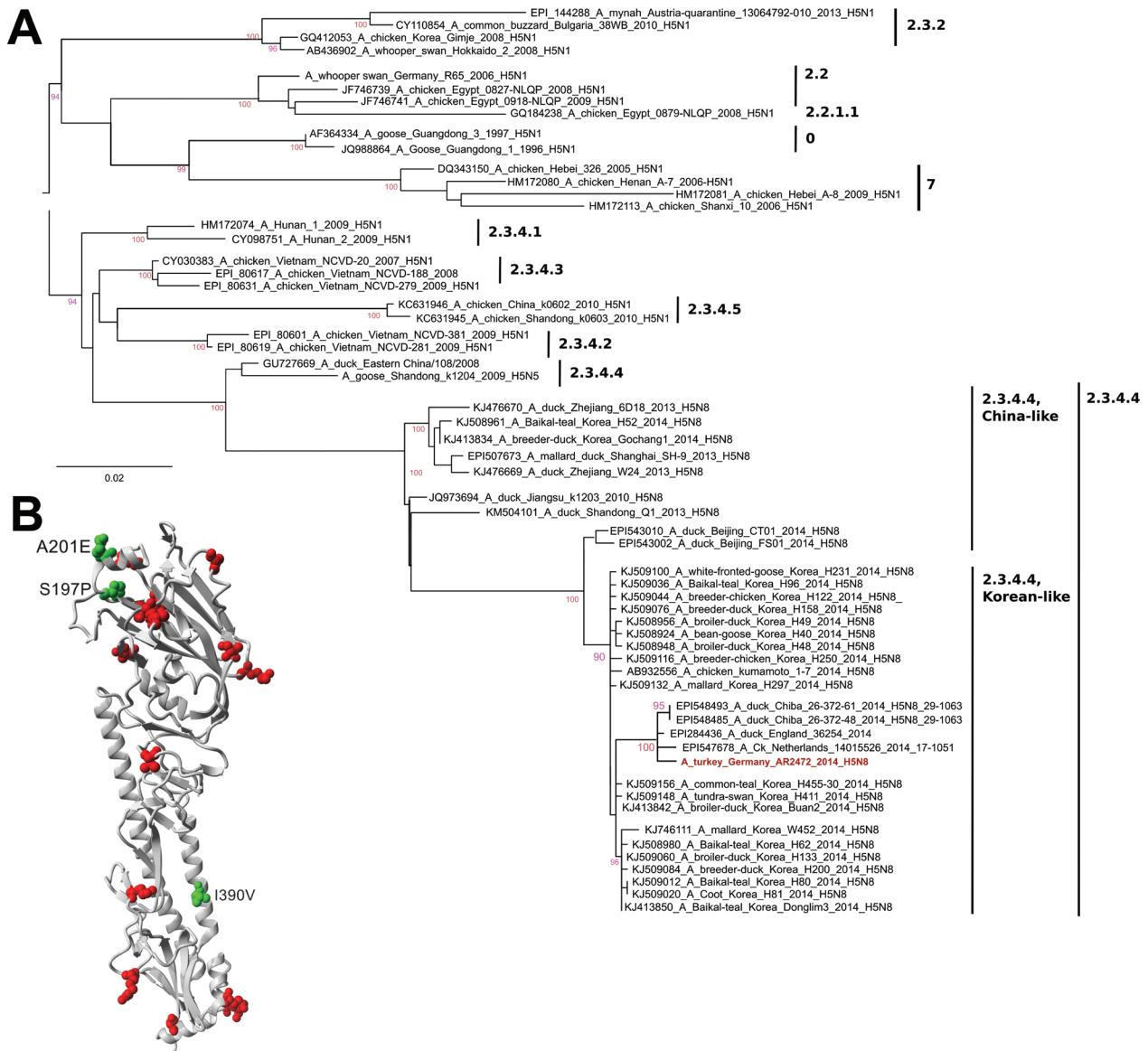


Figure 2. Phylogenetic analysis of hemagglutinin (HA) 1 nucleotide sequences of highly pathogenic avian influenza viruses subtype H5 from Southeast Asia and Germany. Insert shows the structural model of the HA protein of the German H5N8 isolate AR2472/14. A) Nucleotide sequences encoding the membrane-distal part of the HA1 of influenza A(H5N8) viruses were retrieved from public databases, aligned by using MAFFT (<http://mafft.cbrc.jp/alignment/software>) and phylogenetically analyzed by using a maximum-likelihood approach (best fit model: K3Pu+G4) implemented in IQ-Tree (<http://www.cibiv.at/software/iqtree>) (12). Numbers at nodes represent surrogates of branching robustness obtained by an ultrafast bootstrap approach (12). Scale bar indicates nucleotide substitutions per site. B) Model of an HA monomer of AR2472/14 with PDB:3FKU used as template. Green depicts unique mutations distinguishing this virus from other South Korea–origin avian influenza (H5N8) viruses; red indicates additional substitutions relative to the closest vaccine candidate within clade 2.3.4.4. (A/Sichuan/26221/2014 [H5N6]).

Table. Unique amino acid changes found in A/turkey/Germany-MV/R2472/2014 influenza virus compared with A/Baikal teal/Korea/Donglim3/2014 influenza virus*

EpiFlu			
Segment no.	accession no.	Protein	Mutation
1	EPI548431	PB2	V338I R497S K699R
2	EPI548430	PB1	T57K
3	EPI548429	PA	T162I A343V R385K
4	EPI544756	HA	S197P A201E I390V
5	EPI548427	NP	D51N
6	EPI544759	NA	A190T M470T
7	EPI548426		—
8	EPI548428	NS1	V65L

*Numbering according to A/Baikal teal/Korea/Donglim3/2014; sequences were deposited in the GISAID sequence database (<http://gisaid.org>). PB, polymerase basic; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; NS, nonstructural; —, no mutation.

Conclusions

The HPAI outbreak in northeastern Germany in November 2014 resulted from an HPAI (H5N8) subtype virus, represented by isolate AR2472/14, which is closely related to H5N8 subtype viruses that have hitherto been confined to the Far East. Fourteen unique coding mutations of AR2472/14 show differences between this virus and previous isolates from South Korea, but the mutations are shared with the recent H5N8 isolate A/duck/Chiba/26-372-61/2014 from Japan. Epidemiologic and phylogenetic data collected so far are insufficient to establish definite pathways of introduction into Germany. All possible routes, including relay transmission by subclinically infected wild birds, must be thoroughly examined. Enhanced active monitoring of sites frequented by aquatic wild birds and waterfowl is also recommended.

Acknowledgments

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Malaria Imported from Ghana by Returning Gold Miners, China, 2013

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During May–August 2013, a malaria outbreak comprising 874 persons in Shanglin County, China, was detected among 4,052 persons returning from overseas. Ghana was the predominant destination country, and 92.3% of malarial infections occurred in gold miners. Preventive measures should be enhanced for persons in high-risk occupations traveling to malaria-endemic countries.

Malaria is a potentially deadly disease caused by infection with *Plasmodium* spp. parasites, which are transmitted to humans through bites from infected *Anopheles* spp. mosquitoes. As part of global malaria elimination actions by the World Health Organization, in 2010, the government of China initiated the National Action Plan for Malaria Elimination to eliminate malaria by 2020 (1). In recent years, the incidence of malaria in China has decreased sharply to 0.18 cases per 100,000 persons in 2012 (2). However, imported malaria among persons returning from overseas malaria-endemic regions has been documented in some areas of China (3,4). These imported cases present a new challenge to malaria elimination in China. To facilitate formulation of more effective prevention and control measures for imported malaria at a time of rapidly increasing globalization, we describe the epidemiologic characteristics of a large outbreak of imported malaria among Chinese workers returning from overseas countries, in Shanglin County, Guangxi Zhuang Autonomous Region, in 2013.

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The Study

In Shanglin County, since 2006, >10,000 inhabitants have traveled abroad to conduct gold mining work, most of them to Ghana. In late April 2013, the government of Ghana began to strictly regulate the gold mining industry, which forced many gold miners to return to Shanglin County within a short time. In recent years, no locally acquired malaria cases had been reported in Shanglin County; only sporadic cases of imported malaria had been reported. Because Ghana is hyperendemic for malaria, Shanglin County conducted active malaria screening during May 1–August 31, 2013 among 3 groups: 1) persons with an overseas travel history during the previous year, 2) febrile patients visiting hospitals who had no overseas travel history, and 3) asymptomatic local residents who had no overseas travel history but lived in the same household as persons who had malaria.

All persons who had *P. falciparum* infection were treated with artemisinin-based combination therapy; persons who had no glucose-6-phosphate dehydrogenase deficiency and who were infected with *P. vivax* or *P. ovale* were radically cured with chloroquine combined with primaquine; and persons who had *P. malariae* infection were treated with chloroquine. All persons who had malaria were grouped into inpatients and outpatients, and antimalarial treatments differed according to their clinical situations (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-1712-Techapp1.pdf>).

Epidemiologic investigation with a standardized questionnaire was conducted. We also collected data on demographic information, history of overseas travel, dates of illness onset and blood sampling, result of blood testing, clinical features, and treatment.

To evaluate the risk for local transmission of malaria, entomologic investigations using light traps and human landing collections methods were conducted in the 4 villages that had a large number of confirmed malaria cases. The numbers of adult *Anopheles* mosquitoes were recorded, and the species of adult *Anopheles* were distinguished.

During the study period, 6,096 persons were tested for *Plasmodium* spp. infections in Shanglin County: 4,052 persons with histories of overseas travel, 1,316 febrile patients visiting local hospitals, and 728 local residents living with persons who had malaria; no one in the 2 latter groups had traveled overseas (Table 1). We detected 874 persons who had malaria, all of whom had traveled overseas. The attack rate was 216/1,000 persons for the persons returning from overseas.

¹These authors contributed equally to this article.

Table 1. Results of *Plasmodium* spp. screening by microscopic examination, Shanglin County, China, May 1–August 31, 2013

Items	Overall	Persons with overseas travel	Local febrile patients with no overseas travel	Local residents with no overseas travel living with person with malaria
No. persons screened for malaria	6,096	4,052	1,316	728
No. detected malaria infection	874	874	0	0
Attack rate, %	14.3	21.6	0	0
No. <i>Plasmodium</i> species				
<i>P. falciparum</i>	827	827	0	0
<i>P. vivax</i>	42	42	0	0
<i>P. malariae</i>	1	1	0	0
<i>P. ovale</i>	1	1	0	0
<i>Plasmodium</i> spp. co-infection*	3	3	0	0

*All were *P. falciparum* co-infected with *P. vivax*.

Of the 874 persons who had malaria, 871 (99.7%) had returned from Ghana, 2 from Myanmar, and 1 from the Republic of the Congo (Brazzaville). These persons resided in 11 towns in Shanglin County; most lived in 3 towns: 310 (35.5%) in Mingliang, 211 (24.1%) in Dafeng, and 204 (23.3%) in Xiangxian (Figure 1).

Most persons who had malaria were infected with *P. falciparum* (827 [94.6%]). *P. vivax* was responsible for 42 (4.8%) cases; *P. malariae* and *P. ovale* accounted for 1 case each. Three persons were co-infected with different *Plasmodium* spp. (Table 1).

A total of 807 (92.3%) infected persons were gold miners. Nearly all (864 [98.9%]) infected persons were males. Mean age was 36.7 years (range 18–64 years), and most (797 [91.2%]) persons were 20–49 years of age (Figure 2).

A total of 301 (34.4%) *Plasmodium*-positive persons had asymptomatic infections. No deaths occurred. The median interval between return date and diagnosis date was 8 days (range 0–28 days; interquartile range 4–18 days). Among the 369 (42.2%) persons hospitalized for medical treatment, fever $\geq 37.3^{\circ}\text{C}$ (366 [99.2%]), headache (288

[78.0%]), and chills (271 [73.4%]) were the most common symptoms. For hospitalized patients, *P. falciparum* was the predominant species (336 [91.1%]), and *P. vivax* was responsible for 28 (7.6%) cases. Fourteen (3.8%) persons had complicated symptoms. About half (50.7%) of the inpatients had mild to moderate anemia, and 21.1% of those were thrombocytopenic (Table 2).

The median duration of overseas travel was 356 days for persons who had malaria, which did not differ significantly from persons who did not have malaria (median 354 days; $p = 0.7709$, 2-tailed Wilcoxon test). All persons who had malaria reported no history of malaria before going abroad, and 871 (99.7%) had taken no mosquito preventive measures during their stay overseas.

A total of 593 *Anopheles* mosquitoes were collected in the 4 villages that had large numbers of persons who had malaria. All mosquitoes were identified as *An. sinensis*.

Conclusions

We report an unusual, large-scale event of imported malaria among gold miners returning from overseas country to

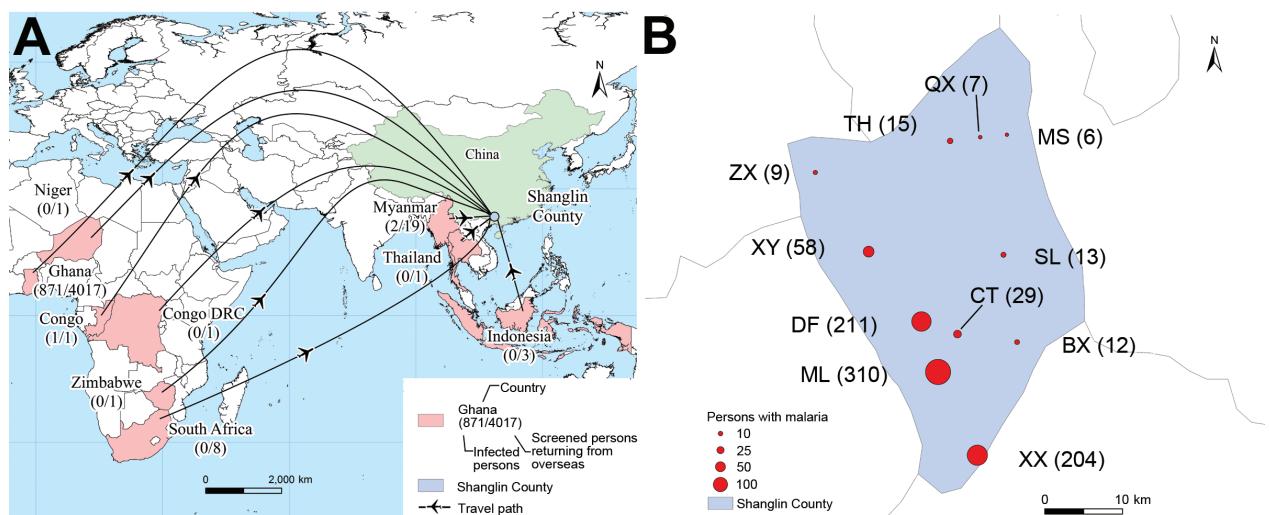


Figure 1. Geographic origin of gold miners returning from overseas (Ghana) and distribution of detected malaria infections, Shanglin County, China, May 1–August 31, 2013. A) Geographic origin of screened miners and persons with malaria. B) Residence of miners who had malaria. DF, Da Feng; ML, Ming Liang; XX, Xiang Xian; CT, Cheng Tai; BX, Bai Xu; SL, San Li; QX, Qiao Xian; MS, Mu Shan; TH, Tang Hong; XY, Xi Yan; ZX-Zhen Xu.

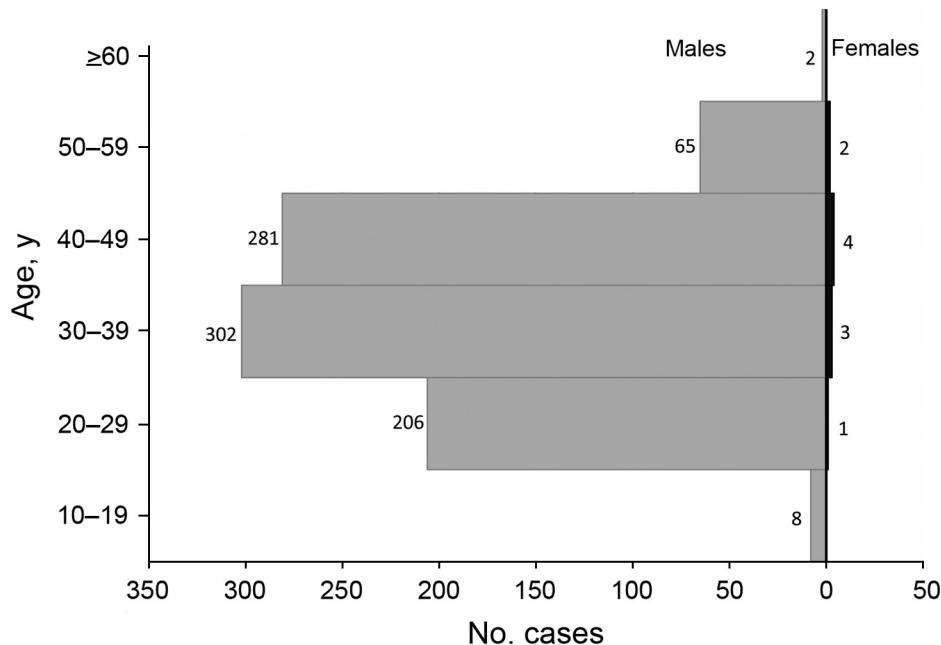


Figure 2. Age and sex of persons who had malaria, Shanglin County, China, May 1–August 31, 2013.

China. Reports have suggested that gold panning activities lead to massive environmental changes, diverting rivers and building basins where vectors can easily breed, thereby increasing the risk for malaria and transmission among gold miners (5–7).

In this outbreak, just over one third (34.4%) of persons who had malaria were asymptomatic. Because asymptomatic carriers with low-level parasitemia can be reservoirs of infection (8,9), the high proportion of asymptomatic malaria among the returning miners would pose challenges to

identifying and treating infection, and transmission interruption in China (10).

In this outbreak, no local malaria transmission was identified. A primary reason is that the local predominant anopheline species is *A. sinensis*, which is refractory to *P. falciparum* (11,12).

One of our study limitations was that chemoprophylaxis and detailed exposure history in Ghana were not well documented because most returning miners lacked knowledge and awareness of malaria. In addition, recall was likely to have been poor, given that the miners had lived and worked overseas for a long time at the time of investigation.

Considering the remarkably increasing volumes of cross-border travel, malaria imported from overseas countries is a new challenge for malaria elimination in China, as illustrated by the outbreak reported here. Measures to prevent mosquito bites and chemoprophylaxis should be addressed among groups at high occupational risk for malaria. Clinicians and public health practitioners should enhance their awareness of malaria infection among groups returning from overseas malaria-endemic areas, regardless of whether they have common symptoms. Additionally, entomologic surveillance should be conducted in areas with high risk for imported malaria to assess the risk for local malaria transmission.

Acknowledgments

We acknowledge Shanglin County Center for Disease Control and Prevention and the clinical institutes in Shanglin County for their assistance in the field investigations and data collection.

Table 2. Clinical manifestation of malaria among hospitalized persons, Shanglin County, China, May 1–August 31, 2013*

Variable	No. cases (%), n = 369
Common signs/symptoms	
Fever, $\geq 37.3^{\circ}\text{C}$	366 (99.2)
Fever $> 38^{\circ}\text{C}$	222 (60.2)
Headache	288 (78.0)
Chills	271 (73.4)
Fatigue	215 (58.3)
Dizziness/nausea	213 (57.7)
Sweating	65 (17.6)
Diarrhea	8 (2.2)
Complicated symptoms	14 (3.8)
Liver function impairment	7 (1.9)
Acute renal dysfunction	2 (0.5)
Gastrointestinal impairment	2 (0.5)
Coma	2 (0.5)
Hemolysis	2 (0.5)
Cerebral lesion	1 (0.3)
Severe anemia	1 (0.3)
Acidosis	1 (0.3)
Blood test result, reference range	
Hemoglobin < 131 g/L, 130–160	187 (50.7)
Platelet count $< 85 \times 10^9$ /L, 85–300	78 (21.1)
G6PD deficiency	45 (12.2)

*G6PD, glucose-6-phosphate dehydrogenase.

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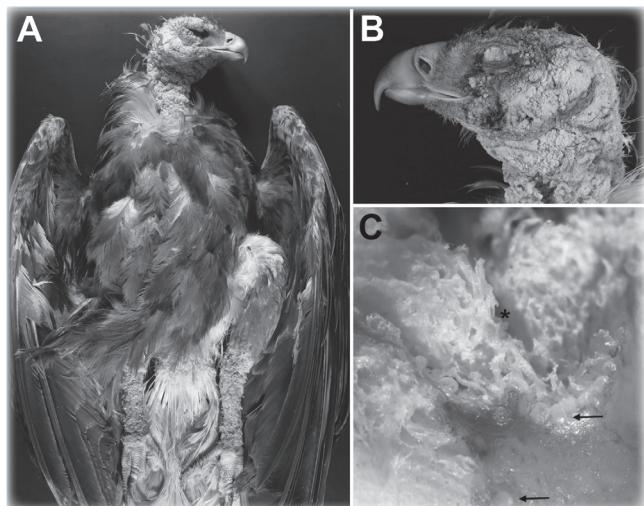
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Knemidocoptic Mange in Wild Golden Eagles, California, USA

Dr. Mike Miller
reads an abridged version
of the article, **Knemidocoptic
Mange in Wild Golden
Eagles, California, USA**



<http://www2c.cdc.gov/podcasts/player.asp?f=8634354>

Canine Infections with *Onchocerca lupi* Nematodes, United States, 2011–2014

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Infections with *Onchocerca lupi* nematodes are diagnosed sporadically in the United States. We report 8 cases of canine onchocercosis in Minnesota, New Mexico, Colorado, and Florida. Identification of 1 cytochrome *c* oxidase subunit 1 gene haplotype identical to 1 of 5 from Europe suggests recent introduction of this nematode into the United States.

The number of human cases of zoonotic filariasis is increasing across industrialized countries (1). In particular, a major zoonotic potential has been recently recognized for *Dirofilaria immitis* and *D. repens* nematodes that infect dogs; both of these nematodes have been reported in cases of human dirofilariasis in the Western Hemisphere and the Old World (1,2).

After the first description of *Onchocerca lupi* nematodes in 1967 in a Caucasian wolf (*Canis lupis cubanensis*) from Georgia (former Union of Soviet Socialist Republics), this nematode has been recognized as the causative agent of canine and feline onchocercosis (3,4). In dogs, the infection occurs in an acute or chronic form characterized by ocular nodules that are often evident on the eyelids, conjunctiva, and sclera (3,5). However, if nematodes localize in the retrobulbar space of the eye, the infection may remain undetected (6). Nonetheless, in disease-endemic areas, *O. lupi* microfilariae may be isolated from skin sediments of apparently healthy dogs (7). Thus, dogs with overt ocular infections might represent only a small portion of the population in which canine onchocercosis occurs in countries such as Hungary, Greece, Germany, and Portugal (3,7).

The role of *O. lupi* nematodes as an agent of infection in dogs in the United States has been suspected. However,

nematodes were previously identified only as *Onchocerca* sp. in California and Utah (8,9) or as *O. lienalis* in Arizona (10). Recent etiologic delineation of *O. lupi* nematodes in dogs and cats in southwestern states (4,11,12) suggested involvement of this parasite in previous cases.

After the first case report of human ocular onchocercosis caused by *O. lupi* nematodes in Turkey (13), interest in this parasite has been renewed, and additional zoonotic cases have been identified in Turkey, Tunisia, and Iran (14). In addition, this parasite has been extracted from the cervical channel of a 22-month-old child in Arizona (12). Information on the epidemiology and life history of *O. lupi* nematodes is still minimal, and data on its distribution in the United States is limited to 6 case reports (4,11).

We report 8 cases of *O. lupi* nematode infection in dogs from Minnesota, New Mexico, Colorado, and Florida. We also compare cytochrome *c* oxidase subunit 1 (*cox1*) gene sequences from 2 nematodes with sequences from parasites in Europe to determine possible recent introduction of this filarioid from Europe to the United States.

The Study

During April 2011–August 2014, a total of 8 privately owned dogs of various ages and sexes were referred to clinical practices in Minnesota (n = 1), New Mexico (n = 4), Colorado (n = 2), and Florida (n = 1) because of different degrees of ocular alterations (Table). At physical examination, nodules were detected in different areas of the eye (Figure 1) and associated with inflammatory reactions ranging from mild scleritis to episcleral swelling and vascular congestion (Table).

All nodules were surgically removed from bulbar conjunctiva or sclera, and white filaria-like parasites were collected and stored in 70% ethanol for morphologic identification. In addition, specimens extracted from 2 dogs (dogs 2 and 3) (Table) were characterized by using molecular techniques. All dogs were treated with macrofilaricides, microfilaricides, antimicrobial drugs, and corticosteroids, which lead to complete resolution of ocular conditions in all except 3 animals (dogs 1, 7, and 8). These 3 dogs had relapses 2, 6, and 12 months, respectively, after surgery.

Nematodes had external, round, transverse ridges and 2 transverse striae per each outer ridge interval, which suggested that they were filarial worms of the genus *Onchocerca*. The ratio between body diameter and distances between ridges (7–10:1) was specific for *O. lupi* nematodes (15). A small piece of nematode was used for molecular

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Table. Characteristics of 8 dogs infected with *Onchocerca lupi* nematodes, United States

Dog no.	Age, y/sex	Geographic origin (travel history)	Clinical signs
1	8/F	Hollywood, Florida (rescued)	Conjunctival and relapsing lesions in both eyes
2	7/M	Oronoco, Minnesota (Durango, Colorado)	Mild scleritis, proliferative eye lesions
3	6/F	Englewood, Colorado (Farmington, New Mexico)	Episcleral swelling and vascular congestion, squinting, scleral indentation in the temporal fundus
4	2/F	Englewood, Colorado (Farmington, New Mexico)	Inflammatory conjunctival follicles, mild epiphora, mild diffuse conjunctival hyperemia, episcleral
5	3/M	Farmington, New Mexico	Moderate blepharospasm and conjunctival hyperemia
6	3/M	Farmington, New Mexico	Moderate chemosis, episcleral flocculent mass
7	9/F	Jerez, New Mexico	Chronic waxing/waning episcleral mass
8	5/M	Farmington, New Mexico	Chronic conjunctivitis, superficial keratitis, episcleral mass, chemosis

identification. Genomic DNA was extracted and partial *cox1* genes were amplified and sequenced as described (13).

In accordance with clinical signs of nodular ocular lesions and morphologic identification, partial *cox1* gene sequence analysis (GenBank accession nos. KP283476 and KP283477) confirmed the identity of the nematode as *O. lupi*, showing 98% nt homology with other sequences of *O. lupi* nematodes in GenBank (KC686701 from Portugal and KC686702 from Greece) and 100% with those derived from dogs and cats from the United States, as well as with a sequence from Greece (EF521409).

Phylogenetic analysis of partial *cox1* gene sequences was performed by using the neighbor-joining method and the Kimura 2-parameter model in MEGA5 (<http://www.megasoftware.net/>). This analysis confirmed that sequences from nematodes examined clustered with *O. lupi* sequences from different areas of the United States (Nevada, California, Colorado, Utah) and with a sequence from Greece (Figure 2). In addition, these sequences were grouped with others from Greece, Hungary and Portugal and formed a paraphyletic clade with other *Onchocerca* species available in GenBank.

Conclusions

Our results indicate that a unique haplotype of *O. lupi* nematodes is circulating in the United States and is endemic to the canine population in this country. Although this onchocercid has been implicated as the causative agent of canine onchocercosis in the United States only recently (11), previous cases attributed to *Onchocerca* spp. have been described in dogs from Arizona, California, and Utah (5,8–10). The cases herein reported from Florida, New Mexico, and Minnesota suggest that the distribution of this nematode is probably wider than previously believed. Detection of *O. lupi* nematodes in Englewood, Colorado, confirms a previous report of infection in a dog from Mancos (11).

