Vol 22, No 2, February 2016

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February 2016

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Pages 193–364

Edward Epp (b. 1950), From the Randal Street Apartment—to the South East—Monrovia, 1981. Watercolor on paper, 22 in x 30 in/55.8 cm x 76.2 cm. Digital image courtesy of Marion Scott Gallery/Kardosh Projects, Vancouver, British Columbia, Canada.

EMERGING INFECTIOUS DISEASES™

Emerging Infectious Diseases is published monthly by the Centers for Disease Control and Prevention, 1600 Clifton Road, Mailstop D61, Atlanta, GA 30329-4027, USA. Telephone 404-639-1960, fax 404-639-1954, email eideditor@cdc.gov.

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

EMERGING INFECTIOUS DISEASES™

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On the Cover

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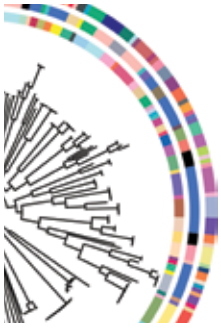
Medscape EDUCATION ACTIVITY



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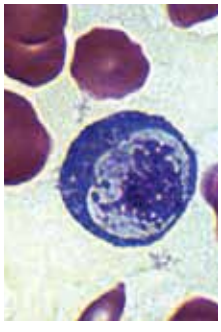

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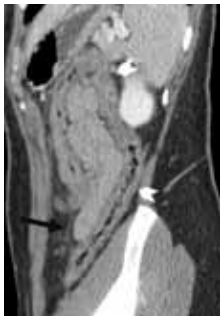

About the Cover

Etymologia

Correction

Ebola and Its Control in Liberia, 2014–2015

Tolbert G. Nyenswah, Francis Kateh, Luke Bawo, Moses Massaquoi, Miatta Gbanyan, Mosoka Fallah, Thomas K. Nagbe, Kollie K. Karsor, C. Sanford Wesseh, Sonpon Sieh, Alex Gasasira, Peter Graaff, Lisa Hensley, Hans Rosling, Terence Lo, Satish K. Pillai, Neal Gupta, Joel M. Montgomery, Ray L. Ransom, Desmond Williams, A. Scott Laney, Kim A. Lindblade, Laurence Slutsker, Jana L. Telfer, Athalia Christie, Frank Mahoney, Kevin M. De Cock

The severe epidemic of Ebola virus disease in Liberia started in March 2014. On May 9, 2015, the World Health Organization declared Liberia free of Ebola, 42 days after safe burial of the last known case-patient. However, another 6 cases occurred during June–July; on September 3, 2015, the country was again declared free of Ebola. Liberia had by then reported 10,672 cases of Ebola and 4,808 deaths, 37.0% and 42.6%, respectively, of the 28,103 cases and 11,290 deaths reported from the 3 countries that were heavily affected at that time. Essential components of the response included government leadership and sense of urgency, coordinated international assistance, sound technical work, flexibility guided by epidemiologic data, transparency and effective communication, and efforts by communities themselves. Priorities after the epidemic include surveillance in case of resurgence, restoration of health services, infection control in healthcare settings, and strengthening of basic public health systems.

In Liberia, Ebola virus disease was first reported from Lofa County on March 30, 2014, a week after cases in Guinea had been reported (*1*–*3*) (Figure 1). Additional cases in May and June heralded the country's severe outbreak (*4*). Events in Liberia drew widespread attention to Ebola as a threat to global health security (*5*) including urbanization of the disease; first-ever infections in expatriate health workers (*6*); international spread to Nigeria, the United States, and Spain with secondary transmission (*7*–*9*); and mathematical model estimates of a future high case load (*10*).

On August 4, 2014, the US ambassador to Liberia declared a disaster; on August 6, the president of Liberia declared a state of emergency; and on August 8, the World Health Organization (WHO) called Ebola in West Africa a public health emergency of international concern (*11*). Ten months later, on May 9, 2015, WHO declared Liberia free of Ebola virus transmission (*12*). However, on June 29, 2015, a postmortem diagnosis of Ebola was made for a 17-year-old boy, and 5 other cases were subsequently confirmed, but no further spread was noted. Liberia was again declared free of Ebola on September 3, 2015 (*13*). We describe the Ebola experience in Liberia and draw conclusions relevant to future responsiveness.

Incident Management System and Coordination of the International Response, July–September, 2014

The government of Liberia initially set up a diverse Ebola Task Force, whose large size and organizational challenges handicapped its effectiveness. In late July 2014, supported by the US Centers for Disease Control and Prevention (CDC), WHO, and other partners, the Liberia Ministry of Health and Social Welfare (MOHSW) implemented an Incident Management System (IMS) with an incident manager devoted exclusively to Ebola (*14*). The IMS ensured streamlined management, clear authority and accountability, structured working groups, and operational follow-up. In September 2014, the IMS moved into an emergency operations center, a location for coordination and oversight of all operations. The incident manager had a deputy empowered to deal with logistics and operational issues and, eventually, an inner core of advisors (including persons from WHO, CDC, the US Agency for International Development, and the UN Mission for Ebola Emergency Response) who conferred daily to coordinate activities. International partners co-chaired IMS technical work groups: case management, contact tracing, safe burials, surveillance, laboratory, and social mobilization (Figure 2). The president of

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DOI: http://dx.doi.org/10.3201/eid2202.151456

Figure 1. Locations of Ebola case-patients and associated facilities, Liberia, 2014–2015. ELWA, Eternal Love Winning Africa; EOC, emergency operations center; ETU, Ebola treatment unit; JFK, John Fitzgerald Kennedy; MoD, Ministry of Defense.