We identified 1 *cox1* haplotype and found that it was identical to all sequences in GenBank from the United States and 1 from Greece. Conversely, up to 5 haplotypes were detected in Greece, Turkey, Iran, and Hungary (7). Genetic variation detected in *O. lupi* nematodes from

Europe, Turkey, and Iran, along with isolation of this parasite from the Caucasian wolf, suggests that the infection probably originated in the Old World and was imported into the United States.

The low genetic distance detected for the *cox1* gene is evidence of a substantially reduced evolutionary rate, which supports relatively recent divergence among specimens found in the Old World and New World. In addition to recent detection of *O. lupi* nematode infections in the United States, circulation of 1 haplotype could also suggest that a unique vector species occurs in areas of the Old World and New World where the infection has been diagnosed.

Given that all reports above are based on clinical signs, the epidemiology of *O. lupi* nematodes in the United States deserves to be thoroughly investigated. In particular, dogs relocated from disease-endemic areas to new areas should be routinely screened for skin-dwelling microfilariae because these parasites might represent a risk for other animals. In addition, because *O. lupi* nematodes circulate among canine populations, the potential role of dogs as reservoirs for human infection should not be underestimated,

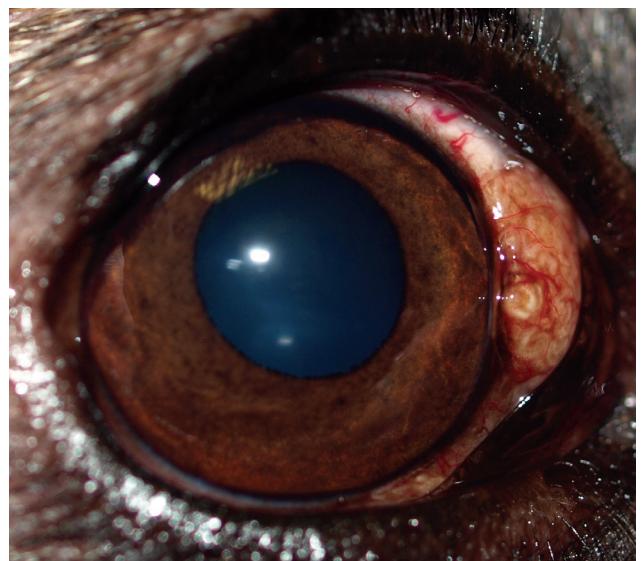


Figure 1. Subconjunctival nodule on the medial canthus of the right eye of dog 2 (Table), Minnesota, USA. This dog was found to be infected with *Onchocerca lupi* nematodes.

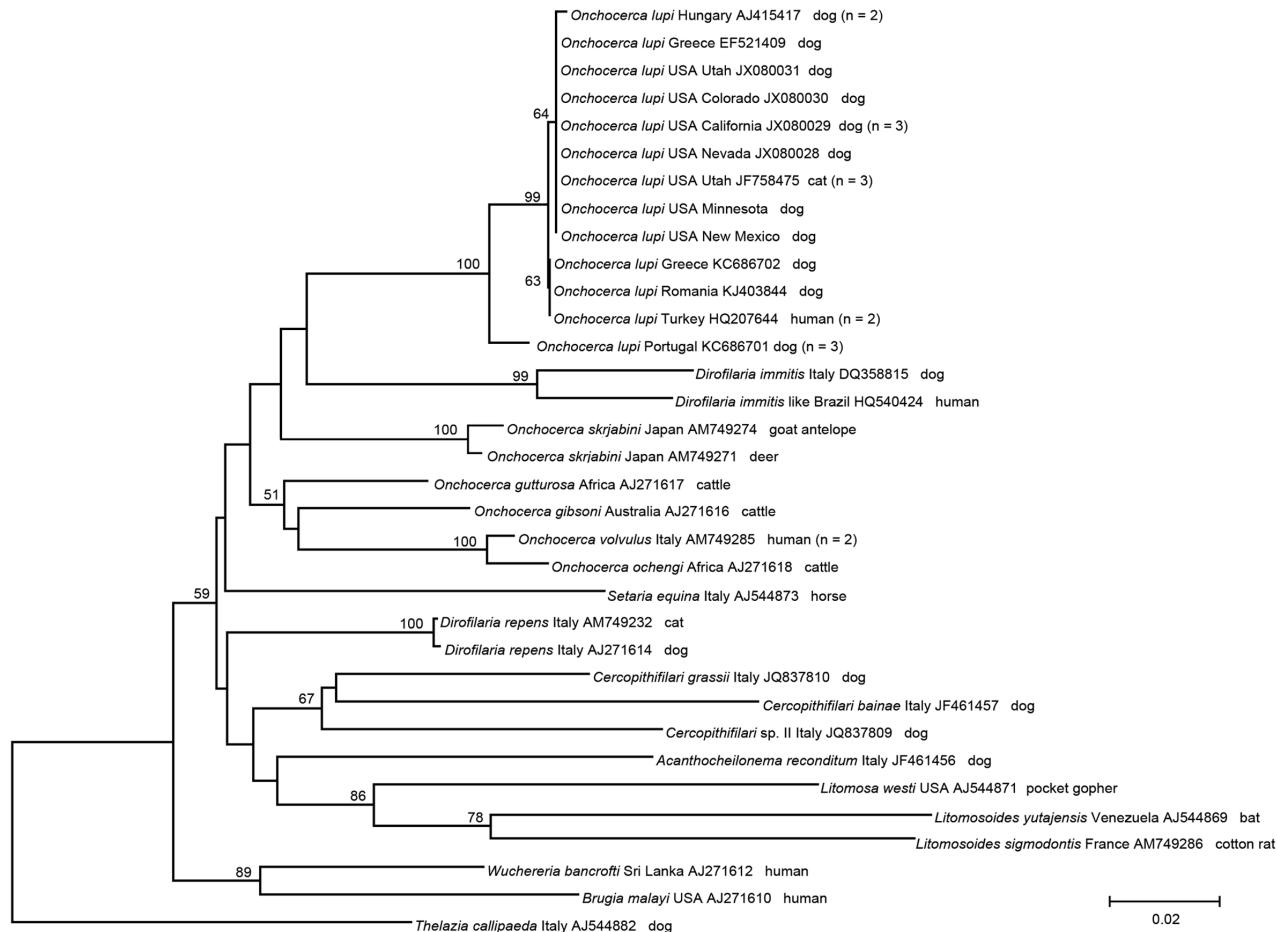


Figure 2. Phylogeny of *Onchocerca lupi* and other filarial nematode species based on partial sequences of the cytochrome c oxidase subunit 1 gene. *Thelazia callipaeda* was used as an outgroup. Bootstrap confidence values (values along branches) are for 8,000 replicates. GenBank accession numbers, number of haplotype sequences (values in parentheses), and geographic origins are shown. Scale bar indicates nucleotide substitutions per site.

as also inferred by zoonotic cases reported in the United States (12). Finally, further studies are urgently warranted toward improving the diagnosis of *O. lupi* nematode infections, which will lead to a better appreciation of its distribution and potential risk for human populations.

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Full-Genome Sequence of Influenza A(H5N8) Virus in Poultry Linked to Sequences of Strains from Asia, the Netherlands, 2014

Ruth Bouwstra, Rene Heutink, Alex Bossers,
Frank Harders, Guus Koch, Armin Elbers

Genetic analyses of highly pathogenic avian influenza A(H5N8) virus from the Netherlands, and comparison with strains from Europe, South Korea, and Japan, showed a close relation. Data suggest the strains were probably carried to the Netherlands by migratory wild birds from Asia, possibly through overlapping flyways and common breeding sites in Siberia.

Highly pathogenic avian influenza (HPAI) A(H5N8) virus probably originated in China, where it was isolated in 2009–2010 (1). Pathogenicity studies showed that the virus was highly virulent in chickens but mildly or moderately virulent in wild ducks. Phylogenetic research demonstrated that it was the product of various reassortment events: the virus's RNA consists of segments that come from other influenza viruses. The backbone of the HPAI (H5N8) virus is formed by parts of the HPAI (H5N1) virus that has circulated in China since 1997 and spread worldwide since 2004.

Beginning in January 2014, H5N8 virus spread rapidly in South Korea, initially mainly among farmed ducks. During the first outbreaks among farmed ducks, numerous dead Baikal teals (*Anas formosa*, a species of migratory wild ducks) were found near the affected farms, leading to the hypothesis that infection was carried by the wild ducks. Genetic analysis of the virus indicated that isolates from infected domesticated ducks and dead Baikal teals in the surrounding area in South Korea strongly resembled earlier isolates from China (2). The analysis also indicated that the HPAI (H5N8) virus in South Korea is a product of reassortment of A/duck/Jiangsu/k1203/2010 (H5N8) and other avian influenza viruses that co-circulated among birds in East Asia during 2009–2012 (3). Kang et al. (4) recently demonstrated by experimental infection of wild ducks (*A. platyrhynchos*) and Baikal teals that HPAI (H5N1) and (H5N8) virus isolates did not cause serious illness or death in these species. Recent phylogenetic studies of HPAI (H5N8) viruses isolated from infected poultry and wild birds in 2014 in South Korea indicate that migrating

birds played a key role in the introduction and spread of the virus in the initial phase of the 2014 outbreak (5). In mid-April 2014, the presence of HPAI (H5N8) virus was demonstrated at a poultry farm in Japan after a rise in the death rate was noted (6). During a monitoring program in November 2014, fecal samples of migrating Bewick's Tundra swans (*Cygnus columbianus bewickii*) tested positive for the HPAI (H5N8) virus. We conducted full-length sequencing to elucidate the origin of the HPAI (H5N8) virus detected in the Netherlands.

The Study

On November 9, 2014, chickens in 1 of 6 poultry houses on a 124,000-bird indoor-layer farm in the Netherlands began dying at an exponentially increasing rate. The dead chickens were submitted for necropsy to the Dutch Animal Health Service (<http://www.gdanimalhealth.com>) on November 14. RNA was extracted from cloacal and oropharyngeal samples from clinically affected hens with positive results from the screening influenza real-time reverse transcription PCR (7), which detects all avian influenza virus subtypes. As a standard procedure, the swab samples were forwarded to the Central Veterinary Institute, the Netherlands' national reference laboratory. Positive screening samples were checked for the presence of H5 and H7 influenza subtypes by real-time reverse transcription quantitative PCR as recommended by the European Union reference laboratory. Hemagglutinin (HA) and neuraminidase (NA) sequence analysis was performed by using PCR fragments that were generated according to previously described protocols (8,9). The HA cleavage site showed polybasic properties RNSPLRERRRKR*GLFGAIA, confirming the high pathogenicity of the virus. In addition, HA and NA sequence results showed that the virus subtype was H5N8.

At the start of the outbreak, preliminary sequencing of the cleavage site showed that it shared high similarity with that of the outbreak strain from Germany. However, complete sequencing was necessary for an investigation of the origin and emergence of this virus in Europe, specifically in the Netherlands. Therefore, we amplified all 8 RNA genome segments of the outbreak virus by using universal 8-segment primers and then directly sequenced the segments (10). Purified amplicons were sequenced at high coverage (average >1,000) by using the Nextera library preparation method and MiSeq system (Illumina, San

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Diego, CA, USA), generating paired-end read lengths of 150 bases. High-quality quality control-passed sequence reads were iteratively mapped by using Bowtie 2 (11) against the genome sequence of the H5N8 virus from South Korea (GenBank accession nos. KJ511809–KJ511816) to generate a majority (>80% evidence) consensus sequence of all segments. The consensus sequences were compared with de novo-assembled sequence reads by using SPAdes version 3 (12); substantial differences were not detected. Most consensus sequences were submitted to the Global Initiative on Sharing Avian Influenza Data (accession no. EPI_ISL_167905). We subsequently performed a molecular phylogenetic analysis on all nucleic acid sequences by using the maximum-likelihood method based on the Tamura-Nei model in MEGA6.0 (13). Accession numbers and sequence providers are listed in the online Technical Appendix (<http://wwwnc.cdc.gov/EID/article/21/5/14-1839-Techapp1.xlsx>).

Genetic analysis shows that the H5N8 virus from the Netherlands (A/chicken/Netherlands/14015526/2014) and viruses from poultry and wild birds from Europe and 2 strains from Japan (A/duck/Chiba/26-372-48/2014 and A/duck/Chiba/26-372-61/2014) detected thereafter are closely

related. Analysis also showed that the viruses are descendants of 3 strains isolated in early 2014: A/broiler duck/Buan2/2014 and A/Baikal teal/Korea/Donglim3/2014 from South Korea and A/chicken/Kumamoto/1-7/2014 from Japan. HPAI (H5N8) virus isolated from a wigeon (*Anas penelope*) in Sakha, northeastern Russia, is a precursor phylogenetically located at the node of European and Chiba viruses (Figure); this virus was isolated in September 2014, but the sequence was released in December 2014 (15).

We determined the number of per site base substitutions between the sequences by using the maximum composite likelihood in MEGA6 (13). Sequences of the 8 genome segments in the H5N8 virus from the Netherlands differed from those of strains A/broiler duck/Korea/Buan2/2014, A/Baikal teal/Korea/Donglim3/2014, and A/chicken/Kumamoto/1-7/2014 by a minimum of 0 and a maximum of 0.009 substitutions. On the basis of data in the National Center for Biotechnology Information Influenza Virus Resource (<http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>) and EpiFlu (<http://www.gisaid.org>) databases, the H5N8 virus from the Netherlands shares the highest similarity with strain A/Baikal teal/Korea/Donglim3/2014 from South Korea.

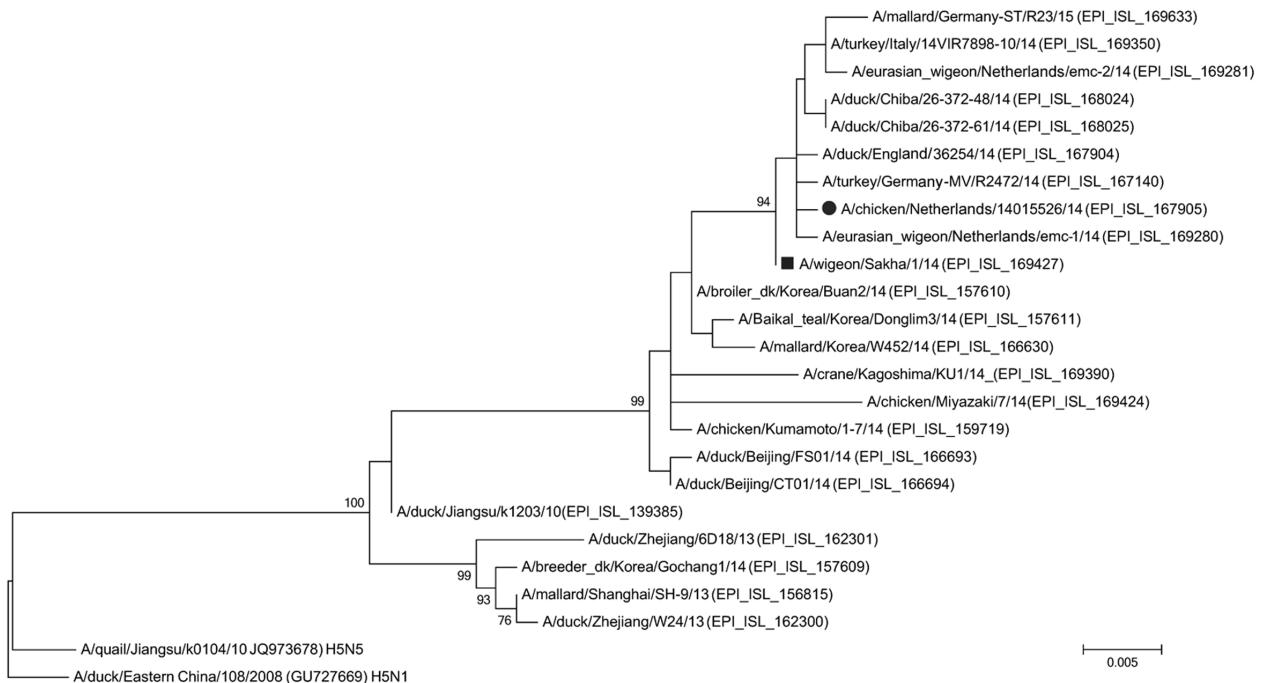


Figure. Phylogenetic tree of hemagglutinin gene of highly pathogenic avian influenza A(H5N8) viruses. The evolutionary history was inferred by using the maximum-likelihood method based on the Tamura-Nei model in MEGA6 (13). The tree with the highest log likelihood is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbor-joining and BIONJ (14) algorithms to a matrix of pairwise distances estimated by using the maximum composite likelihood approach and then selecting the topology with superior log likelihood value. The Tamura-Nei model was used by assuming a gamma distributed rate among nucleotide sites. The tree is drawn to scale; scale bar indicates the number of nucleotide substitutions per site. The analysis involved 25 nt sequences. All positions containing gaps and missing data were eliminated. There were a total of 761 nt positions in the final dataset. Black dot indicates A/chicken/Netherlands/14015526/2014; black square indicates A/wigeon/Sakha/1/2014.

Conclusions

Genetic analysis of influenza A(H5N8) virus from the Netherlands indicates that the virus probably was spread by migratory wild birds from Asia, possibly through overlapping flyways and common breeding sites in Siberia. In addition to the outbreak in the Netherlands, several other outbreaks of HPAI (H5N8) virus infections were reported in Europe at the end of 2014 after exponentially increasing deaths occurred in chicken and turkey flocks. Genetic sequences submitted to the EpiFlu database indicated that the viruses from Europe showed a strong similarity to viruses isolated earlier in 2014 in South Korea, China, and Japan. An H5N8 virus isolated from a wigeon in Russia in September 2014 is located in the phylogenetic tree near the node of all sequences for H5N8 viruses from Europe. In regard to time, this location fits the hypothesized route of H5N8 virus introduction into Europe. Furthermore, for several reasons, it is highly likely that the introduction of HPAI (H5N8) virus into the indoor-layer farm in the Netherlands occurred via indirect contact. First, despite intensive monitoring, H5N8 viruses have never been detected in commercial poultry or wild birds in the Netherlands. Second, when the virus was detected, the Netherlands had no direct trade contact with other European countries or Asia that might explain a route of introduction. Third, because of the severity of disease in galliforms, outbreaks of H5N8 in the Netherlands before November 2014 would have been noticed.

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Dr. Bouwstra is a veterinary virologist and leader of the avian influenza and Newcastle disease project in the Department of Virology, Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, the Netherlands. Her research interests are notifiable animal diseases and One Health.

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Culex torrentium Mosquito Role as Major Enzootic Vector Defined by Rate of Sindbis Virus Infection, Sweden, 2009

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We isolated Sindbis virus (SINV) from the enzootic mosquito vectors *Culex torrentium*, *Cx. pipiens*, and *Culiseta morsitans* collected in an area of Sweden where SINV disease is endemic. The infection rate in *Cx. torrentium* mosquitoes was exceptionally high (36 infections/1,000 mosquitoes), defining *Cx. torrentium* as the main enzootic vector of SINV in Scandinavia.

In Sweden, Finland, Russia, and South Africa, Sindbis virus (SINV; family *Togaviridae*, genus *Alphavirus*) is an etiologic agent for outbreaks of rash and long-lasting polyarthritides (1). Ecologically, SINV is a zoonotic mosquito-borne virus that naturally circulates in bird populations but only incidentally infects humans (1). Previous detections and isolations of SINV from field-collected mosquitoes identified the ornithophilic mosquitoes *Culex pipiens*/*Cx. torrentium* and *Culiseta morsitans* as possible enzootic vectors of SINV and the generalist mosquitoes *Aedes cinereus* and *Ae. rossicus*, which feed on birds and humans, as potential bridge vectors for transmission of the virus from viremic birds to humans (2,3; J.C. Hesson, J.O. Lundström, unpub. data). However, female *Cx. torrentium* and *Cx. pipiens* mosquitoes are morphologically indistinguishable, so all previous virus isolates from these species were from pools that may have contained both species. The distinction between *Cx. torrentium* and *Cx. pipiens* is necessary because vector competence experiments show great differences between the capacities of the 2 species to become infected with and to transmit SINV (4,5). *Cx. torrentium* is highly superior to *Cx. pipiens* as a vector of SINV in the laboratory (4,5), but the extent to which the 2 species are infected in nature is unclear.

We determined the natural SINV infection rates (IRs) in *Culex* mosquitoes, which were identified by using a newly developed molecular method for reliable identification of *Cx. torrentium* and *Cx. pipiens* mosquitoes (6). We also

studied the simultaneous occurrence of SINV in *Cs. morsitans* mosquitoes.

The Study

Every 2 weeks during July 13–September 13, 2009, we collected adult female mosquitoes by using 35 miniature light traps from the US Centers for Disease Control and Prevention (Atlanta, GA, USA) baited with carbon dioxide; the traps were set within the regular mosquito surveillance area of the River Dalälven floodplains in central Sweden (7). Mosquitoes were kept cold on a chilled table during morphologic identification and stored at -80°C. Legs were removed from mosquitoes morphologically identified as *Cx. pipiens*/*Cx. torrentium* and used for DNA extraction, enabling identification of the individual specimens to species by using a previously described molecular method (6).

RNA was extracted from 668 mosquito bodies without legs (301 *Cx. torrentium*, 367 *Cx. pipiens*) and from 74 pools of mosquitoes pooled by collection trap and week (290 *Cs. morsitans*); pool sizes ranged from 1 to 19 mosquitoes. The mosquitoes were processed for RNA extraction, real-time reverse transcription PCR (rRT-PCR), and virus isolation on Vero cells as previously described (3).

First, all samples were screened by SINV rRT-PCR by pooling 2–10 RNA extractions by species and collection week. Of 81 total pools, 14 were positive for SINV RNA. Nine positive pools were from *Cx. torrentium* mosquitoes, and 3 and 2 pools, respectively, were from *Cx. pipiens* and *Cs. morsitans* mosquitoes. Second, all individual samples from the SINV-positive pools from the first screening were subjected to another rRT-PCR, so that individual (*Culex*) or smaller pools of (*Culiseta*) mosquitoes were ultimately tested for SINV RNA. The second rRT-PCR showed 16 samples positive for SINV RNA. One of the positive *Cx. torrentium* pools, which contained samples from 10 mosquitoes, included samples from 3 SINV RNA-positive mosquitoes. Thus, 11 of 301 individual *Cx. torrentium* and 3 of 367 individual *Cx. pipiens* mosquitoes were positive for SINV RNA. For *Cs. morsitans* mosquitoes, 2 of 74 pools were positive for SINV RNA (Table 1).

Because individual *Cx. torrentium* and *Cx. pipiens* mosquitoes were tested, the IRs were calculated as (no. positive individual mosquitoes/total no. tested) × 1,000; differences between the species were tested for significance by using a χ^2 test. The actual species-specific IR differed significantly between species ($p = 0.01$): 36.5, 8.2, and 21 infections/1,000 mosquitoes for *Cx. torrentium*, *Cx. pipiens*,

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Table 1. Ornithophilic *Culex* and *Culiseta* spp. mosquitoes collected for the detection of Sindbis virus, central Sweden, 2009*

Mosquito species	No. collected (no. from which Sindbis virus isolated)					Total	Infection estimates
	Jul 14	Jul 28 and 29	Aug 11	Aug 27	Sep 8		
<i>Culex</i> spp.							
<i>Cx. torrentium</i>	45	183 (6)	62 (4)	9 (1)	2	301(11)	36.5†
<i>Cx. pipiens</i>	38	134 (2)	175 (1)	13	7	367 (3)	8.2†
Total	83	317 (8)	237 (5)	22 (1)	9	668(14)	21.0†
<i>Culiseta</i> sp.							
<i>Cs. morsitans</i>	8	35	93	111 (1)	43 (1)	290 (2)	6.9‡
Total	91	352 (8)	330 (5)	133 (2)	52 (1)	958 (16)	NA

*NA, not applicable.

†Infection rate/1,000 mosquitoes.

‡Minimum field infection rate/1,000 mosquitoes.

and mixed species (*Cx. pipiens* and *Cx. torrentium*), respectively. *Cs. morsitans* mosquitoes were tested in pools, so we calculated the minimum IR (MIR) as (no. positive pools/total no. mosquitoes tested) × 1,000; the calculated MIR was 6.9 infections/1,000 *Cs. morsitans* mosquitoes. A comparison of MIR and maximum-likelihood estimates of infection gave similar estimates.

SINV was successfully isolated from all 16 rRT-PCR-positive mosquito samples (Table 2). Part of the SINV E2 envelope glycoprotein gene was sequenced as previously described (8). The phylogenetic analysis included all genomic sequences obtained in this study together with previously published sequences for 63 other virus strains (8). The results showed that all new strains belonged to the SINV-I genotype and have close relationships with strains from Europe, the Middle East, and South Africa (Figure).

Conclusions

We describe information on the actual occurrence and IR of SINV-I in the enzootic mosquito vectors *Cx. torrentium* and *Cx. pipiens*, reliably identified to species level. The significantly higher SINV-I IR observed for field-caught *Cx. torrentium* than *Cx. pipiens* mosquitoes is a key addition to the previous findings of the extreme susceptibility

of *Cx. torrentium* mosquitoes to SINV-I (4). Experimental transmission studies showed that all infected *Cx. torrentium* mosquitoes could transmit the virus upon refeeding on a susceptible animal (4). Thus, the observed natural IR of 36.5 infections/1,000 *Cx. torrentium* mosquitoes translates to 36.0 mosquitoes/1,000 being able to transmit SINV-I. In contrast, because only one third of infected *Cx. pipiens* mosquitoes can transmit SINV-I upon refeeding, the observed natural IR of 8.2 infections/1,000 *Cx. pipiens* mosquitoes translates to only 2.0 mosquitoes/1,000 being able to transmit the virus (4).

The observed SINV-I IRs are very high for both species, and the IR for *Cx. torrentium* is among the highest ever reported for mosquitoes. This could partly be attributed to the fact that single mosquitoes were analyzed, as compared with the more common technique of pooling. This higher IR would have remained undetected if only pooled mosquitoes were analyzed, even though our original pools consisted of only 10 individual mosquitoes. *Cx. pipiens* mosquitoes are generally considered a secondary enzootic vector of SINV because, as in Sweden, they are less frequently found infected in nature in South Africa, Israel, and Saudi Arabia, where *Cx. univittatus* mosquitoes are the main SINV vector (9–11).

Table 2. Summary of 16 Sindbis virus isolates from ornithophilic *Culex* and *Culiseta* species mosquitoes collected in central Sweden, 2009

Species	Date collected	Geographic coordinates	No. mosquitoes	Strain	GenBank accession no.
<i>Cx. torrentium</i>	Jul 28	60°10.141'N; 16°34.998'E	1	09-M-526-3	KF297644
<i>Cx. torrentium</i>	Jul 28	60°10.141'N; 16°34.998'E	1	09-M-1393-3	KF297639
<i>Cx. torrentium</i>	Jul 28	60°14.698'N; 16°43.592'E	1	09-M-1388-17	KF297643
<i>Cx. torrentium</i>	Jul 28	60°7.506'N; 16°46.828'E	1	09-M-1384	KF297637
<i>Cx. torrentium</i>	Jul 28	60°7.506'N; 16°46.828'E	1	09-M-1394	KF297640
<i>Cx. torrentium</i>	Jul 28	60°6.253'N; 16°45.091'E	1	09-M-1396-1	KF297641
<i>Cx. torrentium</i>	Aug 11	60°10.141'N; 16°34.998'E	1	09-M-1367-3	KF297638
<i>Cx. torrentium</i>	Aug 11	60°9.647'N; 16°30.977'E	1	09-M-564-9	KF297646
<i>Cx. torrentium</i>	Aug 11	60°9.647'N; 16°30.977'E	1	09-M-571-40	KF297636
<i>Cx. torrentium</i>	Aug 11	60°9.638'N; 16°54.428'E	1	09-M-991-1	KF297653
<i>Cx. torrentium</i>	Aug 27	60°26.076'N; 17°22.583'E	1	09-M-358-5	KF297651
<i>Cx. pipiens</i>	Jul 28	60°3.127'N; 16°43.320'E	1	09-M-519-25	KF297652
<i>Cx. pipiens</i>	Jul 29	60°17.846'N; 16°50.486'E	1	09-M-648-2	KF297647
<i>Cx. pipiens</i>	Aug 11	60°9.647'N; 16°30.977'E	1	09-M-564-5	KF297645
<i>Cs. morsitans</i>	Aug 25	60°10.141'N; 16°34.998'E	4	09-M-1169	KF297642
<i>Cs. morsitans</i>	Sep 8	60°7.506'N; 16°46.828'E	3	09-M-887	KF297648

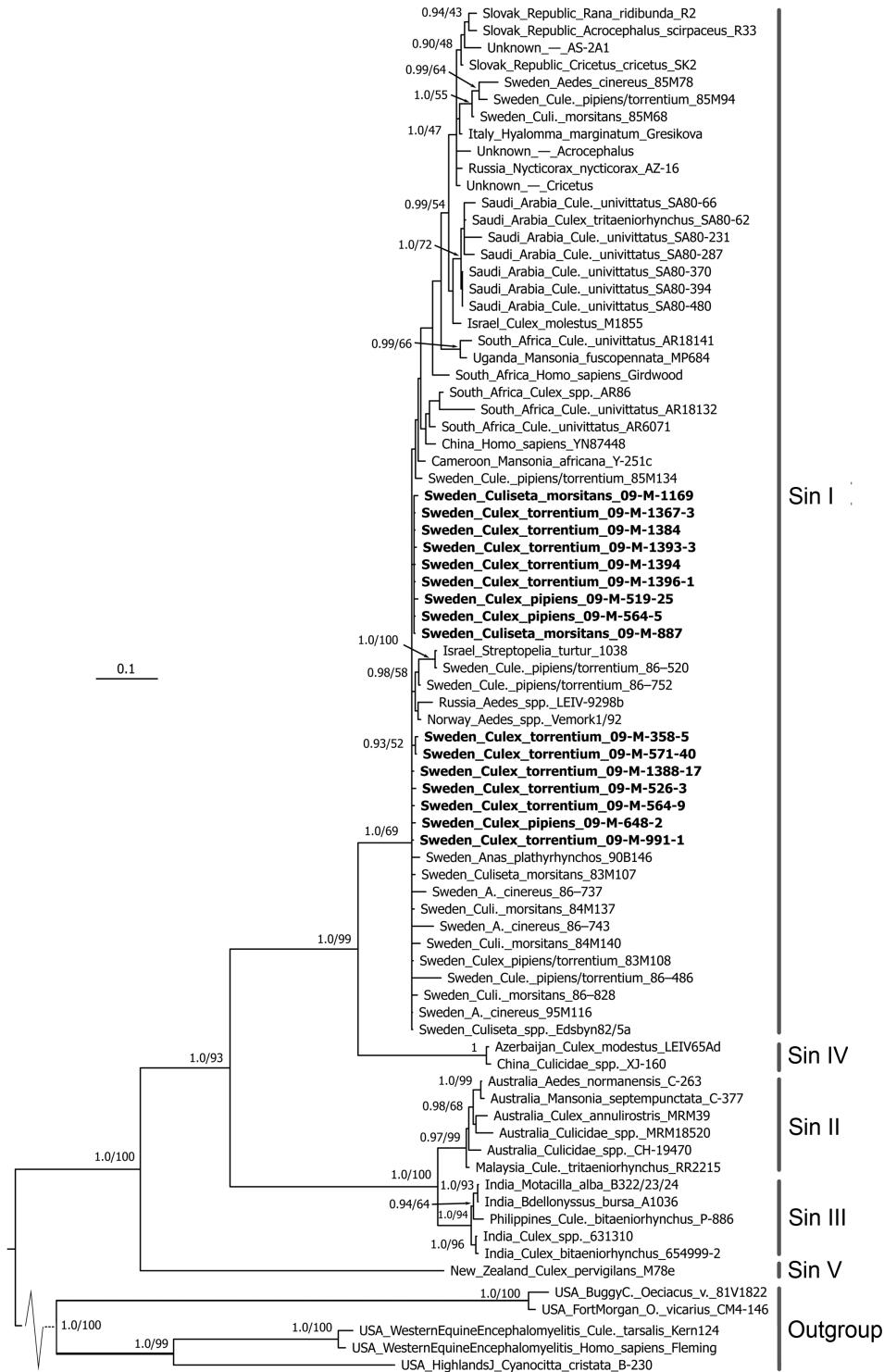


Figure. Consensus tree of the partial E2 envelope glycoprotein gene for Sindbis virus constructed by using MrBayes (<http://mrbayes.sourceforge.net/>). The phylogram includes 16 Sindbis virus strains isolated from mosquitoes collected in central Sweden during July 13–September 13, 2009, against a background of all Sindbis virus strains previously sequenced in the same region. The tree shows that all new strains are of the Sindbis-I virus genotype. Boldface indicates strains isolated during this study. Labels on right indicate Sindbis virus genotypes. Support values at nodes are Bayesian posterior probabilities/Garli maximum-likelihood bootstrap support. Nodes without support values have Bayesian posterior probability of <0.9, and branches are collapsed at 0.5 posterior probability. Scale bar represents average number of substitutions per site.

Because of the exceptionally high SINV IR for field-caught *Cx. torrentium* mosquitoes in Sweden, the outstanding vector competence results for the species in the laboratory (4), and its status as the dominating *Culex* species in SINV-endemic areas of Europe (12), *Cx. torrentium* can now be identified as the main enzootic vector of SINV-I in Scandinavia. In areas of Sweden and Finland where clinical SINV-I infections are most prevalent, *Cx. torrentium* mosquitoes account for >90% of the *Cx. pipiens/Cx. torrentium* population; in central Europe, where the virus is more uncommon in mosquitoes and no human cases have been observed, both species are equally common (12–14). Thus, a large population of *Cx. torrentium* mosquitoes may be a prerequisite for the intense enzootic transmission of SINV-I that is needed to increase the risk for spillover infections in humans. Whether *Cx. torrentium* mosquitoes are also to be considered a vector of other mosquito-borne bird viruses remains to be investigated. In continental Europe, West Nile virus and Usutu virus are emerging, and it is unknown if *Cx. torrentium* has a vector role for these viruses is unknown because of the lack of transmission experiments and isolation attempts from reliably identified female *Cx. pipiens/Cx. torrentium* mosquitoes. Knowledge from such experiments and isolation attempts would be especially valuable for northern and central Europe where *Cx. torrentium* is the dominating candidate enzootic vector species for bird-associated viruses (12).

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Dr. Hesson is a researcher at Uppsala University in Sweden. Her primary research interests are mosquito-borne human pathogens, especially the effect of mosquito ecology on virus transmission.

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Genetic Characterization of Highly Pathogenic Avian Influenza (H5N8) Virus from Domestic Ducks, England, November 2014

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Genetic sequences of a highly pathogenic avian influenza (H5N8) virus in England have high homology to those detected in mainland Europe and Asia during 2014. Genetic characterization suggests this virus is an avian-adapted virus without specific affinity for zoonoses. Spatio-temporal detections of H5N8 imply a role for wild birds in virus spread.

Aquatic birds are considered to be the natural reservoir of low pathogenicity avian influenza viruses of subtypes H1–H16; in these birds, including ducks, they generally do not cause clinical signs (1). In contrast, highly pathogenic avian influenza (HPAI) viruses of certain H5 and H7 strains cause high death rates in poultry with substantial economic losses and are thought to be derived from low pathogenicity avian influenza viruses of wild bird origin. China, Japan, and South Korea have reported outbreaks of highly related HPAI (H5N8) virus in poultry and migratory birds since early 2014 (2,3). Germany and the Netherlands have reported outbreaks among poultry with closely related H5N8 viruses in turkey, chicken, and duck farms since early November 2014. On November 16, 2014, an outbreak of HPAI (H5N8) virus was confirmed on a duck breeding farm in East Yorkshire, England, UK (4). The premises contained 6,000 breeding ducks \approx 60 weeks of age housed in 3 sheds; the ducks showed only mild clinical signs of illness.

The Study

Thirty-seven of 120 individual swab samples and 5 of 12 tissue pools (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/6/14-1954-Techapp1.pdf>) were confirmed positive for avian influenza virus by identification of the matrix (M) gene and H5 real-time reverse transcription PCR (rRT-PCR) as described (5). A highly pathogenic pathotype was confirmed by RT-PCR, then Sanger sequencing of the hemagglutinin (HA) gene as described (5). This process showed the polybasic cleavage site PLRERRRKR/GLF from multiple bird swab and tissue samples from each shed. HPAI virus was isolated in 9- to 10-day-old specific pathogen-free embryonated hens' eggs after incubating 2 days as described (6). A primary isolate, A/duck/

England/36254/2014 (dkEng14), was obtained after egg inoculation with pooled intestinal samples from 1 shed and was typed as H5N8 by using standard protocols (6). We obtained full genome sequence from dkEng14 by next-generation sequencing (online Technical Appendix). Full genome sequences of 2 more isolates, A/duck/England/36038/2014 and A/duck/England/36226/2014, derived from pooled cloacal swabs and pooled viscera from 2 different sheds, respectively, were also assessed by next generation sequencing and analyzed. We entered genome sequences on the Global Initiative on Sharing All Influenza Data EpiFlu database (7) under accession nos. EPI547670–EPI547677, EPI550848–EPI550849, and EPI558000–EPI558013. Initial maximum likelihood phylogenetic analysis of the HA gene was conducted as described (5) and the evolutionary analyses performed by using MEGA6 (8).

Phylogenetic analysis showed 99.9%–100% similarity among the 3 duck isolates from the single premises in England and 99.4%–100% similarity among HPAI (H5N8) sequences from China, Japan, South Korea, Germany, and the Netherlands; all belonged to Asian lineage H5 clade 2.3.4.4. Sequence comparisons based on 1,608 nt of the HA gene from each of the 3 duck isolates identified A/turkey/Germany/MV-R2472/2014 (H5N8) as the closest match, at 99.8% similarity. We implemented further phylogenetic analysis using Bayesian Markov chain Monte Carlo simulation in the BEAST package version 1.7 (9). The maximum clade credibility tree (Figure 1) had a similar topology to that observed for the maximum-likelihood tree.

The time elapsed between the detection of the isolates in England and the most recent common ancestor (MRCA) of the H5N8 virus cluster from Europe and Japan was \approx 5 months (95% highest posterior density [HPD] range 2.7–7.7 months; approximately June 2014). This cluster shares homology of the HA gene with viruses detected in South Korea in early 2014. The ancestor of the viruses from continental Europe, Japan, and Korea occurred \approx 13 months (11–15.5 months, 95% HPD; approximately October 2013) before the detection of the English isolates. The separation between the H5N8 HA sequences from England is attributed to 2 nonsynonymous mutations coding for amino acid substitution S181P and H273Y (HA numbering based on the mature H5 protein). Position 181 is in close proximity to the receptor binding site and antigenic site Sb (10); however, the effect of this condition on antigenicity has not yet been defined.

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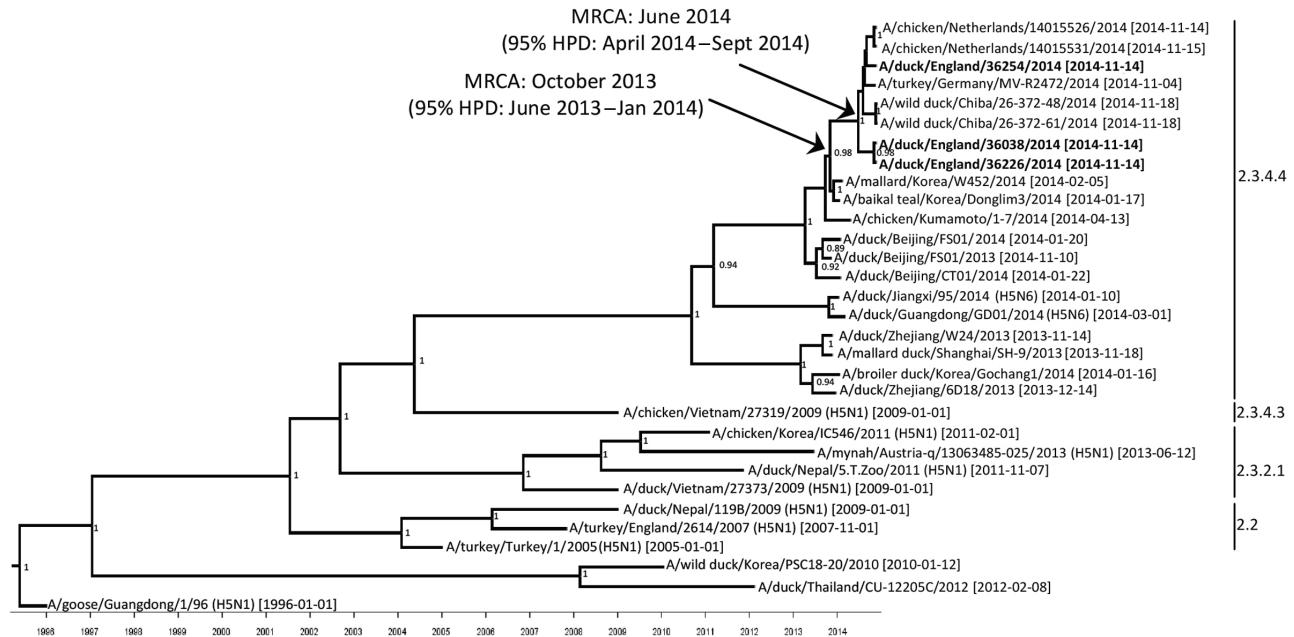


Figure 1. Maximum clade credibility tree of 31 H5 sequences derived from the hemagglutinin gene of avian influenza viruses (1,608 nt). Sampling dates and locations are included on the tip labels; where specific dates were unknown, '01' was assigned. Node labels indicate significant posterior probabilities (>0.75). The dates for the most recent common ancestor (MRCA) of the currently circulating viruses circulating in Europe and Japan are indicated at the relevant nodes with 95% highest posterior density (HPD) levels. Sequences relate to H5N8 subtype unless otherwise noted. Sequences reported in this study are in bold.

The HA sequence variation observed within 1 premises in which ducks were infected in England is potentially the consequence of virus adaptation within the flock subsequent to a single introduction. Full-genome comparison of the 3 viruses in England showed amino acid differences in the HA and NA genes only (Table 1). Further analyses with sequences from the NA gene indicated a similar most recent

common ancestor (≈ 5 months) for the European lineage. However, each of the 3 virus sequences detected in England grouped together with those from the Netherlands (Figure 2).

To investigate the potential zoonotic affinity of dkEng14, sequence data was compared with the H5N1 genetic changes inventory at the US Centers for Disease Control and Prevention (11) to identify single or collective mutations that might influence viral phenotypic characteristics of importance and may indicate adaptation to mammalian species or alter susceptibility to antiviral drugs. A total of 8 point mutations among 114 of interest were identified (Table 2). In addition to the polybasic (PB) cleavage site in the HA protein, positions 133 and 156 each contained an alanine residue (H5 numbering relative to A/Vietnam/1203/2004), which is reportedly related to increased virus binding to $\alpha 2,6$ sialic acid human receptors.

An asparagine that has been associated with reduced susceptibility to amantadine and rimantadine antiviral drugs was present at position 31 in the M2 protein, but we did not detect any signature motifs associated with resistance to antivirals targeting neuraminidase. Aspartic acid at position 30 and alanine at position 215 in the M1 protein together with serine at position 42 and methionine at position 101 in the nonstructural 1 protein were observed; all have been individually linked to increased virulence in mice. A serine residue at position 66 in the PB1-F2 protein

Table 1. Genome sequence comparisons of 3 highly pathogenic avian influenza (H5N8) viruses from domestic ducks, England, November 2014*

Gene segment	Sequence		
	36038, 36226	36038, 36254	36226, 36254
PB2	0	1	1
PB1	0	0	0
PA	1	0	1
HA	0	2 (S181P, H273Y)	2 (S181P, H273Y)
NP	0	0	0
NA	1 (L363I)	4 (S164P, N166S, K186N, L363I)	3 (S164P, N166S, K186N)
MP	0	0	0
NS	0	0	0

*Sequences compared are A/duck/England/36038/2014, A/duck/England/36226/2014, and A/duck/England/36254/2014. Amino acid residue in parentheses indicate that the number of nucleotide differences correspond to a nonsynonymous change. PB2, polymerase basic 2; PB1, polymerase basic 1; PA, polymerase acidic; HA, hemagglutinin; NP, nucleoprotein; NA, neuraminidase; MP, matrix; NS, nonstructural.

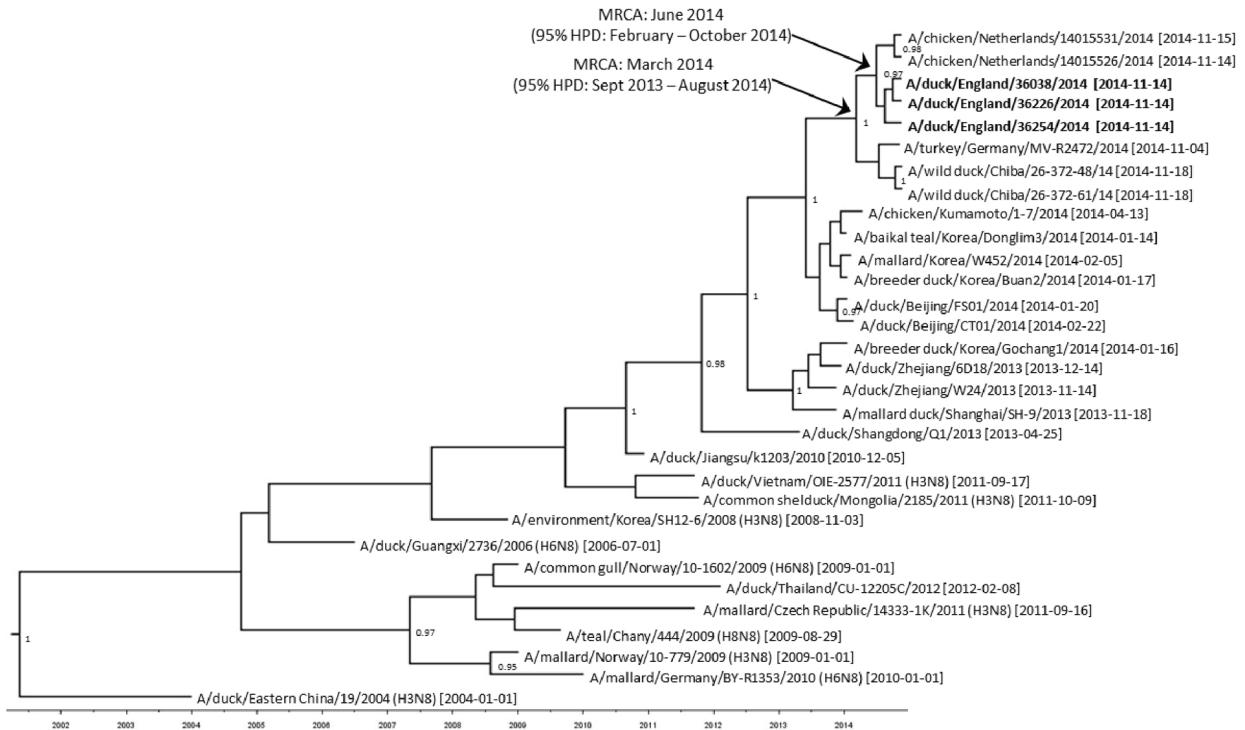


Figure 2. Maximum clade credibility tree of 31 N8 sequences derived from the neuraminidase gene of avian influenza viruses (1,377 nt). Sampling dates and locations are included on the tip labels; where specific dates were unknown, '01' was assigned. Node labels indicate significant posterior probabilities (>0.75). The dates for the most recent common ancestor (MRCA) are indicated at the relevant nodes with 95% highest posterior density (HPD) levels. Sequences relate to H5N8 subtype unless otherwise noted. Sequences reported in this study are in bold.

was also observed. This mutation has also been linked to increased virulence in mice and studies in ducks showed a minor role in pathogenesis. Of interest is the lack of a deletion in the nonstructural 1 protein at amino acid positions 80–84 that is conserved among contemporary H5N1 viruses, possibly decreasing the zoonotic potential of the H5N8 virus. Two mutations in PB2, E627K, and K526R (12), described to be involved with mammalian host adaptation and increased replication in mammalian cells, were not observed. Eight mutations were identified in dkEng14; only 1 (T156A) of the 5 necessary mutations associated with high risk for zoonotic transmission were observed (13,14). The mutations identified in Table 2 are analogous to that of A/mallard duck/Korea/W452/2014 (H5N8), a virus that has been studied for its pathogenic and pandemic potential (15) and was found to result in impaired replication and inefficient contact transmission among ferrets.

Conclusions

The genome of the H5N8 virus isolated in England suggests that it is still predominantly an avian-adapted virus, without any specific increased affinity for humans. Close

genetic homology among the viral genes of the H5N8 viruses detected in England, the Netherlands, and Germany suggest they share a common ancestor with the recent H5N8 viruses isolated from wild ducks in Japan, a result of reassortment estimated to have occurred in June 2014. Reliable interpretation of the topology of the European and Japanese cluster cannot be made with these similar sequences. Phylogenetic analysis of sequences from more viruses will help to resolve these relationships. Detection of H5N8 (HPAI) viruses in 3 countries in Europe over a short time period in different poultry species without the establishment of clear epidemiologic links implicates a role for wild birds in spreading of viruses. The potential for further dissemination of HPAI (H5N8) viruses in Europe is a threat to poultry. Viral sequence analysis from new outbreaks is recommended to monitor virus evolution, understand risk pathways for introduction, and assess the emergence of mutations that may be relevant for veterinary and public health.

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Table 2. Genetic mutations identified in highly pathogenic avian influenza (H5N8) virus isolate A/duck/England/36254/2014 that might result in phenotypic consequences, England, 2014*

Protein, amino acid position/motif	Phenotypic consequences†
PB1-F2, N66S	Increased virulence, replication efficiency and antiviral response in mice
HA S133A T156A 323–330 (R-X-R/K-R)	Increased pseudovirus binding to α 2,6 Increased virus binding to α 2,6 and increased transmission in guinea pigs Polybasic cleavage motif sequence required for high pathogenicity
M1 N30D T215A	Increased virulence in mice Increased virulence in mice
M2 S31N	Reduced susceptibility to amantadine and rimantadine antiviral drugs
NS1 P42S I101M	Increased virulence in mice Increased virulence in mice

*Phenotypic consequences may include an influence on viral phenotypic characteristics of importance, adaptation to mammalian species, or altered susceptibility to existing antiviral drugs. H5N1 numbering based on the mature HA protein relative to A/Vietnam/1203/2004. PB, polymerase basic protein; HA, hemagglutinin; M, matrix; NS, nonstructural.

†The mutation to the right of the amino acid position confers the phenotypic consequence described.

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Getah Virus Infection among Racehorses, Japan, 2014

Manabu Nemoto, Hiroshi Bannai, Koji Tsujimura, Minoru Kobayashi, Takuya Kikuchi, Takashi Yamanaka, Takashi Kondo

An outbreak of Getah virus infection occurred among racehorses in Japan during September and October 2014. Of 49 febrile horses tested by reverse transcription PCR, 25 were positive for Getah virus. Viruses detected in 2014 were phylogenetically different from the virus isolated in Japan in 1978.

Getah virus (genus *Alphavirus*, family *Togaviridae*) is a mosquito-borne virus that was first isolated in Malaysia in 1955 from *Culex* spp. mosquitoes (1). Serologic evidence suggests that Getah virus is widespread from Eurasia to Australasia (1,2). In horses, the virus causes fever, rash on the body, and edema in the legs (2); the virus is also pathogenic to pig fetuses and newborn piglets (3,4) and can cause fever in humans (5). Outbreaks of Getah virus infection have occurred among horses in 1978, 1979, and 1983 in Japan (2) and in 1990 in India (6).

An inactivated whole-virus vaccine (Nisseiken Co., Ltd, Tokyo, Japan) is available to prevent Getah virus infection and is mainly administered to thoroughbred racehorses registered by the Japan Racing Association. It is recommended that this vaccine be administered twice in the first year of registration (mainly 2-year-old horses) and then annually as a booster before each mosquito season. This vaccine contains the MI-110 strain isolated from a febrile horse during the outbreak in 1978.

In mid-September 2014, the number of febrile horses began to increase at the Miho Training Center of the Japan Racing Association in Ibaraki Prefecture; we identified Getah virus infection among these horses. The outbreak of Getah virus infection in 1978 occurred at this facility. We summarize the epidemiologic features and molecular characterization of the epidemic virus.

The Study

Approximately 2,000 horses are stabled at the facility in which this outbreak occurred. In September and October 2014, a total of 36 and 39 horses, respectively, became febrile. The numbers of pyretic horses in September and

October during 2009–2013 were 16.0 ± 4.4 and 17.6 ± 1.7 (mean number \pm SD), respectively.

EDTA-treated blood samples and nasal swab samples from 49 and 48 pyretic horses, respectively, were collected during September 25–November 2. Paired serum samples were collected from 19 febrile horses during the acute (September 15–October 12) and convalescent (2–4 weeks later) phases. Nasal swab samples were suspended in 2.5 mL of transport medium (7). Viral RNA and DNA were extracted from the blood samples and nasal swab samples by using a nucleic acid isolation kit (MagNA Pure LC Total Nucleic Acid Isolation Kit, Roche Diagnostics, Mannheim, Germany). Reverse transcription PCR (RT-PCR) was conducted using a primer pair targeting nonstructural protein 1 (NSP1) of Getah virus (OneStep RT-PCR Kit, QIAGEN, Hilden, Germany) by using the RNA extracted from the blood samples (8). PCR was also used to detect the specific genes of equid herpesviruses 1 and 4 within the blood samples (9). Reverse transcription loop-mediated isothermal amplification was used to detect equine influenza virus in the nasal swab samples (7). Equine arteritis virus was tested by real-time RT-PCR by using the blood samples (10). A virus neutralization test for Getah virus was conducted on Vero cells using the MI-110 strain, which was isolated in 1978 (11) and is the current vaccine strain, as described previously (12) with slight modification. The neutralizing antibody titers were determined as the reciprocal of the highest serum dilution that inhibited viral cytopathic effects. Seroconversion was defined as ≥ 4 -fold increase in the antibody titer between paired serum samples. Serum collected from horses vaccinated after August 1 were not used in this study because the neutralization test cannot distinguish an increase in antibodies induced by natural infection from that induced by vaccination.

The NSP1 and capsid genes in the RT-PCR-positive samples (strain designation Miho-2014) and MI-110 strain were amplified by using previously described primer pairs (8) and were sequenced commercially (Fasmac Co., Ltd., Atsugi, Japan). Sequences were analyzed with the Vector NTI Advance 11 software (Invitrogen, Carlsbad, CA, USA). Phylogenetic analyses of the nucleic acid sequences were conducted with MEGA 5.2 software (13). Phylogenetic trees based on the NSP1 and capsid genes were constructed by using the neighbor-joining method. The statistical analysis of the trees was conducted with the bootstrap test (1,000 replicates). The accession numbers registered in GenBank/EMBL/DDBJ are as follows: the partial sequences of the NSP1 gene Miho-2014 (LC012885) and MI-110

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Table. Number of newly febrile horses and results of RT-PCR and a virus neutralization test, Japan, 2014*

Test	Sep 1–7, n = 3	Sep 8–14, n = 3	Sep 15–21, n = 12	Sep 22–28, n = 15	Sep 29–Oct 5, n = 13	Oct 6–12, n = 16	Oct 13–19, n = 7	Oct 20–26, n = 2	Oct 27–Nov 2, n = 6
RT-PCR	NT	NT	NT	4/5†	11/13	6/16	3/7	1/2	0/6
Neutralization	NT	NT	4/4‡	4/4	5/5	3/6	NT	NT	NT

*NT, not tested; RT-PCR, reverse transcription PCR.

†Number of positive samples/number of blood samples examined.

‡Number of seroconversions/number of paired serum samples examined.

(LC012887); and the partial sequences of the capsid gene Miho-2014 (LC012884) and MI-110 (LC012886).

Of the 49 blood samples tested among horses 2–7 years of age, 25 were positive for Getah virus by RT-PCR (Table). All blood samples were negative for equid herpesviruses 1 and 4 and equine arteritis virus, and all nasal swab samples were negative for equine influenza virus. In the neutralization test, 16 of 19 paired serum samples showed seroconversion (≥ 4 -fold increase) to the MI-110 strain of Getah virus (Table). In total, 33 febrile horses were positive for Getah virus infection by RT-PCR, neutralization test, or both. Seventeen horses had edema in their legs, and 4 had rashes on their bodies; these horses constituted a small percentage of febrile horses. Getah virus randomly infected horses stabled throughout the Miho Training Center. All the horses that were positive for Getah virus infection recovered after treatment of signs.

The first and last samples that were positive by neutralization test, RT-PCR, or both were collected from pyretic horses on September 15 and October 25, respectively. These results show that the Getah virus infection occurred among racehorses at the Miho Training Center from mid-September through late October 2014.

We analyzed the sequences of the NSP1 and capsid genes of 10 positive samples and the MI-110 strain. The nucleic acid sequences of the NSP1 (381 bp) and capsid (552 bp) genes were completely identical among the 10 Getah viruses detected in 2014. The nucleic acid sequence identities between the Getah virus detected in 2014 and

MI-110 were 98.7% for the NSP1 gene and 99.1% for the capsid gene. Phylogenetic analyses were performed with the nucleic acid sequences of the Getah virus NSP1 and capsid genes, including those of isolates from horses, mosquitoes, and pigs (Figure), and showed that the Getah viruses detected in 2014 clustered apart from the MI-110 strain.

Conclusions

The first outbreak of Getah virus infection among racehorses occurred in September–November 1978, and another outbreak in 2014 occurred around the same period at the same facility. In 1978, of 1,903 stabled horses, 722 were affected (11). In 1979 and 1983, several small outbreaks of Getah virus infection occurred among unvaccinated horses at several facilities other than the Miho Training Center. Why an outbreak occurred again in 2014 remains unclear. We have no data on the vector mosquitoes or the climatic conditions in this region. Among the affected horses, eight 2-year-old horses had received the initial vaccination just 1–4 days before disease onset. Clearly, the vaccine against Getah virus could not provide some of the affected horses with sufficient protective immunity. The phylogenetic analyses showed that the currently circulating viruses differ genetically from the MI-110 vaccine strain isolated in 1978. From these results, we infer that this outbreak might be partly attributable to the antigenic differences between the vaccine strain and the currently circulating strain. Serologic studies of the current virus and the vaccine strain are in progress.

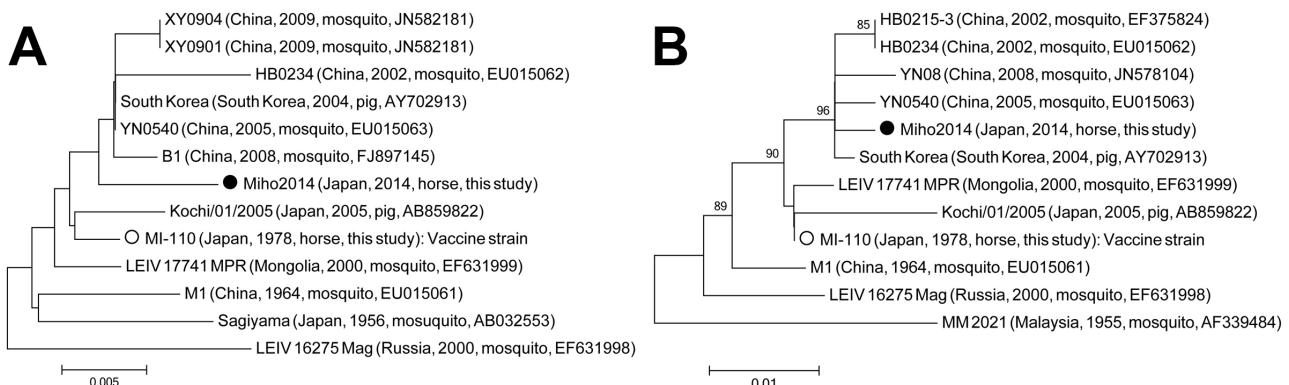


Figure. Phylogenetic analyses of the nucleotide sequences of the (A) nonstructural protein 1 (NSP1) gene (nt 218–598) and (B) capsid gene (nt 7645–8196) of Getah virus isolated in Japan, 2014. The genome positions of the NSP1 and capsid genes correspond to those of Kochi/01/2005 strain (GenBank accession no. AB859822) (14). Closed and open circles represent Miho2014, the strain isolated in this study, and MI-110, the strain isolated in 1978, respectively. The percentage bootstrap support is indicated by the value at each node; values <70 are omitted. Scale bars indicate nucleotide substitutions per site.

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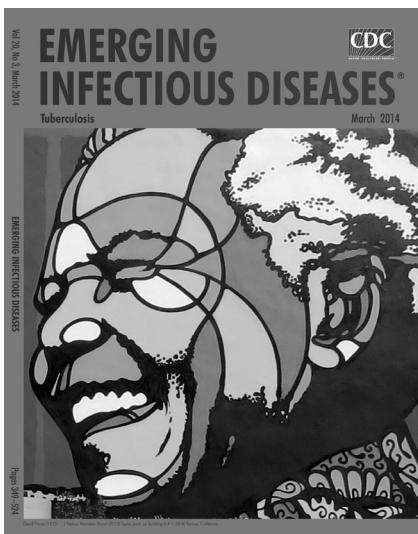
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March 2014: Tuberculosis

Including:

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- Hendra Virus Vaccine, a One Health Approach to Protecting Horse, Human, and Environmental Health
- Possible Role of Songbirds and Parakeets in Transmission of Influenza A(H7N9) Virus to Humans
- Hantavirus Infections among Overnight Visitors to Yosemite National Park, California, USA, 2012

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Novel Eurasian Highly Pathogenic Avian Influenza A H5 Viruses in Wild Birds, Washington, USA, 2014

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Novel Eurasian lineage avian influenza A(H5N8) virus has spread rapidly and globally since January 2014. In December 2014, H5N8 and reassortant H5N2 viruses were detected in wild birds in Washington, USA, and subsequently in backyard birds. When they infect commercial poultry, these highly pathogenic viruses pose substantial trade issues.

The novel Eurasian lineage clade 2.3.4.4 highly pathogenic avian influenza (HPAI) A(H5N8) virus (http://www.who.int/influenza/gisrs_laboratory/h5_nomenclature_clade2344/en/) spread rapidly and globally during 2014, substantially affecting poultry populations. The first outbreaks were reported during January 2014 in chickens and domestic ducks in South Korea and subsequently in China and Japan (1–4), reaching Germany, the Netherlands, and the United Kingdom by November 2014 and Italy in early December 2014 (5). Also in November 2014, a novel HPAI H5N2 virus was reported in outbreaks on chicken and turkey farms in Fraser Valley, British Columbia, Canada (5). This H5N2 influenza virus is a reassortant that contains the Eurasian clade 2.3.4.4 H5 plus 4 other Eurasian genes (polymerase acidic protein subunit, matrix protein, polymerase basic protein subunit [PB] 2, nonstructural protein) and 3 North American wild bird lineage genes (neuraminidase [NA], nucleoprotein, PB1) (5). Taiwan has recently reported novel reassortants of the H5 clade 2.3.4.4 with other Eurasian viruses (H5N2, H5N3).

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The appearance of highly similar Eurasian H5N8 viruses in Asia, Europe, and now the United States suggests that this novel reassortant may be well adapted to certain waterfowl species, enabling it to survive long migrations (6). These appearances also represent a major change in Eurasian H5 virus circulation. After the reported spread of HPAI H5N1 virus in Asia, a large, interagency avian influenza virus (AIV) surveillance effort was implemented throughout the United States during April 2006–March 2011 (7). Of nearly 500,000 wild bird samples tested, none harbored Eurasian subtype H5 AIV. The overall prevalence of AIV was ≈11%, and most viruses (86%) were detected in dabbling ducks (family Anatidae) (8). Although H5N8 subtype viruses have been detected previously in the United States, all have been low pathogenicity AIV of North American wild bird lineage.

Case Reports

After the November 2014 report of H5N2 HPAI outbreaks among poultry in British Columbia, the US Departments of Agriculture and Interior, together with state agency personnel, increased surveillance of poultry flocks, hunter-harvested wild birds, and wild bird die-offs along the US–Canada border. A wild bird die-off was reported on December 1, 2014, at Wiser Lake (48.9039N, 122.4799W) in Whatcom County, Washington, USA. The lake, which has a history of waterfowl deaths caused by lead poisoning and aspergillosis, is located ≈32 km from the location of the index cases in Fraser Valley. Up to 10,000 waterfowl were on the lake when the deaths began. The dead birds consisted primarily of mallards (*Anas platyrhynchos*), American wigeon (*A. americana*), and northern pintail (*A. acuta*), along with smaller numbers of other waterfowl species.

Nine carcasses were submitted to the National Wildlife Health Center (Table 1); 6 were examined in detail. *Aspergillus fumigatus* was isolated from 5 birds with characteristic lesions of airsacculitis (Table 1). In addition, cloacal and/or oral swab samples from 5 birds had molecular assay results positive for influenza A and H5 (Table 1). A Eurasian lineage H5 clade 2.3.4.4 AIV, A/northern pintail/Washington/40964/2014 (H5N2) (GenBank taxon no. 1589662), was isolated from a lung specimen. Whole-genome sequencing indicated the virus was highly similar to the H5N2 reassortant virus from Canada. Both viruses have 3 RNA segments of North American wild bird lineage

¹These first authors contributed equally to this article.

Table 1. Summary of influenza test results for samples from 10 birds from Washington, USA, 2014*

Sample ID no.	Species, common name	Necropsy	AIV status	Diagnostic finding
26080-001	Northern shoveler	Yes	PCR negative, isolation negative	Aspergillosis
26080-002	Northern pintail	Yes	Subtype H5N2	Aspergillosis, HPAI
26080-003	American wigeon	Yes	PCR positive, isolation negative	Aspergillosis
26080-004	American wigeon	No	PCR negative, isolation negative	ND
26080-005	American wigeon	No	PCR negative, isolation negative	ND
26080-006†	Mallard	Yes	PCR positive, isolation negative	Aspergillosis
26080-007†	Mallard	Yes	PCR positive, isolation negative	Aspergillosis
26080-008†	Mallard	No	PCR positive, isolation negative	ND
26080-009	Trumpeter swan	Yes	PCR negative, isolation negative	Emaciation
RW099878	Gyr Falcon	Yes	Subtype H5N8	HPAI

*AIV, avian influenza virus; HPAI, highly pathogenic avian influenza; ID, identification, ND, not determined.

†Specimens tested by the Washington Animal Disease Diagnostic Laboratory.

(PB1, nucleoprotein, and NA) and 5 RNA segments (PB2, polymerase acidic, HA, matrix protein, and nonstructural protein) that showed >99% similarity to 2014 Eurasian clade 2.3.4.4 H5N8 viruses (Table 2). According to World Organisation for Animal Health guidelines (9), the virus was consistent with HPAI on the basis of the amino acid sequence at the hemagglutinin cleavage site and in vivo assay results (intravenous pathogenicity index 2.57).

In a related event, on December 6, 2014, an American wigeon was captured and partially consumed by a captive-reared gyrfalcon (*Falco rusticolus*) in Whatcom County, ≈8 km from Wiser Lake. The wigeon remains were also fed to 3 other gyrfalcon and gyrfalcon–peregrine hybrids at a farm with another 25 raptors and 40 pigeons. The first falcon (RW099878) died on December 8 and was submitted to the National Wildlife Health Center. The second falcon (RX085847) also died on December 8 and the third (RX093091) on December 11; the fourth (RX084955) was euthanized on December 11. Carcasses of these last 3 falcons were submitted to the Washington Animal Disease Diagnostic Laboratory. No further deaths or illnesses have been reported among other raptors at the facility. Histologic

and pathologic findings for the 3 raptors were consistent with those described in previous reports of H5N8 infections (1,6), and the severity of the lesions corresponded to virus concentrations detected in the tissues by molecular assays; results will be further detailed in a subsequent publication.

Molecular assay results for oral and cloacal swab samples and major organ and brain samples from falcon RW099878 were positive for influenza A and H5 viruses. For falcons RW099878 and RX085847, partial HA and NA genes were directly sequenced from brain and oral swab samples. A Eurasian lineage H5 clade 2.3.4.4 AIV, A/gyrfalcon/Washington/41088-6/2014 (H5N8) (GenBank taxon no. 1589663), was isolated from the brain of falcon RW099878. All 8 RNA segments for the strain were >99% similar to those for 2014 Group A H5N8 strains from South Korea (3) (Table 2); the amino acid sequence at the hemagglutinin cleavage site and in vivo assay results (intravenous pathogenicity index 2.65) were consistent with HPAI.

Phylogenetic analysis of the H5 clade 2.3.4.4 viruses detected in the United States resulted in 3 major findings (Figures 1, 2; online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-2020-Techapp1.pdf>). First, the

Table 2. Nucleotide identity between the influenza A(H5N2) and A(H5N8) viruses from Washington, USA, and their nearest homologues in GenBank as of January 8, 2015*

Virus from Washington State	Nearest homologue	% Identity
A/Northern pintail/Washington/40964/2014 (H5N2)		
PB2	A/bean goose/Korea/H40/2014	99.5
PB1	A/bufflehead/California/3118/2011	99.0
PA	A/common teal/Korea/H455-30/2014	99.5
HA	A/crane/Kagoshima/KU1/2014	99.3
NP	A/American green-winged teal/Ohio/13OS2084/2013	99.1
NA	A/bufflehead/California/4935/2012	99.0
MP	A/Baikal teal/Korea/S005/2014	100.0
NS	A/Baikal teal/Korea/Donglim3/2014	99.9
A/gyrfalcon/Washington/40188-6/2014 (H5N8)		
PB2	A/bean goose/Korea/H40/2014	99.6
PB1	A/Baikal teal/Korea/H41/2014	99.4
PA	A/Baikal teal/Korea/Donglim3/2014	99.3
HA	A/crane/Kagoshima/KU1/2014	99.2
NP	A/Baikal teal/Korea/H41/2014	99.5
NA	A/Coot/Korea/H81/2014	99.4
MP	A/Baikal teal/Korea/Donglim3/2014	99.9
NS	A/Baikal teal/Korea/Donglim3/2014	100.0

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

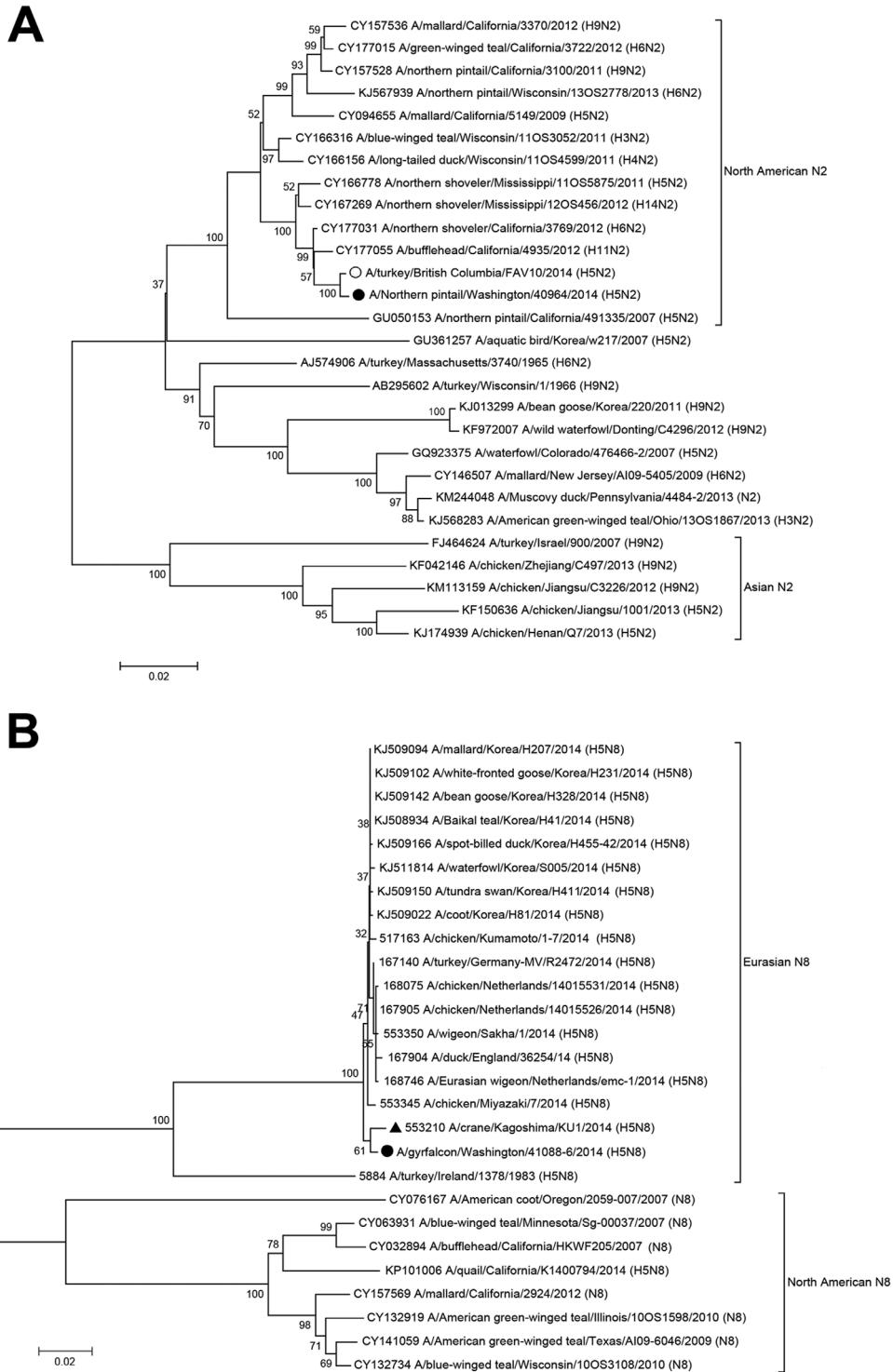


Figure 2. Phylogenetic comparison of the complete neuraminidase genes of highly pathogenic avian influenza A(H5N2) (panel A) and A(H5N8) (panel B) strains from the United States with strains from Asia, Europe, and Canada. Solid circles indicate H5N2 and H5N8 strains from the United States; black triangle indicates H5N8 strain from a crane in Japan. Sequences were aligned by using MUSCLE, and phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 5, using the neighbor-joining tree-building method, with 1,000 bootstrap replicates (70). Analysis was done with viruses that were phylogenetically representative of appropriate lineages (Influenza Virus Resource Database, <http://www.ncbi.nlm.nih.gov/genomes/FLU/FLU.html>). Scale bar indicates nucleotide substitutions per site.

Eurasian lineage avian H5N8 clade 2.3.4.4 virus survived introduction into North America in its entirety. Second, introduction of Eurasian H5N8 virus into North America appears to be independent from introductions of the virus into Europe. Third, the duration of circulation of H5N8 virus in the Pacific flyway (California, Idaho, Nevada, Oregon, Utah, and Washington, USA) is unknown, but it was sufficient for reassortment with low pathogenicity North American lineage wild bird AIV (Figure 1).

Conclusions

The ongoing circulation of these Eurasian HPAI H5 viruses in wild birds considerably alters the potential risks and subsequent consequences for US poultry and wildlife rehabilitation centers. Detection of HPAI H5N8 virus in apparently healthy common teal (*A. crecca*), Eurasian wigeon (*A. penelope*), mallard, spot-billed duck (*A. poecilorhyncha*), and tundra swans (*C. columbianus*) (3,5) suggests that wild birds may contribute to further spread of this HPAI H5 lineage in North America. However, culling and otherwise disturbing wild birds or their habitats has not been shown to be beneficial in the control of avian influenza (11). The scientifically supported management action (11) is to enhance biosecurity to minimize contacts between poultry, wild birds, and their fomites (11). In addition, hunters should be cognizant of risks from handling potentially infected carcasses (http://www.aphis.usda.gov/animal_health/birdbiosecurity/downloads/USDA_HntrCd_Hi.pdf).

Examination of wild bird surveillance samples collected before December 2014 may provide further insight into the timing and route of introduction of the Eurasian clade 2.3.4.4 H5N8 virus into North America. In addition, enhanced and ongoing influenza surveillance in wild birds and poultry will contribute to a better understanding of the geographic distribution and species involved in the spread of these HPAI H5 viruses. Together, these data may enable waterfowl managers and poultry producers to better assess and manage disease risks. Human infections have not been associated with either virus; however, H5 clade 2.3.4 H5N1 virus has caused human death, so caution is warranted. During preparation of this article, H5N8 was reported in wild birds and poultry along the Pacific flyway; novel H5N2 virus was detected in Idaho, Oregon, and Washington; and another novel reassortant H5N1 was detected in Washington and British Columbia (5). These detections have had major effects on US poultry trade (12).

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Characterization of *Shigella sonnei* Isolate Carrying Shiga Toxin 2–Producing Gene

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To the Editor: *Shigella sonnei* causes a bacillary dysentery called shigellosis. Shiga toxins 1 (Stx1) and 2 (Stx2) are mainly produced by Shiga toxin–producing *Escherichia coli* (STEC), but Stx1 can also be produced by *S. dysenteriae* serotype 1 (1). Compared with STEC–producing Stx1, STEC–producing Stx2 has been reported to be more highly pathogenic and to be associated with hemolytic uremic syndrome (HUS) and hemorrhagic colitis (2); the association with HUS especially has been reported for subtypes Stx2a and Stx2c (3).

Stx-converting bacteriophages play a key role in expression of the *stx* gene in *E. coli* and in the lateral gene transfer between the bacteria (4). Although these bacteriophages have not been isolated from *S. dysenteriae* serotype 1, evidence suggests that the bacteria's *stx* gene may be associated with a bacteriophage (5). The *stx*₁ gene, which is located in a bacteriophage, has previously been detected in *S. sonnei* (6). We describe an *S. sonnei* isolate with the *stx*_{2a} gene.

In November 2013, 3 days after her return to Finland from a 2-week visit to relatives in southern Morocco, a 49-year-old woman was admitted to a hospital for bloody diarrhea, fever, and abdominal pain. Her symptoms began with watery diarrhea and fever 5 days before she returned home. The diarrhea ceased after a few days, but symptoms worsened, and diarrhea was visibly bloody after she returned to Finland. On admission, the patient had C-reactive protein and hemoglobin levels of 41 mg/L (reference value <3 mg/L) and 118 g/L (reference value 117–155 g/L), respectively; no thrombocytopenia was observed. No antimicrobial drugs were prescribed because STEC infection was clinically suspected. The patient responded well to intravenous fluid treatment and was discharged from the hospital 3 days after admission. At a follow-up visit 1 week later, she was asymptomatic and had a C-reactive protein level of 19 mg/L.

Shigella spp. was isolated from a feces sample obtained from the patient on illness day 8, and an STEC signal

(based on the PCR-positive *stx*₂ gene) was detected from the same sample. A presumptive STEC strain was subsequently isolated. Both isolates were sent for confirmation and further typing to the Bacteriology Unit, National Institute for Health and Welfare, in Helsinki. The first isolate (FE109024) was confirmed as *S. sonnei* by matching of its biochemical profile to that of the reference strain ATCC 25931 (<http://www.atcc.org/Products/All/25931.aspx>; online Technical Appendix Table, <http://wwwnc.cdc.gov/EID/article/21/5/14-0621-Techapp1.pdf>) and agglutination by antiserum to *S. sonnei* (Denka Seiken, Tokyo, Japan). The agglutination by *S. sonnei* phase I antiserum indicated the smooth form of lipopolysaccharide. Atypical for *S. sonnei*, the isolate did not ferment mannitol (7). The isolate carried the genes encoding invasion plasmid antigen H, the invasion-associated locus, and invasion gene transcription regulator *invE*. The second isolate (FE109046) was initially sent to the National Institute for Health and Welfare as an STEC. However, FE109046 showed biochemical reactions in tubes and when using API 20E (bioMérieux, Durham, NC, USA) that were identical to reactions for isolate FE109024 after an overnight and 3-day incubation (online Technical Appendix Table). Both isolates were nonmotile. FE109046 also agglutinated by *S. sonnei* polyvalent antiserum but was negative for *S. sonnei* phase I antiserum and positive for phase II antiserum, indicating the rough form of lipopolysaccharide. In contrast to FE109024, isolate FE109046 lacked the *Shigella*/enteroinvasive *E. coli*–specific *invE* gene but harbored the STEC-specific *stx*₂ gene. Neither isolate carried other STEC-associated genes. Subtyping of the *stx*₂ gene of isolate FE109046 was performed according to the published protocol (8); the isolate was confirmed to be subtype *stx*_{2a}.

Genomic comparison of the 2 isolates was performed by using pulsed-field gel electrophoresis according to the standard protocol. The isolates showed 96% similarity. A 3-fragment difference suggests that 1 isolate was a variant of the other (Figure); the variant may have arisen through lysogenization with the phage.

The Shiga toxin subtype *stx*_{2a} identified in isolate FE109046 is linked to severe human disease, including HUS (3). The role of Stx in shigellosis is unclear. Stx is not essential for cell invasion or lysis (9). In the previously reported case of *stx*₁–positive *S. sonnei* infection, the patient's symptoms were not severe, and diarrhea lasted 7 days (6).

Shigella spp. can harbor virulence genes found in *E. coli* because both are genetically defined as members of the same species (10). The *stx*₂–positive *S. sonnei* may have emerged in the patient initially infected with a mannitol-negative *S. sonnei* that was subsequently lysogenized by transduction from a STEC co-infection or by a free *stx*₂ phage. *S. sonnei* isolates are not routinely examined for the production of Stx or *stx* genes in clinical



Figure. UPGMA dendrogram of *Xba*I restriction patterns of 2 *Shigella sonnei* isolates from a patient from Finland who became ill during a visit to Morocco. Genomic comparison of the 2 isolates was performed by using pulsed-field gel electrophoresis according to the standard protocol. The isolates showed 96% similarity, and a 3-fragment difference suggests that 1 isolate was a variant of the other. Scale bar represents % similarity. *ial*, invasion-associated locus; *ipaH*, invasion plasmid antigen H; *invE*, invasion gene transcription regulator *invE*; *stx*₂, Shiga toxin 2.

laboratories, so the properties associated with STEC in *S. sonnei* isolates from patients remain undetected. *S. sonnei* with *stx*_{2a} may have potential to cause severe disease, especially in children. This novel and remarkable virulence characteristic in *Shigella* spp. would affect diagnostics, infection control, and prevention.

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Outbreak of *Leishmania braziliensis* Cutaneous Leishmaniasis, Saül, French Guiana

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To the Editor: New World cutaneous leishmaniasis (CL), a zoonotic disease, is increasingly seen among travelers returning from Latin American countries, particularly from Bolivia, Belize, and French Guiana (1). The

epidemiology of CL in the Americas is heterogeneous and has complex variations in transmission cycles, reservoir hosts, and sandfly vectors. Changing human activities that affect these factors may have resulted in the emergence of species with distinct pathogenic potentials and responses to therapy. In the Guianan ecoregion complex, leishmaniasis is endemic, and 5 coexisting *Leishmania* parasite species are known to infect humans: *L. guyanensis*, *L. braziliensis*, *L. amazonensis*, *L. naiffi*, and *L. lainsoni*. Among these species, *L. guyanensis* accounts for $\approx 85\%$ of CL cases (2).

We report an outbreak of 7 cases of *L. braziliensis* CL that occurred among 24 scientists who participated in a field mission at Limonade Creek in Saül, French Guiana, during October 10–25, 2013. Saül is an isolated village in the Amazonian rainforest ($3^{\circ}55'18''\text{N}$, $53^{\circ}18'02''\text{W}$).

Among the 7 patients, 6 were male; mean age was 32 ± 5 years. None of the patients were immunocompromised. The scientists stayed in Saül a mean of 17 (range 12–30) days. The mean time to symptom onset after they left Saül was 19 (range 0–50) days. The mean number of CL lesions was 2.3 (range 1–5). Lesions were localized mainly on lower limbs (11/14 lesions) but also appeared on upper limbs (2/14 lesions) and ears (1/14 lesions). CL was associated with nodular lymphangitis, adenitis, and superficial phlebitis of the affected limb in 2, 3, and 1 patient, respectively. No patients had mucosal involvement, fever, or decline in general health.

Diagnosis of CL was clinically suggested and confirmed by microscope examination of skin scrapings, which revealed typical amastigotes, by a positive *Leishmania* species-specific PCR result, or both. *L. braziliensis* complex was diagnosed by using different molecular techniques, according to the laboratory, and then confirmation of *L. braziliensis* species was conducted by the French National Reference Center for Leishmaniasis on the basis of a putative translation initiation factor α -subunit gene sequence (3). *Leishmania* strain genotyping was performed to explore the epidemiology of the implicated strains. Four single-copy genomic loci were amplified from 5 of 7 patient samples; 1 of the samples had a parasite DNA content that was too low to genotype, and 1 was not analyzed. The genetic analysis of the 4 concatenated sequences showed 5 distinct and nonclustered genotypes (Figure). According to local protocols, patients were treated with 20 mg/kg of intramuscular meglumine antimoniate or with 18–38 mg/kg of intravenous liposomal amphotericin B; at publication time, the patients were still being followed.

This outbreak of *L. braziliensis* CL in French Guiana raises the question of an overall increase in the incidence of this *Leishmania* species. Until now, outbreaks of *L. braziliensis* infection have been observed in Argentina, Brazil, Panama, and Venezuela but not Guiana (5–7). In French Guiana, changes in the epidemiology of CL have been observed since 2006; the emergence of *L. braziliensis*, *L. amazonensis*, and *L. lainsoni* infections represented 8.8%,

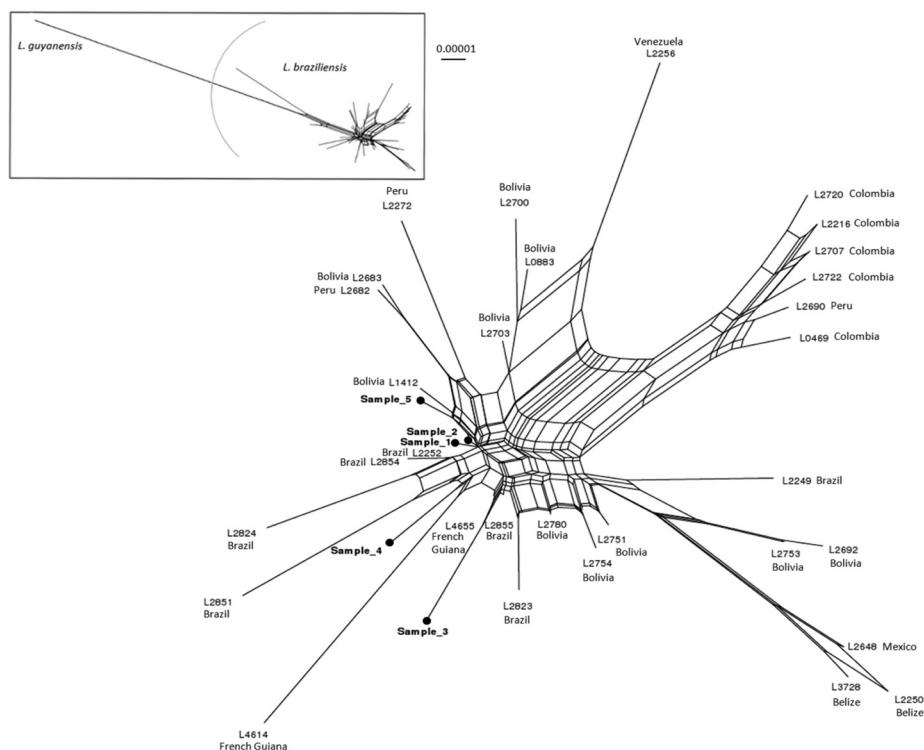


Figure. Data display network showing the genetic diversity of 32 *Leishmania braziliensis* (according to the multilocus enzyme electrophoresis-based taxonomy) compared with 5 strains from clinical samples (boldface) obtained from visitors to the Amazonian forest region of French Guiana. The strains were compared at 4 genomic loci (locus 03.0980, 10.0560, 31.0280 and 31.2610) as previously described (3). The concatenated nucleotide sequences (2,610 bp) were duplicated to avoid information loss due to heterozygous positions (e.g., A to AA or Y to CT). Neighbor-Net analysis was performed with SplitsTree version 4.11.3 (<http://splitsree.org/>) by using p-distances and equal edge lengths (4). Two *L. guyanensis* strains were used as an outgroup. The inset represents the genetic distance between *L. guyanensis* and *L. braziliensis*. Scale bar indicates evolutionary distance.

2.6%, and 1.4%, respectively, of the diagnosed CL cases (8). This trend could be due either to an increase of *L. braziliensis* prevalence in the forests of Guiana or to a greater presence of humans (e.g., military personnel, scientists, and tourists) in deep forest areas with hot spots of transmission. Favorable environmental conditions in a well-delimited zoonotic microfocal hot spot might have contributed to this high rate of transmission. However the relative genetic diversity of strains we observed among the 5 analyzed patients was unexpected, given the relatively small spatial and temporal scale of the transmission area, and indicates that the reservoirs in this restricted area were infested by distinct genotypes. Development of a peridomestic cycle, perhaps with specific reservoirs (pets) and vectors, cannot be excluded in the Saül area.

This case series suggests that caution should be taken in the diagnosis and treatment of CL in patients returning from the Amazonian rainforest, and a species-specific approach based on molecular identification should be proposed to provide appropriate medical management (9). Indeed, although *L. braziliensis* parasites cause <10% of CL acquired in French Guiana, this species is noteworthy for its involvement of the mucous membranes of the lips, nose, soft palate, or larynx. Also, *L. braziliensis* parasites usually fail to respond to pentamidine isethionate, the first-line treatment of *L. guyanensis* CL in French Guiana; instead, treatment of *L. braziliensis* infection relies on parenteral meglumine antimoniate or liposomal amphotericin B (1).

In summary, the geographic extension of and numeric increase in *L. braziliensis* cases in the Guiana ecoregion complex, as observed in the rest of South America, are worrisome, and continuous epidemiologic surveillance is needed. Infection with *L. braziliensis*, which is emerging and has potential to disseminate, must be considered in cases of CL acquired in this region. These issues have key implications for leishmaniasis treatment, which should be directed to the identified species (10).

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Ciprofloxacin-Resistant *Shigella sonnei* Associated with Travel to India

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To the Editor: Shigellosis is an uncommon infection in many industrialized countries, and many cases are linked to travel to *Shigella* spp.–endemic countries. The epidemiology of *Shigella* infections in developing countries is changing. *S. sonnei* seems to be replacing the more antigenically diverse *S. flexneri* in regions undergoing economic development and improvements in water quality (1).

In 2012, a total of 29 cases of shigellosis were reported in Ireland through the Computerized Infectious Disease Reporting system (crude incidence rate 0.63 cases/100,000 population). Isolates from 20 (69%) of those 29 cases were submitted to the National Reference Laboratory in Galway, Ireland, for additional typing. In 2013, a total of 43 isolates

were submitted for typing, more than double the 20 isolates submitted for 2012. This increase may be associated with a change in diagnostic methods: the increasing use of molecular methods for primary testing (2). During 2010–2013, the most common isolates were *S. sonnei* (54%) and *S. flexneri* (38%).

Isolate identification was confirmed by using VITEK 2 (bioMérieux, Marcy l'Etoile, France) and serotyping performed by using slide agglutination with commercial antisera (Sifin, Dusseldorf, Germany, and Mast, Liverpool, UK). Antimicrobial drug susceptibility testing was performed with disk-diffusion tests or Etests (2000–2009) and by broth microdilution (2010–2013) (Sensititre, Trek Diagnostic Systems, Cleveland, OH, USA). Susceptibility to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, naladixic acid, ciprofloxacin, gentamicin, ceftazidime, cefpodoxime, and cefotaxime was assessed by using criteria from the European Committee on Antimicrobial Susceptibility Testing (http://www.eucast.org/clinical_breakpoints). Since October 2013, testing has also included azithromycin, tigecycline, meropenem, and cefepime. Pulsed-field gel electrophoresis (PFGE) was performed on all *S. sonnei* isolates by using the PulseNet method developed by the Centers for Disease Control and Prevention (3). Fisher exact test was applied to assess the significance of the association of ciprofloxacin resistance with reported travel to the subcontinent of India.

Although infection with *S. sonnei* is generally self-limiting, antimicrobial drug therapy is necessary for some

patients and may reduce duration of shedding in feces (4). Ciprofloxacin is widely recommended for use in the absence of susceptibility test results. Alternative agents for therapy include ceftriaxone and azithromycin.

For 2000–2009, none of the 65 *S. sonnei* isolates submitted for typing were resistant to ciprofloxacin. For 2010–2013, the number of ciprofloxacin-resistant *S. sonnei* isolates and the total number of *S. sonnei* isolates submitted for testing were 6/17 (2010), 2/20 (2011), 4/12 (2012), and 12/23 (2013). All 24 ciprofloxacin-resistant isolates were co-resistant to trimethoprim, and all but 2 were also resistant to streptomycin, sulfamethoxazole, and tetracycline. Cefotaxime resistance in 1 isolate was associated with extended-spectrum β -lactamase production. Azithromycin resistance has not been detected since testing for this resistance began in October 2013.

All 24 isolates had indistinguishable or closely related (>92%) *Xba*I-PFGE profiles (Figure). The *Xba*I cluster also included 21 of 50 ciprofloxacin-susceptible *S. sonnei* isolates submitted during 2000–2013. Use of a second enzyme (*Bln*I) on a subset of the 24 isolates confirmed the close relationship among these 24 isolates (data not shown).

Data from the Computerized Infectious Disease Reporting system (2010–2013) identified 72 reported cases of *S. sonnei* infection, of which 24 were ciprofloxacin resistant. Of 15 isolates associated with travel to the subcontinent of India, 11 were ciprofloxacin resistant, but of 47 other isolates for which the country of infection was reported, only 9 were ciprofloxacin resistant, a significant difference ($p < 0.0001$).

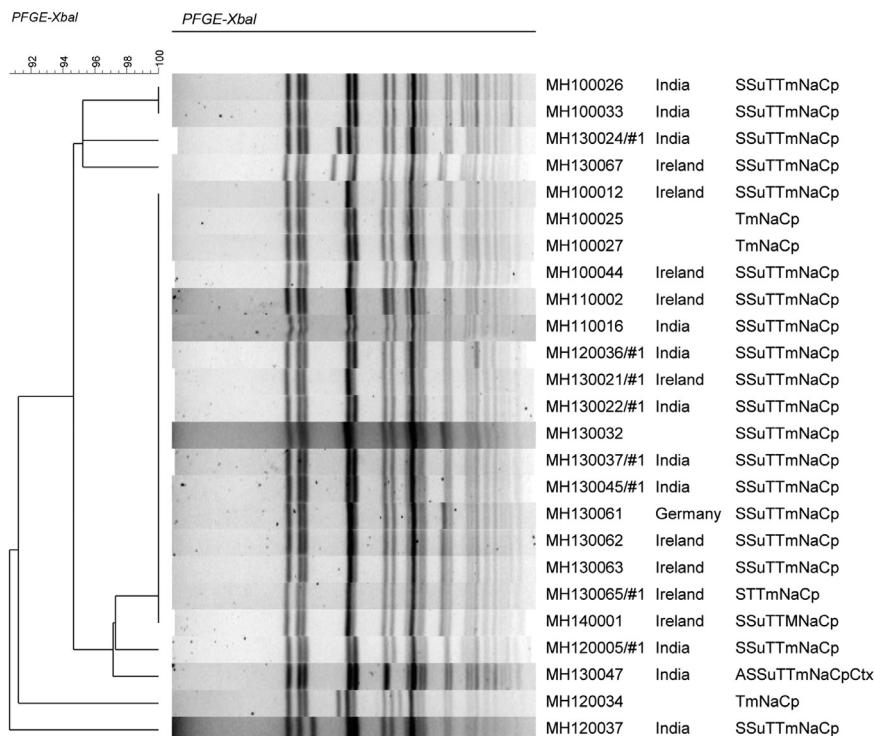


Figure. Dendrogram of ciprofloxacin-resistant *Shigella sonnei* digested with *Xba*I enzyme. Isolate identification numbers and country location for origin of infection are shown. In column on far right, antibiogram abbreviations indicate resistance to antimicrobial drugs: A, ampicillin; S, streptomycin; Su, sulfamethoxazole; T, tetracycline; Tm, trimethoprim; Na, naladixic acid; Cp, ciprofloxacin; Ctx, cefotaxime. Scale bar indicates evolutionary distance. PFGE, pulsed-field gel electrophoresis.

International concern is growing regarding antimicrobial drug resistance in *Shigella* infections associated with India. Fluoroquinolone resistance emerged in *S. dysenteriae* in 2002, in *S. flexneri* in 2004, and in *S. sonnei* in 2007 (5). Studies from Japan have also reported an association between travel to India and infection with an *S. sonnei* clonal group that was multidrug resistant, including resistance to nalidixic acid (6). Furthermore, ciprofloxacin-resistant *S. sonnei* isolates from foodborne outbreaks in India in 2009 and 2010 (7) had *Xba*I- PFGE types and resistance profiles visually indistinguishable from those reported in our study. A study of *S. sonnei* isolates in Bhutan showed that this clonal group was also common there (8). Furthermore, a 2010 outbreak of ciprofloxacin-resistant *S. sonnei* in Canada associated with men who have sex with men showed *Xba*I- and *Bln*I-PFGE patterns that appear similar to the patterns for isolates in this study (9).

Antimicrobial drug resistance is a major global problem that is likely to be exacerbated in places with poor sanitation and intensive use of antimicrobial drugs in humans and animals. These factors have contributed to increased ciprofloxacin resistance in *Salmonella enterica* serovars Typhi and Paratyphi A (10).

A review of published literature and informal communication indicates that our observation of ciprofloxacin resistance in *S. sonnei* infections associated with travel to India is part of a general global trend. This increasing resistance suggests that ciprofloxacin may no longer be suitable for empiric therapy for *S. sonnei* infection, particularly for patients with a history of travel to the subcontinent of India.

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Fatal *Balamuthia mandrillaris* Meningoencephalitis in the Netherlands after Travel to The Gambia

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To the Editor: *Balamuthia mandrillaris* is a free-living amoeba that has a worldwide distribution in soil and was first reported in 1990 (1). Approximately 200 *B. mandrillaris* meningoencephalitis cases have been described, mostly from warm climate areas in South America. Its prevalence in the United States is estimated to be 1 case/year (2). However, *B. mandrillaris* meningoencephalitis

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has not been reported in Africa, and only 4 cases have been reported in Europe (3–6). Transmission occurs through the respiratory tract or the skin or by organ transplant, and the incubation period varies from weeks to months after primary infection (7). After an indolent, subacute phase with aspecific symptoms, the amebae invade the central nervous system, and illness rapidly progresses, leading almost invariably to death (7). Because *B. mandrillaris* is difficult to detect in soil, its specific geographic distribution around the world is unknown and is estimated on the basis of where illnesses have been reported (7). This report addresses fatal *B. mandrillaris* meningoencephalitis in a woman from the Netherlands who had visited The Gambia.

In December 2013, a previously healthy 61-year-old white woman in the Netherlands sought care for fever, headaches, and muscle pains she had experienced for 1 week. That year, she had traveled 4 times to The Gambia, the last visit being 1 month before her hospitalization (online Technical Appendix Table 1, <http://wwwnc.cdc.gov/EID/article/21/5/14-1325-Techapp1.pdf>). After she returned from her visit in September 2013, fatigue, diarrhea, fever, and pustular skin lesions on her back and lower extremities developed. A wound swab culture showed *Staphylococcus aureus*, for which she was treated successfully with oral clarithromycin and topical fucidin ointment.

On admission in December, her physical and neurologic examination results were unremarkable. Malaria was excluded; because of persisting headaches, a cerebral computed tomography scan without contrast was performed but showed no abnormalities. In the following days, high fevers, altered mental status, and nuchal rigidity without focal neurologic deficits developed. Cerebrospinal fluid (CSF) examination showed mononuclear pleocytosis, highly elevated protein levels, and low glucose levels (online Technical Appendix Table 2). Serial cerebral computed tomography and magnetic resonance imaging scans showed development of an asymmetric hydrocephalus and diffuse leptomeningeal and subependymal contrast enhancement,

especially around the brainstem, without signs of intracerebral mass lesions (online Technical Appendix Figure).

Presumed diagnosis was tuberculous meningitis, and she was treated with tuberculostatic drugs (isoniazid, rifampin, pyrazinamide, and ethambutol) combined with intravenous acyclovir, ceftriaxone, and co-trimoxazole for other infectious causes of meningoencephalitis. Despite external lumbar and ventricular (both lateral ventricles and fourth ventricle) CSF drainage, her neurologic condition deteriorated. Multiple cranial nerve palsies developed, and she became comatose and died 11 days after admission.

Informed consent for postmortem examination was obtained, and macroscopic pathologic examination showed uncal and cerebellar herniation caused by increased intracranial pressure. Microscopic brain tissue examination showed signs of acute granulomatous inflammation, multiple hemorrhagic infarctions, and angiitis in the presence of numerous amebic trophozoites and cysts (Figure), which showed granulomatous hemorrhagic necrotic amebic meningoencephalitis. Real-time PCR and subsequent sequencing on brain biopsy and CSF specimens showed *B. mandrillaris* to be the causative ameba (8,9).

The infection could have been acquired in The Gambia or the Netherlands because the patient had intensive soil contact in The Gambia, where she frequently cultivated land, and in the Netherlands, where she worked in glass horticulture. She may have been infected through the skin after contact with contaminated soil, but her skin lesions were atypical for *B. mandrillaris*, and postmortem examinations failed to identify *B. mandrillaris* except in the central nervous system.

The lack of reported *B. mandrillaris* cases from Africa might indicate a low number of postmortem examinations and little access to advanced diagnostics, rather than a low environmental prevalence of *B. mandrillaris*. The few reported cases in Europe might be related to lack of awareness and to clinical signs and symptoms that mimic tuberculous meningitis: a lymphocytic pleocytosis with an elevated protein level and a low glucose level in CSF, together with a hydrocephalus and subependymal and

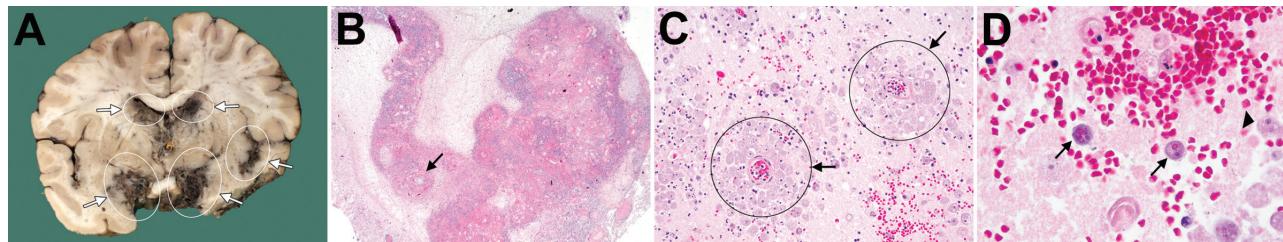


Figure. Postmortem pathologic findings for woman in the Netherlands who died of *Balamuthia mandrillaris* meningoencephalitis after returning from travel to The Gambia. A) Macroscopic coronal central section scan showing hemorrhagic necrotizing lesions of the subependymal, meningeal, and parenchymal areas of the parietotemporal lobes (circles and arrows). B) Low-power microscopic scan showing hemorrhagic necrotizing angiitis of the meningeal vessels (arrow) (original magnification $\times 25$). C) Medium-power microscopic scan (original magnification $\times 200$) showing perivascular trophozoite cuffing (arrows) and granulomatous inflammation. D) High-power microscopic scan (original magnification $\times 630$) showing encysted amebae (arrows) and free trophozoites (arrowhead). Hematoxylin and eosin stains.

leptomeningeal contrast enhancement on magnetic resonance imaging (10). Also, *B. mandrillaris* meningoencephalitis imaging findings are often nonspecific, including cerebral edema, hydrocephalus, multiple space-occupying and ring-enhancing lesions, leptomeningeal enhancement, or formation of mycotic aneurysms (2). Furthermore, amebic trophozoites are seldom detected in CSF by microscopy (2,3). Consequently, *B. mandrillaris* meningoencephalitis could be underdiagnosed, especially where this infection has no or only sporadic reports.

B. mandrillaris should be considered in refractory or unexplained cases of meningoencephalitis, even outside the Americas and in immunocompetent patients. Detecting *B. mandrillaris* by PCR in CSF seems most likely to enable early diagnosis and timely treatment. However, appropriate therapy is not well defined; success has been sparsely reported with the simultaneous use of azoles, flucytosine, pentamidine, sulfazidine, macrolide antimicrobial drugs, phenothiazines, and miltefosine (2,7,10).

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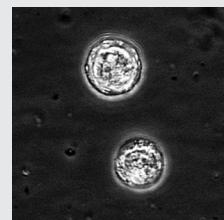
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etymologia

Balamuthia mandrillaris [bal"ə-moo'the-ə man"dril-a'ris]

A free-living ameba naturally found in the environment, *Balamuthia mandrillaris* can cause a serious infection of the brain, other organs (skin, liver, kidneys), and rarely, spinal cord. Originally isolated from the brain of a mandrill that died of meningoencephalitis at the San Diego Zoo, *Balamuthia mandrillaris* is named for the late professor

William Balamuth of the University of California at Berkeley, for his contributions to the study of amebae. More recently, *B. mandrillaris* has been shown to be transmissible through organ transplantation.



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Citizens' Actions in Response to Chikungunya Outbreaks, Réunion Island, 2006

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To the Editor: To get ready for the spread of chikungunya, health authorities in North, South, and Central America and the Pacific Islands are developing preparedness and response plans (1,2) that contain vector control, epidemiologic surveillance, medical education, and communication components. They might consider the experience of Réunion Island, an overseas department of France, where a chikungunya outbreak affected 38.5% of its 800,000 inhabitants during the first 3 months of 2006 (3). Although the island was unprepared to deal with such a massive outbreak (4), the disease was under control by the middle of 2006; only a few sporadic cases occurred during the following years. In addition to taking recommended public health measures, public health officials in France created a task force with physicians (including intensive care unit doctors, pediatricians, and obstetricians), specialists in public health and social sciences, virologists, immunologists, entomologists, and pathologists (5) to develop a multidisciplinary approach to the outbreak.

Some citizens' initiatives complemented the official measures. First, associations of chikungunya virus-infected patients helped families (through means that included psychological and friendly support and home visits) and updated mass media with regard to disease complications, persistent symptoms, and administrative difficulties (including receiving long-term sick leave and disability, recognition of professional exposure, and free analgesic medication). Second, citizens created a chikungunya-dedicated website (<http://www.chikungunya.net>) that included citizens' frequently asked questions and university-affiliated physicians' responses and patients' forums. Third, citizens actively supported the twice-yearly *Kass moustik* (Creole for "to break mosquitoes") operations, which involved vast community mobilizations to educate persons on mosquitoes' role in spreading chikungunya and to destroy breeding sites near homes. The operations also involved mobilizing community-based and municipality groups, making door-to-door visits, and lobbying for government funds (each operation cost US \$60,000). After implementation of these initiatives, telephone operators

sent health messages to all cell phones on the island. These actions demonstrate that citizens have a place in their countries' response to chikungunya outbreaks.

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Loa loa Infection in Pregnant Women, Gabon

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To the Editor: *Loa loa*, the African eye worm, is a filarial pathogen of Central African rainforest regions. As of 2013, it had affected an estimated 2–3 million persons in Central Africa (1,2). Adult worm migrations in humans may intermittently cause Calabar swelling, and microfilariae are commonly found in blood and body fluids. Loiasis is a chronic infection persisting for many years; a considerable proportion of women in loiasis-endemic regions are infected during gestation. To date, the epidemiology of loiasis in pregnant women has not been investigated, and the effects of loiasis on maternal and fetal health outcomes are unknown. We investigated the epidemiology of loiasis in a cohort of pregnant women participating in a drug trial for preventing malaria during pregnancy.

This study was conducted at the Centre de Recherches Médicales de Lambaréné, Albert Schweitzer Hospital, Lambaréné, Gabon, and at the Ngounié Medical Research Centre, Fougamou, Gabon, during September 2009–April 2012 (3). The filarial pathogens *L. loa* and *Mansonella perstans* are endemic to the study region, which is in the equatorial rainforest, and malaria is hyperendemic in the region (1,4). Study participants were HIV-negative pregnant women in a clinical trial assessing intermittent preventive treatment of malaria during pregnancy (clinical trials identifier: Malaria in Pregnancy Preventive Alternative Drugs [MiPPAD]; NCT00811421) (5). Pregnant women were recruited before their third trimester. After providing written informed consent, they were randomly allocated to receive treatment with either sulfadoxine/pyrimethamine or mefloquine. Ethical clearance was obtained from the Comité d’Ethique Régional Indépendent de Lambaréné.

Women for whom ≥ 1 microscopic examination of blood revealed microfilariae during the course of their pregnancy were classified as *L. loa* infected. Women were considered afilemic if ≥ 2 blood examination results were negative. Microfilariae were detected by examination of thick and thin blood smears or by saponin leukoconcentration. Examination for placental infection was performed by impression smear of a fresh placental biopsy. Baseline demographic and anthropometric characteristics were recorded at the first antenatal visit and at delivery. Data were double-entered into an electronic database for statistical analysis (STATA/SE 12.1; StataCorp LP, College Station, TX, USA).

Of 1,184 women participating in the antimalarial drug trial, 1,004 contributed data for our analysis. Women who had no record of delivery ($n = 120$) or who had multiple births ($n = 60$) were excluded. Of these 1,004 women, *L. loa* microfilariae were found in peripheral blood of 179 (18%); of those, microfilariae were found in placental blood of 24 (13%). No microfilariae were found in the placenta of women with amicrofilaric peripheral blood. Loiasis prevalence was higher among older women (≥ 30 years of

age) than among adolescents (14–17 years of age; odds ratio 2.1, 95% CI 1.2–3.9). Microfilaremia was more common among multigravid women than among primigravid women ($p = 0.06$) but was not associated with other maternal baseline characteristics or with low infant birthweight, preterm births, or adverse delivery outcomes (Table). No histologic evidence of intervillous inflammation, infarcted areas, or chorioamnionitis was observed in the placenta of women with loiasis, and no microfilariae were observed in any examined cord blood samples.

In this study, we attempted to characterize the epidemiology of *L. loa* infection during pregnancy in a highly *L. loa*-endemic region of Central Africa. Microfilaremia was associated with the women’s age, a finding indicating that prevalence increases because of the long duration of infection and continued exposure. The age-related increase in the prevalence of *L. loa* infection aligns with previously reported prevalence and contrasts with age-related prevalence of other parasitic infections (6,7). Anecdotal evidence suggests the potential of *L. loa* worms to invade the placenta (8). In this systematic investigation, microfilarial invasion of the placenta occurred in 13% of microfilaric patients. However, histopathologic analysis showed no evidence for pathologic alterations of the placenta, and risk for adverse birth outcomes did not increase. Transgression of microfilariae into cord blood was not observed.

This study has limitations. First, misclassification of occult infection is possible because infection status was classified on the basis of the presence of microfilaremia. Also, antimalarial drugs routinely administered during pregnancy may have influenced the course of loiasis, as has been shown for other helminth infections (9,10). Further, the observational study design creates difficulties in establishing a causal relationship between infection status and birth outcomes because of possible confounding factors. Additional research is needed to disentangle the association among pregnancy outcomes, socioeconomic conditions, and pathophysiologic consequences of *L. loa* infection in pregnant women.

Results of this prospective study show a high prevalence of loiasis among pregnant women in a loiasis-endemic region in Central Africa. The invasion of microfilariae into the intervillous space of the placenta is a newly described feature of pregnancy-associated loiasis. These data can be used as a starting point for further epidemiologic and clinical research activities investigating this neglected filarial infection in pregnant women in Central Africa.

Acknowledgments

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Table. Baseline characteristics of microfilaremic and amicrofilaremic pregnant women, Gabon, September 2009–April 2012

Characteristic	Microfilaremic, no. (%), n = 179	Amicrofilaremic, no. (%), n = 825	p value*
Age, y, n = 1,004			0.156
14–17	17 (9.5)	129 (15.6)	
18–20	43 (24.0)	191 (23.2)	
21–24	40 (22.3)	168 (20.4)	
25–30	34 (19.0)	175 (21.2)	
31–49	45 (25.1)	162 (19.6)	
Gravidity, n = 1,004			0.057
First pregnancy	40 (22.3)	214 (25.9)	
1–3 previous pregnancies	73 (40.8)	381 (46.2)	
≥4 previous pregnancies	66 (36.9)	230 (27.9)	
Literacy, n = 1,004			0.151
Yes	141 (78.8)	687 (83.3)	
No	38 (21.2)	138 (16.7)	
Delivery outcome, n = 1,004			0.432
Live birth	172 (96.1)	781 (94.7)	
Stillbirth or abortion	7 (3.9)	44 (5.3)	
Maternal malarial infection at delivery, n = 867†‡			0.453
Yes	6 (3.7)	36 (5.1)	
No	156 (96.3)	669 (94.9)	
Anemia at delivery, hemoglobin <11 g/dL, n = 903†			0.364
Yes	77 (46.1)	368 (50.0)	
No	90 (53.9)	368 (50.0)	
Premature delivery, n = 886†			0.082
Yes	14 (8.5)	36 (72.0)	
No	151 (91.5)	685 (95.0)	
Low birthweight, n = 905†			0.465
Yes	24 (14.3)	90 (12.2)	
No	144 (85.7)	647 (87.8)	

*By χ^2 test.

†Maternal malarial infection, anemia, prematurity, and birthweight were assessed for live births only in this analysis.

‡Low birthweight occurred in 7 (17%) of 41 babies born to malaria-infected mothers compared with 98 (14%) of 721 babies born to non-malaria-infected mothers.

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Melioidosis in Trinidad and Tobago

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To the Editor: Melioidosis refers to infection caused by the facultative intracellular gram-negative bacterium *Burkholderia pseudomallei*. The clinical manifestations of melioidosis span a wide spectrum, from asymptomatic exposure or localized cutaneous infection to septic shock with multi-organ failure. Melioidosis usually occurs in residents of or travelers to disease-endemic areas in northern Australia and Southeast Asia; however, an increasing number of confirmed melioidosis cases are being reported from the Caribbean. We report a case of melioidosis acquired in Trinidad and Tobago.

In February 2014, a 17-year-old male student was admitted to a tertiary care hospital in Vancouver, British Columbia, Canada, with catecholaminergic polymorphic ventricular tachycardia and electrical storm. He had a 9-month history of dry cough that was unresponsive to multiple and prolonged courses of treatment for community-acquired pneumonia. During the 6 months before his admission, the patient had hemoptysis and radiologic evidence of pneumonia that were treated with courses of cephalosporins without resolution of symptoms. Bronchoscopy and culture of lavage samples had revealed infection with *Staphylococcus aureus* and an organism most closely related to *Actinomyces graevenitzi*.

The patient had no history indicative of risk factors for recurrent sinusitis or pneumonia (e.g., cystic fibrosis, chronic granulomatous disease, Job syndrome), and no risk factors for tuberculosis or infection with dimorphic fungi. He was up to date on his vaccinations and had no pets. He was born in Jamaica, had moved to Canada at age 4, and had not traveled anywhere other than Trinidad and Tobago, Canada, and England. He had traveled to visit family in

Trinidad for 2 months during the rainy season in 2012, at which time he also visited Tobago.

On day 5 of hospital admission, the patient became febrile (39.6°C), and an infectious diseases specialist was consulted. Examination revealed that the patient was clinically stable but emaciated at 45 kg. His oxygen saturation while breathing room air was 98%. Physical examination, including cardiorespiratory examination, was unremarkable. Laboratory results showed a normal hemoglobin concentration of 133 g/L; elevated leukocyte count of 22.8×10^9 cells/L; neutrophils 19.4×10^9 cells/L; normal platelet count of 295×10^9 /L; and normal creatinine of 54 μ mol/L. Test results for HIV-1 and blood cultures were negative. Computed tomography scan showed dilated bronchi and dense consolidation of the right and left lower lobes. Piperacillin/tazobactam was started for presumed hospital-acquired pneumonia.

The patient underwent diagnostic bronchoscopy with bronchoalveolar lavage. Gram staining of specimens showed occasional gram-negative bacilli, and aerobic cultures grew gram-negative bacilli. Further testing with the Vitek 2 (bioMérieux, Laval, Quebec, Canada) (96%) and RapID NF (Oxoid, Nepean, Ontario, Canada) (99.9%) systems identified *B. pseudomallei*, but matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Vitek MS, bioMérieux) did not. Phenotypic confirmation was performed at the provincial public health and reference laboratory. Antimicrobial drug susceptibility testing performed by broth microdilution according to Clinical and Laboratory Standards Institute recommendations (1) and by Etest (bioMérieux) showed susceptibility to amoxicillin/clavulanic acid, ceftazidime, imipenem, doxycycline, and trimethoprim/sulfamethoxazole. The patient's condition improved after 2 weeks of intravenous meropenem, and antimicrobial therapy was changed to oral trimethoprim/sulfamethoxazole.

The *B. pseudomallei* isolate was sent to the Public Health Agency of Canada's National Microbiology Laboratory for molecular typing. Query of 7 standard multilocus sequence typing loci (<http://bpbseudomallei.mlst.net/>) identified the isolate as a novel multilocus sequence type. The sequence type (1,1,2,1,5,6,1) closely resembled that of *B. pseudomallei* previously isolated from the Caribbean (2).

Although melioidosis was first described in the Caribbean in 1947 (3), most case reports of the disease in the area are from the past 2 decades. This case report suggests progression of the range of melioidosis to include Trinidad and Tobago. A recent study documented the presence of *B. pseudomallei* in soil samples and high seroprevalence rates among contacts of persons with melioidosis in Puerto Rico (4). If examined, this pattern of regional melioidosis endemicity may also be found on other Caribbean islands.

Increased clinical awareness of and improved surveillance for *B. pseudomallei* infection may partly explain

emergence. Nonetheless, underascertainment probably occurs in rural areas with limited access to advanced diagnostic support and in urban areas when *B. pseudomallei* infection is not suspected because of lack of travel to

classic disease-endemic areas. Because *B. pseudomallei* is a Biosafety Level 3 agent, when infectious disease specialists consider melioidosis in their differential diagnoses, they should alert the microbiology laboratory to confirm

Table. Published case reports of melioidosis from the Caribbean*

Ref.	Site of origin (year)	Age, y/sex	Type of exposure	Concurrent condition	Clinical manifestation	Diagnostic method	Treatment (duration)	Outcome
(3)	Panama (1947)	31/M	Fall on buttock, TR	Polio, spinal meningitis	Buttock abscess	Abscess culture	Sulfathiazole, sulfapyridine, streptomycin, penicillin	Survived
(5)	Panama (1948)	25/F	UNK, TR	None	Retroperitoneal abscess, sepsis	Abscess culture	Penicillin, streptomycin	Died
(5)	Panama (1960)	20/M	UNK	None	Acute septic arthritis	Synovial fluid culture	Chloramphenicol, novobiocin, sulfisoxazole	Survived
(5)	Puerto Rico (1982)	62/F	UNK	Diabetes, SLE, cirrhosis	Septic meningitis	Blood and CSF culture	Penicillin, chloramphenicol, moxalactam, amikacin	Died
(5)	Mexico (1986)	72/M	UNK	None	Pneumonia, splenic abscess	Blood and sputum culture	Cefoxitin, gentamicin	Died
(5)	Martinique (1995)	66/M	UNK, resident	Diabetes	Sepsis	Blood and urine culture	IV ceftazidime, then oral TMP/SMX and doxycycline (2 mo)	Survived
(5)	Guadeloupe (1997)	4/M	UNK, TR	None	Pneumonia, pleural effusion, peritonitis	Pleural fluid culture	IV ceftazidime and TMP/SMX(1 mo), then oral TMP/SMX (6 mo)	Survived
(5)	Puerto Rico (1997)	11/M	UNK, resident	X-linked CGD	Mediastinitis, lymphadenitis	Supraclavicular and hilar biopsy culture	Imipenem and doxycycline (6 weeks), oral cefixime and doxycycline (3 wk)	Died†
(5)	El Salvador (2001)	UNK	UNK, TR	UNK	Cerebral abscess	UNK	UNK	Survived
(4)	Puerto Rico (2003)	55/F	Flood water, resident	Diabetes	Pneumonia, septic shock	Blood and sputum culture	Imipenem, amikacin, azithromycin	Died
(6)	British Virgin Islands (2006)	17/M	UNK, resident	CF	Pneumonia	Sputum culture, PCR	UNK	Survived
(7)	Aruba (2009)	7/F	UNK, TR	CF	Pneumonia	Oropharyngeal and induced sputum culture	Imipenem and ceftazidime (14 d), then inhaled meropenem (28 d) and long-term oral TMP/SMX	Survived
(4)	Puerto Rico (2009)	88/M	Ditch digging, resident	CAD, PVD	Pneumonia	MLST	Doxycycline (20 wk), oral TMP/SMX	Survived
(8)	Guadeloupe (2010)	15/F	UNK, TR	Asthma, dengue fever	Adenopathy, tumefaction	Tumefaction culture PCR	IV ceftazidime (10 d), then oral TMP/SMX (12 wk)	Survived
(9)	Martinique (2010)	35/M	UNK, TR	None	Diarrhea, pneumonia	Blood culture; PCR	Imipenem, G-CSF	Died
(10)	Aruba (2012)	46/F	Water exposure, TR	None	Breast abscesses	Abscess culture	Meropenem (14 d), then oral TMP/SMX (12 wk)	Survived
(4)	Puerto Rico (2010)	38/M	Landscaping, resident	None	Pneumonia, hepatitis, myocarditis, septic shock	Immunohistochemistry with polyclonal Ab; PCR	None	Died
(4)	Puerto Rico (2012)	60/M	Agricultural work, resident	Diabetes	Diabetic ketoacidosis	Blood culture; MLST	Amoxicillin/cloxacillin (10 d), then oral TMP/SMX (12 weeks)	Survived
This study	Trinidad and Tobago (2014)	17/M	Rainy season, TR	CPVT	Chronic pneumonia	BAL culture; MLST	Meropenem (2 weeks), then oral TMP/SMX (8 mo to date)	Survived

*Ab, antibody; BAL, bronchoalveolar lavage; CAD, coronary artery disease; CGD, chronic granulomatous disease; CPVT, catecholaminergic polymorphic ventricular tachycardia; CSF, cerebrospinal fluid; G-CSF, granulocyte colony-stimulating factor; IV, intravenous; MLST, multilocus sequence typing; PVD, peripheral vascular disease; Ref., reference; SLE, systemic lupus erythematosus; TMP/SMX, trimethoprim/sulfamethoxazole; TR, travel related; UNK, unknown.

†Cause of death unknown.

species identification and ensure that staff use proper biosafety measures.

A total of 19 cases of melioidosis acquired in the Caribbean have been reported (Table). Nine of these were travel related, suggesting that melioidosis may be emerging as a travel health issue. Travelers with known risk factors for melioidosis, such as diabetes mellitus and chronic lung disease, should be informed of their increased infection risk. Physicians should include *B. pseudomallei* in the differential diagnosis of travelers with pneumonia or sepsis who are returning from the Caribbean, particularly when they have a history of travel during the rainy season, soil-contaminated wounds, or known risk factors for melioidosis.

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Probable Toxic Cause for Suspected Lychee-Linked Viral Encephalitis

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To the Editor: Paireau et al. (1) reported a spatiotemporal association between unexplained outbreaks of suspected acute encephalitis in children in northern Vietnam and the harvesting of lychee (litchi) fruit. The clinical, biologic, and immunologic characteristics of the patients suggested a viral etiology (1). However, the lychee-associated acute brain disorder, which has also been reported in Bangladesh and India (Bihar and West Bengal), could also result from ingestion of phytotoxins present in lychee fruit, specifically a-(methylenecyclopropyl)glycine (2), the lower homologue of the neurotoxic L-amino acid hypoglycine (3,4).

As previously described (5), ingestion of the hypoglycine-rich fruit of ackee, a relative of lychee, can induce a dose-dependent toxic hypoglycemic encephalopathy in poorly nourished children. The syndrome is best known from Jamaica, where ackee is widely eaten, and occurs most frequently in 2- to 10-year-old children, who develop severe hypoglycemia and metabolic acidosis. Clinical manifestations of Jamaican vomiting sickness include headache, thirst, sweating, vomiting, lethargy, seizures, coma, and death over a span of hours to days. Patients may be mildly to moderately febrile, and emesis may not be present in all cases. Heavy ingestion of the immature aril (fruit) of ackee (*Blighia sapida*) or other members of the soapberry family (Sapindaceae), including lychee (*Litchi sinensis*), rambutan (*Nephelium lappaceum*), and longan (*Dimocarpus longan*), by an undernourished child with low glycogen/glucose stores probably has the potential to result in toxic hypoglycemic syndrome.

Assessment of finger-prick blood glucose levels, which may be markedly depressed in children with severe Sapindaceae fruit poisoning, provides a rapid and convenient screening tool to identify suspected cases. Intravenous administration of glucose is the first line of treatment, along with serial monitoring of glucose, serum aminotransferase, and serum creatinine levels. Restoration of body fluid, electrolytes, glucose, and pH balance is the goal of supportive treatment.

Note added in proof. Subsequent to the submission of this letter, a description was published of recent outbreaks

of unexplained acute hypoglycemic encephalopathy in young children in Muzaffarpur, Bihar, coinciding with local lychee harvests (6).

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Pin-Site Myiasis Caused by Screwworm Fly, Colombia

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To the Editor: Myiasis is the infestation of humans or animals with dipterous insect larvae (1). The term pin-site myiasis was recently adopted for a rare and emerging

parasitic infection after treatment of open fractures with external metal fixators (pins). Myiasis can also occur as a result of invasion of larvae deposited by flies in wounds adjacent to these fixators (1,2). We describe a patient with pin-site myiasis caused by the *Cochliomyia hominivorax* screwworm fly associated with external fixators used for treatment of an open fracture of the femur.

In September 2014, a 26-year-old male soldier from the Department of Meta in central Colombia was admitted to a primary medical unit for treatment of an open fracture of the right femur after a traffic accident. The patient had no relevant medical history. After multiple surgical interventions and external fixation of the fracture, he was discharged. Two weeks later, he returned to the medical unit with edema, redness, and warmth in the area surrounding the metallic fixators. At this time, 50 larvae were observed in the surgical wound (Figure, panel A).

The patient was referred to Hospital Militar Central in Bogota, Colombia, where surgical cleansing of the wound was performed and 30 additional larvae were obtained (Figure, panel B). Extracted larvae were sent to the Parasitology Laboratory of the Universidad Nacional de Colombia in Bogota, Colombia for identification. The larvae were taxonomically classified as those of the *C. hominivorax* screwworm fly.

Treatment with oral ivermectin and intravenous ampicillin/sulbactam was initiated. The next day, surgical cleansing showed signs of osteomyelitis. A culture of bone tissue was positive for multidrug-susceptible *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia* susceptible to trimethoprim/sulfamethoxazole (TMP/SMX). At this time, antimicrobial drug therapy was changed to intravenous ciprofloxacin (400 mg every 12 h) and oral TMP/SMX (160/800 mg every 12 h). The patient completed 2 weeks of treatment in the hospital and showed no signs or symptoms of infection or infestation by larvae. He was discharged, prescribed oral TMP/SMX, and followed up by the Orthopedics and Infectious Diseases Service of Hospital Militar Central.

Bacterial infection in insertion sites of metallic pins is usually the most frequent complication when external fixators are used in treatment open fractures and represents

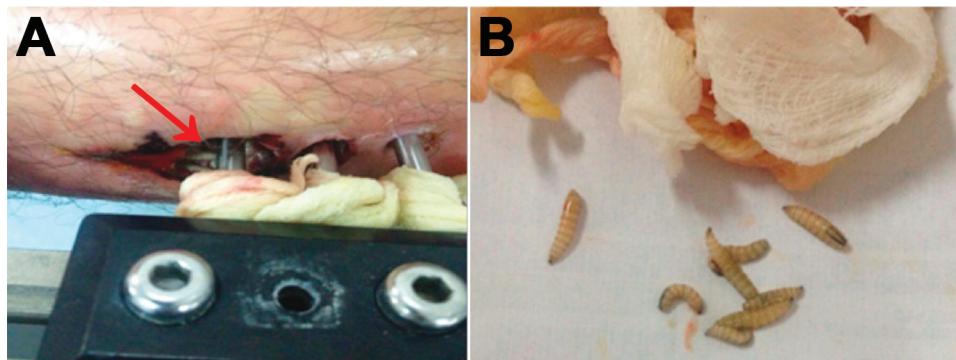


Figure. Pin-site myiasis in a 26-year-old male soldier, Colombia. A) Larvae of *Cochliomyia hominivorax* screwworm fly around an external metallic fixator (arrow). B) Larvae isolated from the insertion wound of the external metallic fixator. A color version of this figure is available online (<http://wwwnc.cdc.gov/EID/article/21/05/14-1680-F1.htm>).

10%–40% of complications, followed by loss of pins (5%), pain/edema (3.3%), and vascular or nervous injury (1.7%) (3). In the past decade, pin-site myiasis has been described as a new complication; 6 cases have been reported (1 in the United States, 2 in Venezuela, and 3 in Greece) (2,4–6). All case-patients had predisposing risk factors for parasitic infestation, such as diabetes mellitus, immobilization, alcohol and drug use, or decreased immune status. Our patient had the same risk factor (previous surgical interventions) as that reported for a patient in Venezuela (2). Also, the anatomic region (leg) involved and the larvae species (*C. hominivorax*) identified for our patient were observed in other reported cases (2,5,6).

C. hominivorax screwworm fly is the main species involved in wound myiasis in the New World (1). Wound myiasis is initiated when female flies oviposit on or near a wound (≤ 300 larvae/wound). Upon hatching, larvae, which have small spines on each body segment that resemble the threads of a screw, penetrate head first into the tissues, burrow deeper perpendicular to the skin surface (resembling a screw), and cause extensive destruction of tissue and a bloody discharge (1). *C. hominivorax* larvae differ from larvae of other fly species because they feed only on living flesh (7). The anatomic site around a lesion becomes swollen, and local tissue destruction can cause pain and secondary bacterial infection (1).

Our patient was co-infected with *P. aeruginosa*, which was similar to a patient with pin-site myiasis reported by Paris et al. (5). Removal of the metallic fixators (a necessary procedure in 50% of reported cases) (1) was not required for our patient. Surgical cleansing, extraction of all larvae, and antimicrobial drug therapy resulted in resolution of the infection.

After a screwworm eradication program was developed by the Animal and Plant Health Inspection Service of the US Department of Agriculture, screwworm was eradicated in the United States in 1966, in Mexico in 1991, in Belize and Guatemala in 1994, in El Salvador in 1995, in Honduras in 1996, in Nicaragua in 1999, in Costa Rica in 2000, and in Panama in 2006 (7). Current distribution of *C. hominivorax* screwworm flies is limited to South America and some Caribbean Islands (1). However, physicians should be aware of the possible reemergence of myiasis as a complication of surgery and use of metal fixators.

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East/Central/South African Genotype Chikungunya Virus, Brazil, 2014

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To the Editor: Chikungunya virus (CHIKV) is an arthropod-borne alphavirus (family *Togaviridae*) comprising 3 genotypes: West African, East/Central/South African, and Asian (1). This zoonotic pathogen originated in Africa and since 2004 has caused outbreaks in several countries on different continents (2). In 2013, CHIKV reached the Americas and caused an explosive epidemic that has already caused 1,231,077 cases in 43 countries (3).

In Brazil, autochthonous cases of chikungunya were confirmed in September 2014 in Feira de Santana (FSA), a city of 612,000 residents (4) near the eastern edge of Bahia State in east-central Brazil. Surprisingly, the CHIKV genotype was determined to be East/Central/South African and not the Asian genotype that is circulating in the Americas; this finding was based on sequence data obtained from a cell culture viral isolate using an Ion Torrent platform (3,5).

Dengue is endemic/epidemic to FSA, and the first cases of chikungunya were mistakenly reported as dengue. Beginning in July 2014, when dengue virus transmission is low, an increased number of suspected cases of dengue from a FSA neighborhood caught the attention of local surveillance officials. CHIKV infection was suspected because results of laboratory tests for dengue (nonstructural 1 and IgM ELISA) were negative, and the patients complained mainly of high fever and intense bilateral joint pain accompanied by swelling (4). IgM ELISA and quantitative reverse transcription PCR conducted at the Instituto Evandro Chagas (Ananindeua, Brazil) confirmed the cause of illness as CHIKV. The sequences obtained in this study were deposited in the GenBank under accession nos. KP164567–KP164572.

Data from epidemiologic investigations suggested that the index case-patient could have been a Brazilian citizen living in Luanda, Angola, who visited his family in FSA. He went to an emergency health unit in FSA on May 28, reporting intense joint pain and high fever. His laboratory tests (nonstructural 1 and IgM) for dengue were negative. On June 4 (epidemiologic week [EW] 23), another person sought care for similar symptoms, and new cases emerged, all in residents in that same neighborhood (4). The epidemic peaked in EW 39, when 200 cases were reported. Cases then decreased, and in EW 48 only 10 cases were reported (Figure).

In FSA, of the 1,346 chikungunya cases (219.9/100,000 residents) reported through EW 48, a total of 52.4% (1,498.1/100,000) patients lived in the same neighborhood as the index patient. However, the other 77 neighborhoods in FSA also recorded cases. Twice as many cases occurred among female patients (67.1% of cases) as among male patients. All age groups were affected; incidence was highest in persons 20–49 years of age (56.2%; 267.1 cases/100,000 inhabitants). The main clinical manifestations were high fever, arthralgia and arthritis with edema, headache, myalgia, rash, and itching. As of EW 48, no deaths were recorded (4).

CHIKV is transmitted by *Aedes aegypti* and *Ae. albopictus* mosquitoes, but FSA has *Ae. aegypti* only, and the Premise Index was 1.1% on January 2014 (6). Thus, during EW 36, the surveillance service of FSA began intense actions to combat that vector (5) by using integrated environmental management (7): elimination of breeding sites,

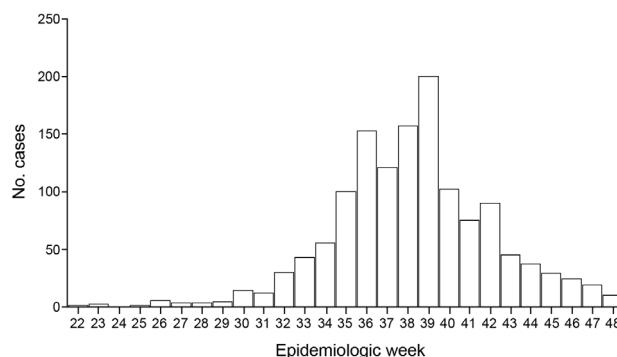


Figure. Reported cases of chikungunya fever, by epidemiologic week. Feira de Santana, Bahia State, Brazil, 2014.

applications of larvicide in water bodies, spraying insecticide (ultra-low volume), mobilization, and community education. However, cases continued to be diagnosed in neighborhoods in FSA, and transmission was detected in another municipality 77 km from FSA (391 cases through EW 48). Isolated cases imported from FSA were detected in other municipalities of Bahia State (8).

This epidemic had some unusual aspects. First, it was not caused by the Asian genotype circulating in affected countries of the Americas, which maintain intense tourism and trade with Brazil. Second, it occurred during the dry season, when little dengue transmission was occurring. The introduction of a person from a country reporting CHIKV activity (9) into an area infested by *Ae. aegypti* mosquitoes and having a population immunologically naive to CHIKV created favorable conditions to establish a local transmission cycle with quick production of many cases.

Concurrently with the outbreak in FSA, chikungunya cases were detected in Oiapoque municipality (10), Amapá State (northern Brazil bordering French Guiana); these cases were caused by the Asian genotype (genotype determined by nearly complete genome sequencing using an Ion Torrent sequencer). The picture so far suggests that expansion of the epidemic to other places in Brazil can be caused both by internal movement of persons and by new cases imported from other countries.

Chikungunya fever is a health problem that threatens Brazilian society and poses a challenge for health authorities. CHIKV produces epidemics of great magnitude, is highly debilitating, and does not have any specific treatment or vaccine. This situation is creating serious social and economic consequences for low- and middle-income countries because of the excessive demand on health services and the social security programs used by much of the population. Therefore, the global spread of chikungunya fever highlights the need to mobilize national and international efforts to focus scientific research on developing tools to prevent this disease.

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Chikungunya, Dengue, and Malaria Co-Infection after Travel to Nigeria, India

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To the Editor: Arboviral infections, such as chikungunya and dengue, are endemic to South Asia. Concurrent infection of these viral infections with another vector-borne parasitic disease, malaria, is uncommon in India and would pose a challenge for medical diagnosis because of overlapping clinical symptoms. We present a case of multiple co-infections in a young man attending college in India after his return from Nigeria, a region endemic for chikungunya, dengue, and malaria.

After spending a 1-month vacation in Nigeria, a 21-year-old male asymptomatic Nigerian national arrived in Bengaluru, India, on September 5, 2014, to resume college. He developed febrile illness, chills, abdominal discomfort, headache, epigastric pain, and myalgias 6 days after his arrival. High-grade fever (103°F), icterus, and vomiting subsequently developed. He received treatment for his symptoms, and a physical examination revealed general weakness, pulse rate of 100 beats/min, and blood pressure of 140/70 mm Hg.

Various tests to assess his medical condition were conducted. A complete blood count showed a reduced platelet count of 68,000/mm³ (reference 1.5–5.0 ×10⁵ mm³); findings of an abdominal ultrasonography were normal. Comprehensive kidney and liver function tests showed elevated values (blood urea, 53 mg/dL [reference 15–45 mg/dL]; serum creatinine, 1.66 mg/dL [reference 0.6–1.2 mg/dL]; aspartate aminotransferase, 67 IU/L [reference 5–34 IU/L]). Because the man had visited and returned from a region endemic for chikungunya, dengue, and malaria (*I*) and had a reduced platelet count, diagnostic tests for these infections were conducted. Accordingly, dengue nonstructural 1 antigen detection rapid test conducted on blood collected 2 days after symptom onset was positive. Microscopic observation of thick and thin blood smears (also from blood taken 2 days after symptom onset) showed the malaria parasite *Plasmodium falciparum*. Reverse transcription PCR (RT-PCR) on serum collected 2 days after symptom onset was conducted to detect chikungunya and dengue viral genomes. Test results were positive for both chikungunya and dengue viruses. However, IgM antibody capture–ELISA (MAC-ELISA) for

detecting chikungunya and dengue viral antibodies was negative for both infections. In accordance with World Health Organization travel guidelines, a blood sample, taken 3 days after symptoms onset, was tested at the National Institute of Virology (Pune, India) for Ebola virus disease. This test used RT-PCR and real-time RT-PCR to detect Ebola virus nucleoprotein and polymerase genes and ruled out Ebola virus disease.

These tests were repeated with standard positive and negative controls to ensure no contamination and no false-positive results. RT-PCR for chikungunya and dengue viruses was performed by using virus gene-specific primers. RT-PCR for Japanese encephalitis and West Nile viruses also were conducted to rule out these cross-reacting arboviral infections that share common clinical manifestations with chikungunya and dengue. The failure of MAC-ELISA to detect chikungunya virus- and dengue virus-specific IgM was attributed to collection of the blood on day 2 after symptom onset, and thus the IgM would not have been generated to be detected by MAC-ELISA.

Fever similar to those common with malaria and typhoid are often exhibited with any of the arboviral infections that are endemic to Nigeria (1). Often these fevers are misdiagnosed as malarial fevers, and the opportunity to test for arboviral infections is missed. Dual infections of chikungunya and dengue are becoming more common in India (2,3), and there were earlier reports of dengue and malaria co-infection (4). Because these diseases are endemic to both Nigeria and India and because the incubation periods of infections vary, we do not know the exact location where the patient acquired any or all of these infections. Multiple infections in a single patient would drastically change the spectrum of clinical manifestations and thus complicate the diagnosis process. Our study particularly draws attention to understanding emerging arboviral infections and emphasizes the need for a multidimensional diagnostic approach in such clinical situations.

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Chikungunya Virus Outbreak, Dominica, 2014

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To the Editor: Chikungunya is a dengue-like mosquito-borne viral disease that has caused outbreaks in Africa, Asia, and the Pacific Islands (1). St. Martin reported the first documented occurrence of autochthonous transmission of chikungunya in the Caribbean islands in December 2013 (2). Dominica reported its first case on January 17, 2014 (3). This report describes the outbreak of chikungunya in Dominica through July 12, 2014.

Cases were characterized by using guidelines issued by the Centers for Disease Control and Prevention (CDC) and the Pan American Health Organization (4). Surveillance of chikungunya cases began on January 16, 2014, and data were collected on patients' age, sex, residence, date of illness onset, clinical features, and travel history.

The virus was detected at the Caribbean Public Health Agency (CARPHA) laboratory in Trinidad by using a real-time PCR (rPCR) developed by CDC; some testing was also done at CDC's Arboviral Diseases Branch in Fort Collins, Colorado, USA, by using an IgM ELISA and a plaque-reduction neutralization test, as appropriate. All suspected infections were laboratory confirmed through April 30, 2014, when community transmission was established. Thereafter, testing was done only for patients hospitalized >48 hours, women in their third trimester of pregnancy, patients who died, or patients thought to be infected and coming from geographic areas where chikungunya transmission was not yet established.

During December 15, 2013–July 12, 2014, a total of 3,559 chikungunya cases were reported in Dominica, of which 141 were confirmed by laboratory testing (134 [95%] by rPCR, 7 [5%] by serologic methods). The remaining 3,418 patients were considered infected (Figure), indicating an overall attack rate of 5% (on the basis of Dominica's census population for 2011, 71,293). Retrospective investigation showed that the 2 index patients experienced onset of illness during the week beginning December 15, 2013, and 1 of the patients had recently traveled from St. Martin. The 2 patients were unrelated and resided far apart.

Of the 141 confirmed patients, 78 (55%) were female and 60 (43%) were male; data on sex was unavailable for 3 patients. Mean age of the patients was 34 years (range 13 days–87 years; median 30 years). Thirty (21%) of the patients were children ≤ 9 years of age; 76 (55%) were 19–49 years of age. Most patients experienced fever (95%) and arthralgia (72%), and 21% of patients experienced rash. No deaths associated with chikungunya infection in Dominica were reported during the study period.

Across all age groups, more patients were female than male, as reported in previous outbreaks (5,6). This trend may suggest that, compared with men and boys, women and girls have greater health-seeking behaviors, greater levels of skin exposure, and potentially greater exposure due to peridomestic activities (7).

In this study, a disproportionate number of patients were ≤ 9 years of age, unlike findings for chikungunya outbreaks in Indonesia and Réunion Island, where children ≤ 9 years of age were least affected (7). Of all confirmed patients, 55% were 19–49 years of age, suggesting that the outbreak had economic effects because workplace productivity may substantially decrease if disease sequelae (e.g., arthralgia and arthritis) cause those affected to take time off from work.

Genotypic sequencing identified the Asian genotype of chikungunya as the strain currently circulating in the Caribbean (8,9). The East/Central/South African genotype was responsible for the Réunion Island outbreak, and an overall attack rate of 35% was reported after retrospective and active case detection (6). Differences in transmission and pathogenicity between genotypes require further investigation.

In response to the Dominica outbreak, a risk communication plan was developed and implemented on January 17, 2014, and consisted of 2 phases: an onset emergency phase and a control phase. Both phases targeted audiences through audio, print, and social media. To control and reduce the mosquito population in and around the homes of chikungunya patients, vector control activities (i.e., source reduction, application of larvicides, and fogging) were intensified with assistance from CARPHA and Yale University, New Haven, CT, USA. In addition, CARPHA and the Pan American Health Organization arranged for delivery of insecticide-treated bed nets for use in hospitals and other health care settings.

Although the introduction of chikungunya into the Caribbean islands may have been anticipated because of the broad distribution of the *Aedes aegypti* mosquito vector and suitable climatic conditions, our findings show that this outbreak could not be prevented. The continuing geographic spread of the disease emphasizes the ongoing challenge posed by mosquito-borne viral infections resulting from globalization and indicates a need for innovative prevention and control strategies.

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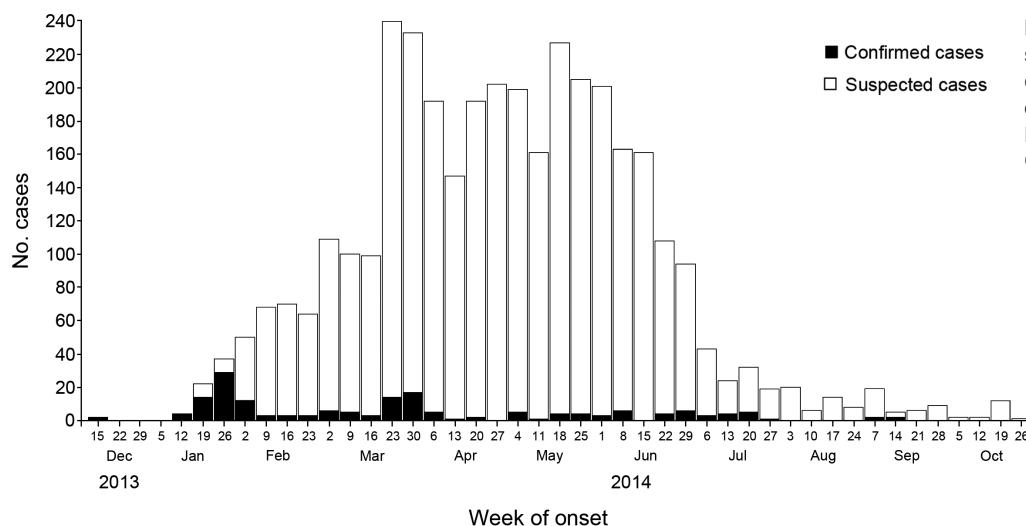


Figure. Confirmed and suspected chikungunya cases, by week of illness onset, Dominica, December 15, 2013–October 26, 2014.

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Acute Zika Virus Infection after Travel to Malaysian Borneo, September 2014

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To the Editor: Zika virus (ZIKV), a mosquito-borne flavivirus, causes Zika fever, a self-limiting febrile and exanthematic arthralgia syndrome closely resembling

dengue fever. Most often, signs and symptoms are maculopapular rash, fever, arthralgia, myalgia, headache, and conjunctivitis; edema, sore throat, cough, and vomiting occur less frequently (1). The virus, which was initially isolated from a rhesus monkey (*Macaca mulatta*) in 1947 in Uganda, has come to attention recently after a large outbreak occurred in the western Pacific region, including French Polynesia, New Caledonia, Easter Island, and the Cook Islands (2). Travel-related imported infections have thus been increasingly reported from the western Pacific and sporadically also in travelers to other regions of the world, including Thailand, Indonesia, and Senegal (2,3). ZIKV is transmitted by different *Aedes* mosquito species, and nonhuman primates play a role as reservoirs (1). After the beginning of the ZIKV epidemic in late 2013, a 20-fold increase of Guillain-Barré syndrome incidence was noted in French Polynesia; 1 patient was infected a week before neurologic symptoms started (4). We report an acute ZIKV infection in a traveler returning from Malaysian Borneo who experienced bilateral hearing difficulties during the course of illness.

On September 1, 2014, a 45-year-old woman was seen in an outpatient clinic in Heidelberg, Germany for fever of up to 39°C and maculopapular rash covering her trunk, arms, and legs. Fever had started on August 30, which was 6 days after she had returned from a 3-week vacation to peninsular Malaysia and Sabah, Malaysian Borneo. Laboratory analyses showed a slightly elevated C-reactive protein level of 5.2 mg/L (reference range <5.0), but liver function test and complete blood count results were within reference range. During the next 3 days, the fever subsided, but the patient experienced a sore throat, bilateral conjunctivitis, and a burning sensation of the palms and soles. These symptoms were accompanied by swelling of the hands and increasing arthralgia of the wrists, palms, and fingers. There was no lymphadenopathy. An indirect immunofluorescence assay for ZIKV (3) demonstrated an IgM titer of 1:640 and an IgG titer of 1:320 (cutoff <1:20) on day 6 of illness (Figure). An indirect immunofluorescence assay for dengue virus demonstrated an IgG titer of 1:80 and no IgM (cutoff <1:20).

Two days later, the patient experienced sudden bilateral dull and metallic hearing; in her left ear, she experienced a very short delay between a sound and her perception of the sound. Follow-up ZIKV serologic testing on day 11 of illness showed a decreased IgM titer of 1:160 and an increased IgG titer of 1:2,560 (Figure). Viral neutralization testing (3) of the same sample demonstrated the presence of ZIKV-specific neutralizing antibodies. Chikungunya virus serology results were negative. An archived serum sample from day 3 of illness studied by ZIKV serology and a ZIKV-specific real-time reverse transcription PCR (3) was negative (Figure). Hearing difficulties lasted for 10 days and resolved gradually (Figure).

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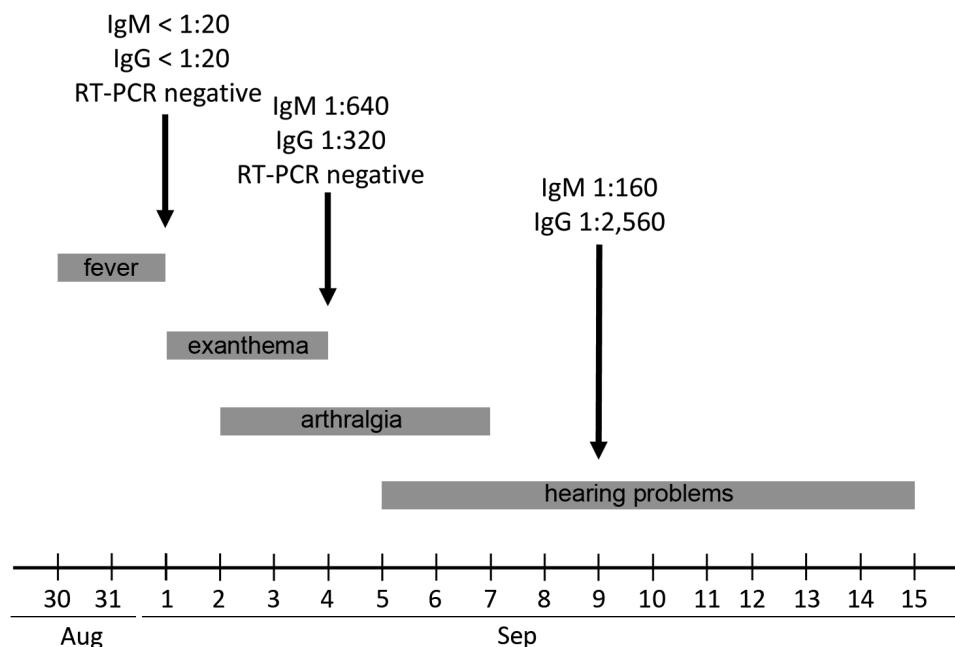


Figure. Clinical course and laboratory results (reverse transcription-PCR [RT-PCR]) for a patient with Zika virus (ZIKV) infection acquired from Malaysian Borneo. Length of gray box indicates duration of symptom.

During her journey to several cities and villages in Sabah, Malaysian Borneo, the patient had noticed several mosquito bites even though she had used repellents. She had stayed in hotels, private homes, and remote church homes under various conditions (online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/14-1960-Techapp1.pdf>).

In Asia, Zika fever has been described sporadically in Cambodia, Thailand, and Indonesia (Java and Lombok) (1,3,5,6). On the basis of the incubation time of ≈ 6 days in returning travelers (2,3), we assumed that the patient became infected in Keningau or surrounding villages, in northern Borneo. Although ZIKV was detected in *Ae. aegypti* mosquitoes in peninsular Malaysia in 1969 (7) and antibodies against ZIKV were demonstrated in serum samples from 15 of 79 patients on peninsular Malaysia and 9 of 50 patients in Borneo in 1953 (8), Zika fever in peninsular Malaysia or Borneo has not been reported. In 2001, ZIKV seropositivity was demonstrated in a native Bornean, 2 migrants to Borneo, and 2 Bornean orangutans (*Pongo pygmaeus*) (9). A later study found an additional 8 Bornean orangutans to be seropositive for antibodies against ZIKV (10). Thus, in Borneo, either the virus only rarely infects humans or the disease is mistaken for dengue fever.

Neurologic complications of ZIKV infections had previously been reported only as Guillain-Barré syndrome, and hearing difficulties in Zika fever patients have not been reported. Because this symptom resolved spontaneously, no audiometry or auditory brainstem response testing was performed, and the cause of the disorder remains unclear. Because of increasing travel and migration and heightened clinical and laboratory awareness, more ZIKV infections are likely to be diagnosed outside of epidemic events.

Acknowledgments

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Ebola and Psychological Stress of Health Care Professionals

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To the Editor: Providing medical care for Ebola virus-infected patients entails physical and psychological stress, extended shift times, and risk for infection. In addition, the wearing of personal protective equipment impairs communication and performance of diagnostic and therapeutic procedures. Lessons learned from outbreaks of other infectious diseases indicate that such challenging treatment environments require the monitoring of health care professionals for psychological distress (e.g., anxiety, depression, fatigue, and social isolation) to prevent personal exhaustion and reduced job performance (1).

In August 2014, the first patient in Germany known to have Ebola virus disease was admitted to the University Medical Center Hamburg-Eppendorf (2) and received treatment in the isolation facility for 18 days. We hypothesized that health care professionals working in the isolation unit who had direct contact with the Ebola patient would show more signs of psychological distress than those not working in the isolation unit.

To test our hypothesis, we conducted a cross-sectional controlled study by using validated self-report scales (1,3–5) and open-response questions. Seven days after the Ebola patient was admitted, we distributed questionnaires to the 46 health care professionals (17 physicians, 29 nurses) who had direct contact with the patient (Table).

Of the 46 health care professionals, 30 participated in the study. During patient contact, these staff members wore

Astro-Protect pressurized suits (Asatex, Bergheim, Germany). As a control group, 40 health care professionals from other wards in the same department were recruited and participated in the study. Providers in the control group cared for terminally ill patients and for patients with reduced consciousness, but they had no direct contact with the Ebola patient. The control participants were not recruited from intensive care units because, at the time of the study, the patient was not receiving intensive care treatment. The 2 groups were balanced with respect to age and occupational characteristics (Table). There was no special psychological support service for health care workers in this hospital. Staff members had received mandatory biweekly training, which included decontamination procedures, technical aspects of diagnostic procedures, and emergency care.

In contrast to our hypothesis, no significant differences emerged between the 2 groups with respect to the severity of somatic symptoms, anxiety, depression, and fatigue (Table). Moreover, mean total scores for both groups were at a comparable level to mean scores for the general population (3–5). However, health care professionals who had direct contact with the Ebola patient reported significantly greater social isolation and felt significantly more need for shorter shift hours. The open responses of participants who experienced social isolation suggested that their spouses, children, and other relatives had infection-related concerns. Additionally, half of the participants who did not have direct patient contact reported feeling a need for psychological preparation (Table). Nevertheless, almost all health care professionals (97% of those with direct patient contact, 93% of those without direct patient contact) believed that the health care facilities of the hospital were safe.

Our investigation of the psychological stress of health care professionals in a Western tertiary care center showed that a well-trained and dedicated team can cope well with the stress of caring for a severely ill Ebola patient. Of note, the direct patient contact group tended to comprise more male participants and more participants living with partners, which may have influenced the experience of psychological stress. No staff member refused to participate in the treatment of the Ebola patient, which underlines the high level of motivation within the team and may render direct comparison to other centers difficult.

While the patient was in the isolation unit, working shifts lasted up to 12 hours, consisting of 2 periods with 3–4 hours of work while wearing personal protective equipment in addition to time spent disinfecting. Most respondents felt that these shifts were too long. We therefore suggest that shift durations should be decreased to 8 hours comprising 2 blocks of 2 hours each for direct patient contact. Shorter shifts should improve staff satisfaction with the working conditions and potentially increase the personal safety of all health care personnel involved in direct patient contact.

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Table. Demographic characteristics, self-reported symptoms, and evaluation of working conditions of health care professionals with and without direct contact with an Ebola patient, Germany, 2014*

Characteristic	Health professionals		Group differences	
	With direct patient contact, n = 30†	Without direct patient contact, n = 40‡	t-value or odds ratio§	p value
Demographic				
Mean (SD) age, y	38 (8.3)	35 (9.9)	1.10	0.29
Male sex, no. (%)	16 (55)	12 (30)	0.35	0.05
Living with partner, no (%)	22 (76)	22 (55)	0.39	0.08
Occupation, no. (%)				
Physician	9 (31)	11 (28)	0.89	1.00
Nurse	19 (66)	26 (65)		
Self-report scale, mean (SD)¶				
Somatic symptom severity, SSS-8	5.03 (3.4)	4.74 (4.9)	0.30	0.77
Anxiety severity, GAD-7	2.43 (2.7)	2.41 (2.0)	0.03	0.98
Depression severity, PHQ-9	3.52 (3.3)	3.38 (3.0)	0.18	0.86
Fatigue symptoms, Facit	12.88 (9.1)	13.32 (8.1)	-0.20	0.84
Social isolation	0.62 (0.9)	0.00 (0.0)	3.70	<0.001
Evaluation of working conditions, no. (%)				
Had confidence in health care facilities	29 (97)	26 (93)	2.20	0.61
Desired psychological preparation	7 (26)	16 (52)	0.33	0.06
Desired shorter shift durations	16 (70)	5 (28)	5.70	0.01
Experienced treatment with Ebola patient as an exceptional circumstance	22 (85)	18 (64)	2.99	0.12

*Facit, Functional Assessment of Chronic Illness Therapy; GAD-7, Generalized Anxiety Disorder Scale-7; PHQ-9, Patient Health Questionnaire-9; SSS-8, Somatic Symptom Scale-8.

†Because of missing values, no. patients varied between 26 and 30.

‡Because of missing values, no. patients varied between 37 and 40.

§t-values (and corresponding p values) for continuous data and odds ratios (and corresponding p values) for categorical data.

¶Mean (SD) of total scores. Higher means indicate more severe symptoms.

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Enterovirus D68–Associated Acute Respiratory Distress Syndrome in Adult, United States, 2014

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To the Editor: Each year, nonpolio enteroviruses cause 10–15 million infections in the United States (1). Enterovirus D68 (EV-D68) is an uncommon strain of

nonpolio enterovirus that emerged in Illinois and Missouri in August 2014 in association with severe respiratory infections in children and spread across the United States (2). On August 23, 2014, the infection control department for Comer's Children's Hospital at the University of Chicago initially notified the Centers for Disease Control and Prevention of an increased number of children hospitalized with unusually severe respiratory illness (3). From mid-August to December 4, 2014, there were 1,121 laboratory-confirmed cases of EV-D68 in the United States (2). Almost all EV-D68 infections have occurred in children, many of whom had a history of asthma or wheezing (2).

One day before the first report (August 22, 2014), a 26-year-old obese woman with an unremarkable medical history was transferred to the medical intensive care unit at Saint Francis Medical Center, a tertiary care medical center in Peoria, Illinois, USA, with severe acute respiratory distress syndrome (ARDS). The transfer was from a nearby community hospital where she had sought care 4 days earlier for influenza-like symptoms consisting of cough, wheezing, progressive shortness of breath, nausea, and vomiting. In the community hospital emergency department, she mentioned that 2 children at home had similar symptoms and that her mother had recently been hospitalized with an acute respiratory illness. Despite treatment with supplemental oxygen, nebulized albuterol, and intravenous antimicrobial drugs for community-acquired pneumonia, her condition deteriorated, and she was intubated on hospital day 2, after which the antimicrobial drug treatment was changed from intravenous ceftriaxone and azithromycin to intravenous vancomycin and piperacillin/tazobactam. Results of bronchoscopy performed on hospital day 4 were unremarkable, and bacterial cultures of alveolar lavage samples were negative.

Her transfer to St. Francis Medical Center was prompted by persistent mechanical ventilation requirements of 100% fraction of inspired oxygen; positive end-inspiratory pressure of 12 mm/H₂O consistent with classic ARDS (hypoxemia, indicated by a ratio of arterial oxygen partial pressure to fractional inspired oxygen <200 mm Hg); and bilateral infiltrates on chest radiograph (Figure) without evidence of left heart failure (4). On hospital day 8 (cumulative), a nasopharyngeal swab sample was tested by FilmArray Respiratory Panel multiplex PCR (BioFire Diagnostics, Salt Lake City, UT, USA); results were positive for rhinovirus/enterovirus. That day, intravenous methylprednisolone therapy was initiated.

During a prolonged hospital stay, the patient required mechanical ventilation for 32 days, underwent a second bronchoscopic evaluation, required a percutaneous tracheostomy (and subsequent decannulation), and underwent endoscopic gastrostomy tube placement (and removal). She

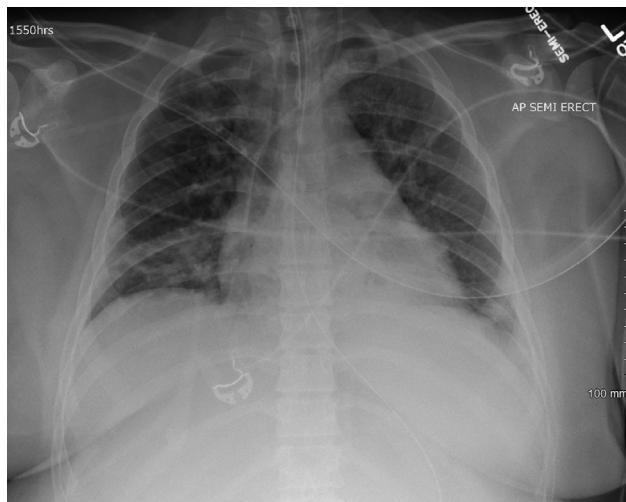


Figure. Chest radiograph obtained (with portable machine) of semirecumbent adult patient with enterovirus D68–associated acute respiratory distress syndrome on hospital day 3.

was discharged from the hospital after 55 days and ultimately recovered completely.

To determine the etiology of the clinical syndrome for the patient reported here, molecular diagnostic testing of respiratory tract clinical specimens was required. Institutional review board approval was obtained for molecular diagnostics and sequencing of the patient's nasopharyngeal swab specimens and bronchoalveolar lavage (BAL) fluid samples. The FilmArray platform is capable of detecting enteroviral infections caused by EV-D68 but cannot differentiate between rhinoviruses and enteroviruses (5). Confirmation of EV-D68 requires EV-D68–specific PCR (6).

A novel, research-based diagnostic modality that is capable of rapid identification of viral pathogens directly from clinical specimens is the combination of PCR and electrospray ionization mass spectrometry (ESI-MS) (7), which was instrumental in early recognition of the novel pandemic strain of influenza A(H1N1) virus that emerged in 2009 (8). For a variety of viral pathogens, PCR/ESI-MS sensitivity is 94% and specificity is 98% (9). In this case, PCR/ESI-MS detected a human enterovirus from the right middle lobe and left lingular segment BAL fluid samples. For the assay, 2 primer pairs were used; both confirmed the presence of human enterovirus, but only 1 matched the signatures for EV-D68.

For confirmation, we pursued testing with EV-D68–specific PCR, which was performed by the Special Projects Laboratory of the Washington University Department of Pediatrics. This assay amplifies a segment of the viral protein 1 gene, which enables discrimination of EV-D68 from other enteroviruses and rhinoviruses (K.M. Wylie et al., unpub. data). The nasopharyngeal swab sample and the right middle lobe and lingula BAL fluid specimens were positive for EV-D68.

PCR/ESI-MS of BAL fluid followed by EV-D68-specific PCR testing of 1 nasopharyngeal swab and 2 BAL fluid samples confirmed our clinical suspicion of ARDS secondary to EV-D68 in an adult. The patient's history of contact with sick family members and clinical signs (non-productive cough, nausea, and vomiting) were suggestive of a viral infection. Lessons learned from the emergence of swine-origin influenza A(H1N1)pdm09 virus and recognition (in the midst of the pandemic) that younger age and obesity were risk factors for severe disease were also suggestive of a viral respiratory infection.

We are developing a specific rapid molecular assay for EV-D68, which should help clinicians recognize when EV-D68 is present in the community. During those times, EV-D68 infection should be included in the differential diagnosis of severe respiratory infection. Documentation of EV-D68 infection may help with clinical management for individual patients and minimize unnecessary use of antimicrobial drugs within communities.

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Enterovirus D68–Associated Severe Pneumonia, China, 2014

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To the Editor: Over the past 4 years, outbreaks caused by enterovirus type D68 (EV-D68) infection have occurred in many parts of the world (1); this virus can cause severe respiratory tract infections (RTIs) in children. This public health concern has been boosted by the recent outbreaks of EV-D68 infection in the United States (<http://www.cdc.gov/non-polio-enterovirus/outbreaks/EV-D68-outbreaks.html>). Outbreaks associated with novel EV-D68 have also been reported during 2006–2012 in China (2,3). However, since 2012, no EV-D68 infections in China have been reported. Whether the EV-D68 outbreaks in the United States affected those in China is unclear. Continuous characterization of EV-D68 epidemics is therefore necessary for purposes of early alert and for facilitating control measure decisions.

To determine EV-D68 prevalence in China, we screened for EV-D68 infections in 2014 in Beijing, China. We tested patients with RTI during August–November 2014, reported by the Respiratory Virus Surveillance System, established by Beijing Center for Disease Prevention and Control. The System covers 30 sentinel hospitals in all 16 districts of Beijing. We obtained 1,478 clinical specimens (1,034 nasopharyngeal swab and 444 sputum). Patient ages ranged from 8 months to 93 years (median 33.5 years, mean 37.9 years). Enteroviruses and other known respiratory viruses were detected by real-time PCR (4). A total of 70 enterovirus-positive samples were identified. Other respiratory viruses detected were 89 rhinoviruses, 87

influenza viruses, 70 human parainfluenza viruses (types 1–4), 43 human coronaviruses, 26 respiratory syncytial viruses, 29 adenoviruses, 9 bocaviruses, and 2 metapneumoviruses. Among the EV-positive samples, 1 was positive for EV-D68 according to PCR amplification of the viral protein 1 (VP1) gene (5); no other respiratory viruses were detected in this patient.

The EV-D68-positive patient was a 5-year-old girl with no underlying disease. On August 5, 2014, she had fever (highest temperature 40°C), cough, breathing difficulty, abdominal pain, diarrhea, and vomiting. Her condition deteriorated, and on August 11, she was hospitalized in Beijing Children's Hospital with a diagnosis of severe

pneumonia. A nasopharyngeal swab sample was collected at the time of admission. She was released on August 16, after receiving symptomatic supportive treatment in the general ward; she had not required mechanical ventilation. She and her family had no history of travel in the months before she became ill.

To further characterize this virus strain, Beijing-R0132, we amplified the genome sequence directly from a nasopharyngeal swab sample by using overlapping primers designed according to the reference sequence (GenBank accession no. KM892501; online Technical Appendix, <http://wwwnc.cdc.gov/EID/article/21/5/15-0036-Techapp1.pdf>). The termini of the genome were independently

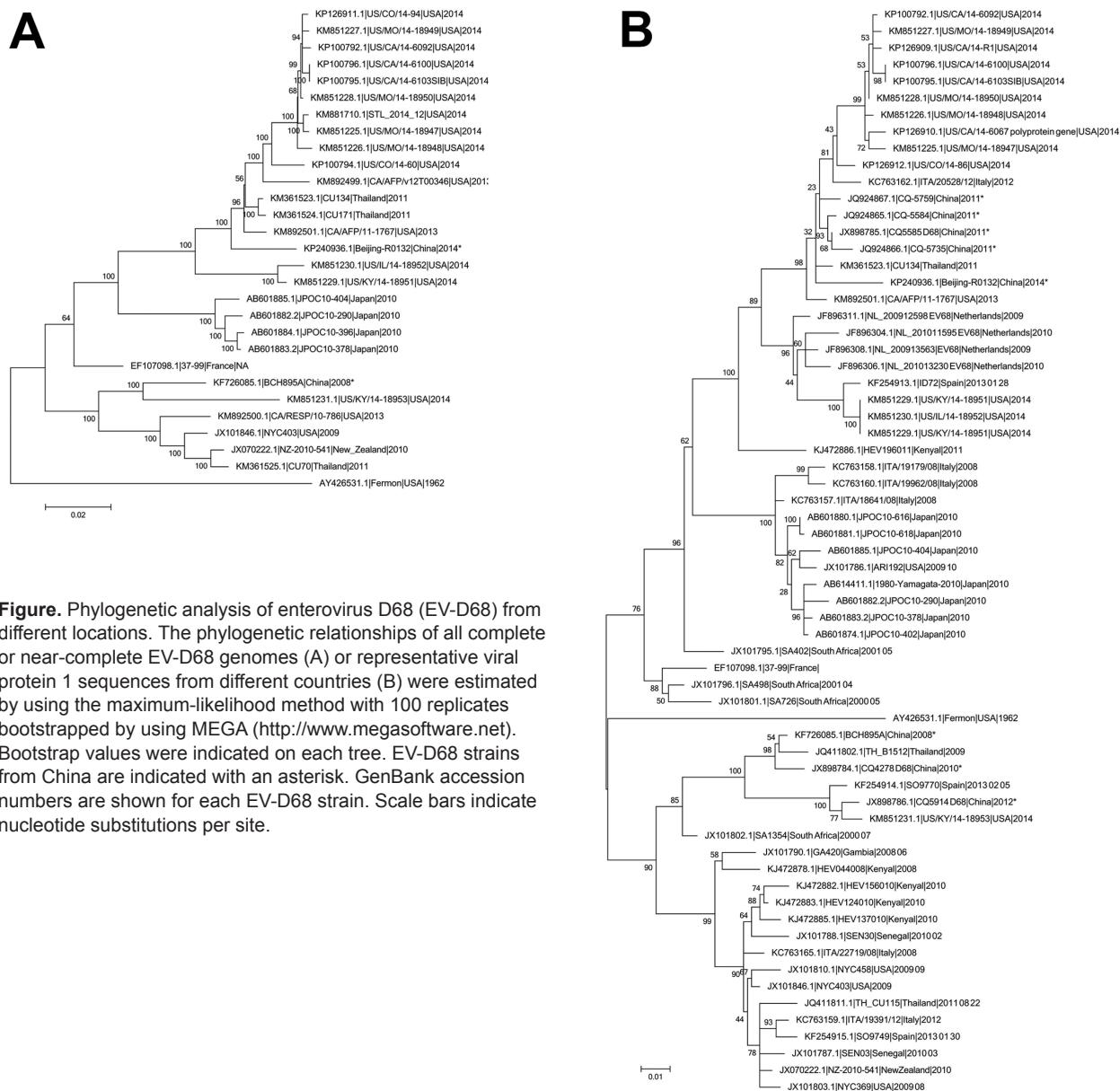


Figure. Phylogenetic analysis of enterovirus D68 (EV-D68) from different locations. The phylogenetic relationships of all complete or near-complete EV-D68 genomes (A) or representative viral protein 1 sequences from different countries (B) were estimated by using the maximum-likelihood method with 100 bootstrapped replicates by using MEGA (<http://www.megasoftware.net>). Bootstrap values were indicated on each tree. EV-D68 strains from China are indicated with an asterisk. GenBank accession numbers are shown for each EV-D68 strain. Scale bars indicate nucleotide substitutions per site.

determined by using the RACE System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Each amplicon was sequenced 4 times by using the Sanger method, and the forward and reverse sequences agreed well. Sequences were assembled by using DNA-Star software (Lasergene, Madison, WI, USA) and deposited in GenBank (accession no. KP240936). The genome of Beijing-R0132 was 7,334 nt long, including 699 nt in 5'-untranslated regions (5'-UTRs), 6,567 nt in open reading frame (ORF), and 68 nt in 3'-UTR. Beijing-R0132 shared 96% nt sequence identity with the virus circulating in the United States in 2014, US/CO/14-60. In contrast to the prototype EV-D68 (Fermon strain, AY426531), deletions of the CTCAAAACCTCCAGTACATAACA sequence in the 5'-UTR and TTATTTATAACA sequence in the front of the ORF of Beijing-R0132 were observed, corresponding with nt 682–704, and nt 721–732 of the Fermon strain, which were similar to those identified in the United States in 2014.

We then used MEGA software version 6.06 (<http://www.megasoftware.net>) to analyze the phylogeny of the whole genome and the VP1 gene with EV-D68 sequences available in GenBank (Figure). Beijing-R0132 was clustered with most of the EV-D68 strains that circulated throughout the United States during 2014. The strains identified from Beijing in 2008, represented by BCH895A/2008, belong to another distinct lineage according to genome phylogeny (Figure, panel A). Similar relationships were observed in the phylogenetic tree of the VP1 gene (Figure, panel B). Beijing-R0132 and some EV-D68 strains from China identified in 2011 grouped with most of the strains obtained from the United States in 2014. These findings demonstrate that the EV-D68 strain circulating in Beijing was closely related to strains circulating in the United States.

The origin of the 2014 EV-D68 outbreaks in the United States is unclear. This Beijing-R0132 genome sequence provides information for tracking EV-D68 as it spreads throughout the world and for evaluating the sequence diversity in circulating EV-D68 strains. In contrast to EV-D68

detection during the outbreaks in the United States, positive detection of EV-D68 in this study was limited, although the circulating virus strains were closely related. The reason for this disparity warrants further investigation. However, the severe pneumonia caused by EV-D68 reported here underlies the need for intensive attention to surveillance and control of EV-D68 in vulnerable populations, such as young children. In addition, for a megacity with very high population density and mobility, such as Beijing, the city-wide implementation of the Respiratory Virus Surveillance System is critical for monitoring the epidemic or potential outbreaks of EV-D68 and other respiratory viruses and for enabling early warning.

Acknowledgment

We thank sentinel hospitals of the Respiratory Virus Surveillance System in Beijing for collecting samples and investigating cases.

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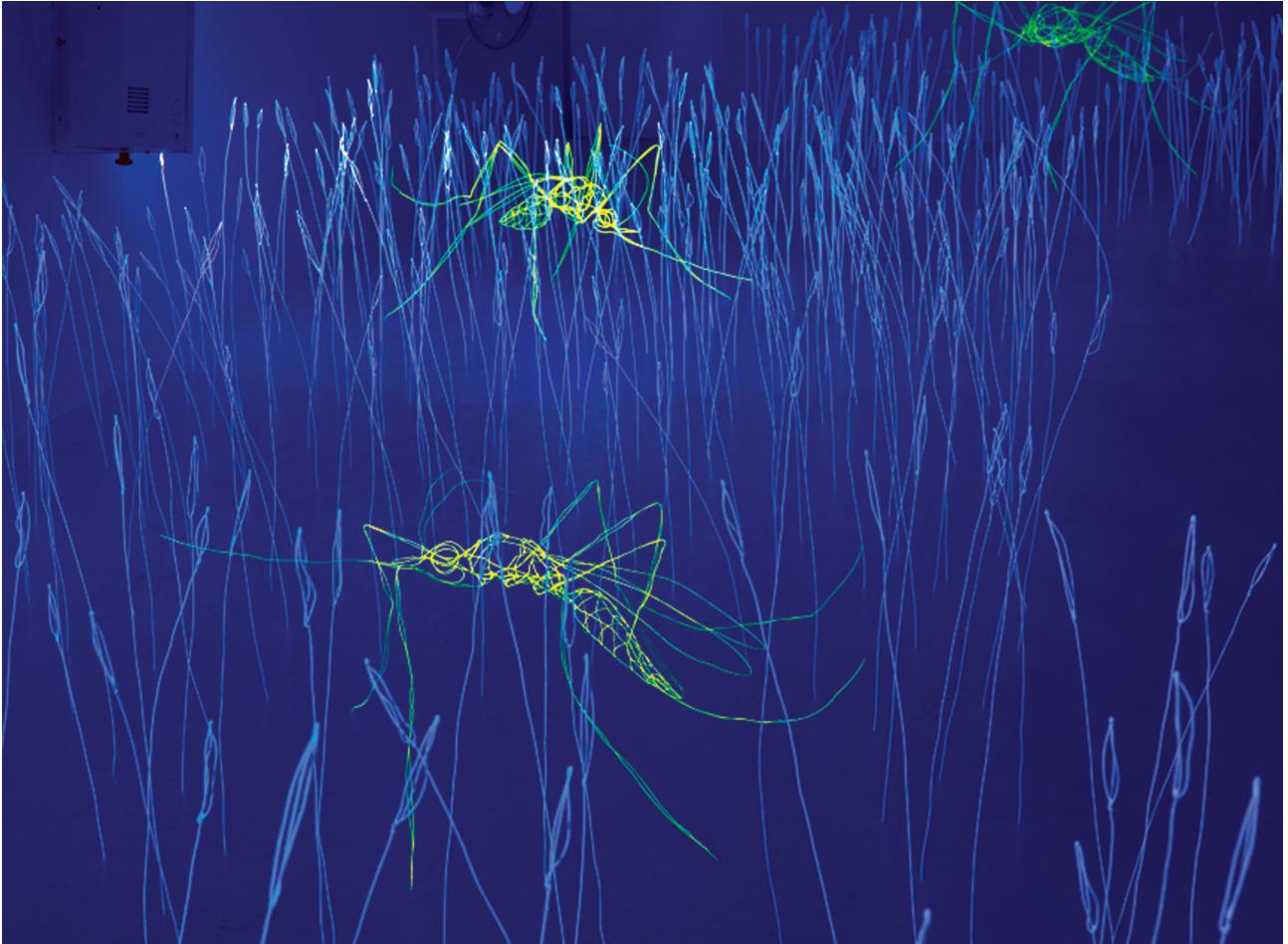
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Correction: Vol. 21, No. 4

The Continuing Medical Education quizzes for 2 articles were inadvertently omitted from the April issue. The quizzes are printed at the back of this issue, and

the complete articles and quizzes are available online (http://wwwnc.cdc.gov/eid/article/21/4/14-1479_intro and http://wwwnc.cdc.gov/eid/article/21/4/14-1033_intro).



Stefan Waibel (1970–) *Ideal Nature Machine*, 2012. Installation (metal wire, epoxy resin paint, UV light, wind machines). Gallery Mauroner Vienna and Salzburg, Austria (<http://www.galerie-mam.com/>).

The Mosquito—a Cog in the Ideal Nature Machine

Carmen C.H. Petrosian-Husa and Byron Breedlove

“You stagger as well as you may.
Your own imponderable weightlessness
Saves you, wafts you away on the very
draft my anger makes in its snatching.”

—The Mosquito by D.H. Lawrence

The installation *Ideal Nature Machine* by Austrian artist Stefan Waibel is a minimalistic depiction of a piece of turf where several mosquitoes are resting among the

grass blades. The installation can be adjusted to fit different rooms and can measure from 5 to 350 square meters depending on the venue. Its presentation in a dark setting that is illuminated by UV light emphasizes the work’s artificiality; wind machines, which cause the grass blades and the mosquitoes to slightly sway, make the work seem more lifelike. (Installations built for an outdoor presentation are made of sturdier materials than are those built for indoor exhibition and do not use the wind machine because the grass blades and mosquitoes are naturally exposed to wind and weather.)

The noise from the wind machines adds to the illusion of a hot night, when the whizzing sound of a single mosquito can escalate from awareness to annoyance. The

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dark surroundings paired with the intensive colors created by the UV light suggest the sweltering atmosphere of a tropical night, the kind of night when a layer of heat and humidity covers everything and a fan stirring the air cannot prevent beads of perspiration from developing on the skin. This installation, thus, evokes not only nature but also those natural sensations.

The grass blades are minimalistic but measure about half a meter, so they are just as tall as those in an unmown meadow. The mosquitoes, though, measure ≈ 1.5 m and are 300 times as large as the actual average (5 mm) insect. In *Ideal Nature Machine*, Waibel does not depict any other animals, just the mosquitoes, but he invites the visitors to participate in his installations, to step in and walk around, and in this way to become part of it.

As viewers, we do not know among what kind of grass blades we are walking, nor do we know what kind of mosquitoes are hiding there. Perhaps these mosquitoes are resting, which they do for a large portion of their gonotrophic cycle (blood feeding, egg maturation, and oviposition, a cycle which is repeated several times throughout the life of adult females). These are likely exophytic mosquitoes, spending this resting period outside human dwellings, usually preferring shady areas such as among tall grass blades.

Nature may be viewed as an ideal, but multifaceted machine that functions without concern for consequences. Mosquitoes are an efficient and even graceful component; they provide food for bats and other insectivorous animals. Some mosquitoes are effective vectors that contribute to the spread of disease-causing agents that have serious and widespread consequences for humans. Mosquitoes transmit five species of *Plasmodium* parasites that cause malaria in humans and a constellation of infectious agents that can cause yellow fever, West Nile virus disease, chikungunya virus infection, Rift Valley fever, Japanese encephalitis, lymphatic filariasis, and dengue hemorrhagic fever.

Stefan Waibel regards mosquitoes as the plankton of the air, thus, codifying their abundance as a cog within

nature's and the machine's cogwheels. Waibel's series of Ideal Nature Machines installations underscore that the "Machine Nature" would not work without its myriad cogs and components, mosquitoes included. Efforts to track, study, and prevent diseases by controlling mosquitoes and other vectors, as described in many of this issue's articles, also underscore the complex, intertwined relationships that fuel creative thought in both art and science.

In 1970 Stefan Waibel was born in Lustenau, Province Vorarlberg, Austria. Because of an accident, he had to give up his goal of becoming a musician (organist). He instead became a commercial trainee and had experience as a social worker and in some other fields. In 1994 he started to study at the Academy of Fine Arts in Vienna, finishing in 1999 with a diploma in painting and graphic arts. Nature was always an important topic in his work. After completing his initial panel paintings, he started his first installations in 2007. In Vienna, his atelier is situated close to the river Danube. In the summer, his atelier is often inundated by mosquitoes, which—so the artist claims—are attracted by the linseed oil he uses for his paintings. Sometimes his canvases are covered in drowned and drowning mosquitoes to such an extent that he has to throw them away, which might explain his preoccupation with mosquitoes.

Carmen C.H. Petrosian-Husa is an independent anthropologist and art historian. Her areas of expertise include Asia and the South Pacific (especially Micronesia) and modern art.

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EMERGING INFECTIOUS DISEASES[®]

Upcoming Issue

Cost-effectiveness of Chlamydia Vaccination Programs for Young Women

Acquired Drug Resistance and Poor Outcomes among Patients with Multidrug-Resistant Tuberculosis

Ebola Risk Perception in Germany, 2014

ST-4821 Clonal Complex Serogroup B *Neisseria meningitidis* in China, 1978–2013

Global Burden of Invasive Nontyphoidal *Salmonella* Disease, 2010

Oral Cholera Vaccine Coverage, Barriers to Vaccination, and Adverse Events, Haiti, 2013

Risk Factors for Development of Drug Resistance during Multidrug-Resistant Tuberculosis Treatment, Russia, 2005–2008

Pneumonia Outbreak Caused by *Chlamydomphila pneumoniae* among US Air Force Academy Cadets, Colorado, USA

European Rabbits as Reservoir for *Coxiella burnetii*

Acute Middle East Respiratory Syndrome Coronavirus Infection in Livestock Dromedaries, Dubai, 2014

KPC and NDM-1 Genes in Related *Enterobacteriaceae* Strains and Plasmids from Pakistan and the United States

Salmonella enterica Serotype Enteritidis in French Polynesia, South Pacific, 2008–2013

Extensively Drug-Resistant New Delhi Metallo- β -Lactamase–Encoding Bacteria in the Environment, Bangladesh, October 2012

Mycobacterium bovis in Panama, 2013

Invasion Dynamics of White-Nose Syndrome, Midwestern United States, 2012–2013

MRSA *spa* t1081, a Highly Transmissible Strain Endemic to Hong Kong, in the Netherlands

Multibacillary Leprosy in an Active Duty Military Member

Bacteremia Associated with Myiasis Caused by *Lucilia sericata* Fly, United Kingdom

Reducing the Risk for Waterborne Nosocomial Neonatal Legionellosis

Fatal Nosocomial MDR TB Identified through Routine Genetic Analysis and Whole-Genome Sequencing

Complete list of articles in the June issue at
<http://www.cdc.gov/eid/upcoming.htm>

Upcoming Infectious Disease Activities

May 14–17, 2015

SHEA
The Society for Healthcare
Epidemiology of America
Orlando, FL, USA
<http://shea2015.org/attendees/registration/>

May 30–June 2, 2015

American Society for Microbiology
General Meeting
New Orleans, LA, USA
<http://gm.asm.org/>

August 10–21, 2015

14th International Dengue Course
Havana, Cuba
<http://instituciones.sld.cu/ipk/14thdenguecourse/>

August 24–26, 2015

ICEID
International Conference
on Emerging Infectious Diseases
Atlanta, GA, USA

August 29–September 2, 2015

IDBR
20th Annual Infectious Disease
Board Review Course
McLean, VA, USA
<http://smhs.gwu.edu/cehp/activities/courses/idbr>

August 6–8 2015

Third International Congress on Pathogens
at the Human-Animal Interface (ICOPHAI)
Chiang Mai, Thailand
Veterinary Public Health and Biotechnology
(VPH-Biotec)
Global Consortium
<http://icophai.org/>

September 17–21, 2015

ICAAC
Interscience Conference on Antimicrobial Agents
and Chemotherapy
San Diego, CA, USA

March 2–5, 2016

ISID
17th International Congress
on Infectious Diseases
Hyderabad, India

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Announcements may be posted on the journal Web page only, depending on the event date.

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Article Title Recent US Case of Variant Creutzfeldt-Jakob Disease—Global Implications

CME Questions

1. You are consulting for a public health department regarding vigilance for emergence of variant Creutzfeldt-Jakob disease (vCJD). According to the case report and review by Maheshwari and colleagues, which of the following statements about the clinical presentation of vCJD is correct?

- A. vCJD usually presents with rapidly progressive dementia
- B. Development of chorea, ataxia, or other neurologic manifestations supports a diagnosis of sporadic CJD (sCJD) or vCJD
- C. Painful sensory complaints are typically reported with sCJD but not vCJD
- D. Compared with sCJD, vCJD begins at an older age

2. According to the case report and review by Maheshwari and colleagues, which of the following diagnostic findings is most likely to occur in vCJD?

- A. Diffusion restriction and T2 hyperintensity of the cortex and basal ganglia on magnetic resonance imaging
- B. Periodic sharp wave complexes on electroencephalogram

- C. Elevated 14-3-3/Tau levels in cerebrospinal fluid
- D. Detection rate of PrPSc, indicating tonsil biopsy as alternative to brain biopsy

3. Which of the following statements about the global implications of the report by Maheshwari and colleagues of a new US case of vCJD would most likely be correct?

- A. The incidence of vCJD is increasing globally
- B. vCJD should not be diagnosed if the patient has not lived in a country with a known endemic case of vCJD
- C. Iatrogenic transmission of vCJD is unlikely
- D. Because the incubation period may be several decades, we can reasonably anticipate the international occurrence of additional vCJD cases

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree

1

2

3

4

Strongly Agree

5

2. The material was organized clearly for learning to occur.

Strongly Disagree

1

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3

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Strongly Agree

5

3. The content learned from this activity will impact my practice.

Strongly Disagree

1

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Strongly Agree

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4. The activity was presented objectively and free of commercial bias.

Strongly Disagree

1

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Strongly Agree

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Article Title

Animal-Associated Exposure to Rabies Virus among Travelers, 1997–2012

CME Questions

1. Your patient is a 52-year-old man planning a vacation to China. According to the study by Gautret and colleagues, which of the following statements about the demographic characteristics of travelers exposed to potentially rabid animals that might differentiate them from other ill travelers seeking medical care is correct?

- A. Most persons who reported to GeoSentinel requiring rabies postexposure prophylaxis (PEP) were middle-aged adults
- B. Most persons who reported to GeoSentinel requiring rabies PEP were business travelers visiting high-income regions
- C. This study showed that only children were at risk for animal bites requiring PEP
- D. Travelers exposed to potentially rabid animals did not have specific demographic characteristics differentiating them from other ill travelers seeking medical care

2. According to the study by Gautret and colleagues, which of the following travel-related characteristics is most likely to be associated with potential exposure to rabid animals?

- A. Long duration of stay
- B. Travel to South America
- C. Travel to Asia
- D. Travel during the winter

3. Which of the following statements about the clinical implications of findings from the study by Gautret and colleagues would most likely be correct?

- A. Regardless of demographics or length of stay, international travelers to Asia and other rabies-endemic regions should be informed about potential rabies exposure and pretravel vaccination
- B. Study findings support current recommendations from the US Centers for Disease Control and Prevention stating that preexposure rabies vaccine recommendations should be based, at least in part, on longer durations of stay
- C. Bats are the leading animal responsible for exposure among travelers
- D. Travelers exposed to nonhuman primates (NHPs) do not require rabies PEP because rabies cannot be transmitted from NHPs to humans

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	

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Article Title

Deaths Associated with Respiratory Syncytial and Influenza Viruses among Persons ≥5 Years of Age in HIV-Prevalent Area, South Africa, 1998–2009

CME Questions

1. Which of the following general trends was identified in respiratory deaths in South Africa in the current study?

- A. Gradual decline in the rate of respiratory deaths between 1998 and 2009
- B. Gradual increase in the rate of respiratory deaths between 1998 and 2009
- C. Sharp increase in the rate of respiratory deaths between 1998 and 2004, followed by a diminution
- D. Sharp increase in the rate of respiratory deaths maintained after 2004

2. Which of the following statements regarding death related to influenza infection in the current study is most accurate?

- A. The highest rates of death were found among young adults, who had the highest rates of concomitant HIV infection
- B. The presence of HIV was associated with a nearly 8-fold increase in the risk for influenza-related death

- C. Influenza A H1N1 particularly increased influenza-related mortality rates among older adults
- D. There was limited difference in the rate of influenza-related mortality based on age

3. Which of the following statements regarding mortality related to respiratory syncytial virus (RSV) in the current study is most accurate?

- A. Mortality rate related to RSV was highest among children and adolescents
- B. There was a spike in RSV-related mortality rate among individuals 75 years and older
- C. Concomitant HIV infection had an insignificant effect on the risk for RSV-related mortality
- D. Nearly 90% of individuals who died of RSV had concomitant HIV infection

Activity Evaluation

1. The activity supported the learning objectives.

Strongly Disagree

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Strongly Agree

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2. The material was organized clearly for learning to occur.

Strongly Disagree

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Strongly Agree

5

3. The content learned from this activity will impact my practice.

Strongly Disagree

1

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4

Strongly Agree

5

4. The activity was presented objectively and free of commercial bias.

Strongly Disagree

1

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Strongly Agree

5

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Title Page. Give complete information about each author (i.e., full name, graduate degree(s), affiliation, and the name of the institution in which the work was done). Clearly identify the corresponding author and provide that author's mailing address (include phone number, fax number, and email address). Include separate word counts for abstract and text.

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Text. Double-space everything, including the title page, abstract, references, tables, and figure legends. Indent paragraphs; leave no extra space between paragraphs. After a period, leave only one space before beginning the next sentence. Use 12-point Times New Roman font and format with ragged right margins (left align). Italicize (rather than underline) scientific names when needed.

Biographical Sketch. Include a short biographical sketch of the first author—both authors if only two. Include affiliations and the author's primary research interests.

References. Follow Uniform Requirements (www.icmje.org/index.html). Do not use endnotes for references. Place reference numbers in parentheses, not superscripts. Number citations in order of appearance (including in text, figures, and tables). Cite personal communications, unpublished data, and manuscripts in preparation or submitted for publication in parentheses in text. Consult List of Journals Indexed in Index Medicus for accepted journal abbreviations; if a journal is not listed, spell out the journal title. List the first six authors followed by "et al." Do not cite references in the abstract.

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Figures. Submit editable figures as separate files (e.g., Microsoft Excel, PowerPoint). Photographs should be submitted as high-resolution (600 dpi) .tif or .jpeg files. Do not embed figures in the manuscript file. Use Arial 10 pt. or 12 pt. font for lettering so that figures, symbols, lettering, and numbering can remain legible when reduced to print size. Place figure keys within the figure. Figure legends should be placed at the end of the manuscript file.

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Perspectives. Articles should not exceed 3,500 words and 40 references. Use of sub-headings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles should provide insightful analysis and commentary about new and reemerging infectious diseases and related issues. Perspectives may address factors known to influence the emergence of diseases, including microbial adaptation and change, human demographics and behavior, technology and industry, economic development and land use, international travel and commerce, and the breakdown of public health measures.

Synopses. Articles should not exceed 3,500 words and 40 references. Use of sub-headings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. This section comprises concise reviews of infectious diseases or closely related topics. Preference is given to reviews of new and emerging diseases; however, timely updates of other diseases or topics are also welcome.

Research. Articles should not exceed 3,500 words and 40 references. Use of sub-headings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Report laboratory and epidemiologic results within a public health perspective. Explain the value of the research in public health terms and place the findings in a larger perspective (i.e., "Here is what we found, and here is what the findings mean").

Policy and Historical Reviews. Articles should not exceed 3,500 words and 40 references. Use of subheadings in the main body of the text is recommended. Photographs and illustrations are encouraged. Provide a short abstract (150 words), 1-sentence summary, and biographical sketch. Articles in this section include public health policy or historical reports that are based on research and analysis of emerging disease issues.

Dispatches. Articles should be no more than 1,200 words and need not be divided into sections. If subheadings are used, they should be general, e.g., "The Study" and "Conclusions." Provide a brief abstract (50 words); references (not to exceed 15); figures or illustrations (not to exceed 2); tables (not to exceed 2); and biographical sketch. Dispatches are updates on infectious disease trends and research that include descriptions of new methods for detecting, characterizing, or subtyping new or reemerging pathogens. Developments in antimicrobial drugs, vaccines, or infectious disease prevention or elimination programs are appropriate. Case reports are also welcome.

Another Dimension. Thoughtful essays, short stories, or poems on philosophical issues related to science, medical practice, and human health. Topics may include science and the human condition, the unanticipated side of epidemic investigations, or how people perceive and cope with infection and illness. This section is intended to evoke compassion for human suffering and to expand the science reader's literary scope. Manuscripts are selected for publication as much for their content (the experiences they describe) as for their literary merit. Include biographical sketch.

Letters. Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research, are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 figure or table and should not be divided into sections. No biographical sketch is needed.

Commentaries. Thoughtful discussions (500–1,000 words) of current topics. Commentaries may contain references (not to exceed 15) but no abstract, figures, or tables. Include biographical sketch.

Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

Etymologia. Etymologia (100 words, 5 references). We welcome thoroughly researched derivations of emerging disease terms. Historical and other context could be included.

Announcements. We welcome brief announcements of timely events of interest to our readers. Announcements may be posted online only, depending on the event date. Email to eideditor@cdc.gov.