Liberia interacted directly with the incident manager. Separately, the president convened the Presidential Advisory Committee on Ebola, a small group of senior officials and international partners who provided advice about sensitive matters and policy.

Surveillance, Epidemiology, and Laboratory Diagnosis, July 2014–May 2015

An early priority for directing the response was surveillance. Using WHO case definitions for suspected, probable, and confirmed cases, the 15 counties of Liberia reported Ebola cases to MOHSW. Reporting modalities included case investigation forms; mobile phone, text, and email messages; reports from Ebola Treatment Units (ETUs); laboratory results; and reports from burial teams, case investigators, and contact tracers. Timely reconciliation of data from multiple sources proved challenging. Constraints included shortage of trained staff, lack of communication and information technology, poor internet and mobile phone coverage, and lack of transport from remote locations.

Choice of data management platforms proved difficult. Initially, case data were entered into an application based on Epi Info version 7, developed by CDC for hemorrhagic

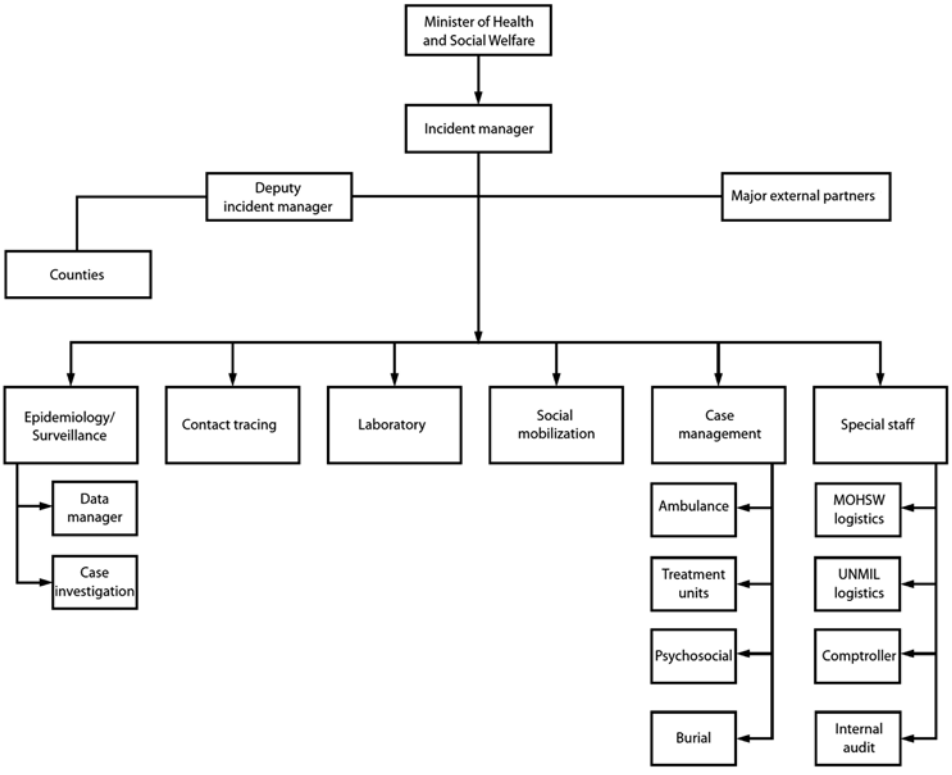

Figure 2. Organizational flowchart for Ebola response Incident Management System, Liberia Ministry of Health and Social Welfare (MOHSW), August 2014. UNMIL, United Nations Mission in Liberia. Source: http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6341a4.htm

fever outbreaks (https://epiinfovhf.codeplex.com). However, in the face of a widespread epidemic, this software had limitations. In mid-December 2014, MOHSW changed to District Health Information Software 2 (https://www.dhis2.org/), an open-source software platform enabling web-based data entry. Contact tracing generally depended on paper and Excel (Microsoft, Redmond, WA, USA) spreadsheets maintained at the county level. At the national level, daily situation reports were compiled manually from aggregate data received from the counties. Although these reports were instrumental in guiding the response, they were incomplete, contained duplicates, and could not be analyzed in real time. Reconciliation of all available data is ongoing.

In July 2014, only 1 laboratory, at the Liberia Institute for Biomedical Research outside Monrovia, was able to conduct Ebola testing, with support from the US National Institutes of Health and the US Army Medical Research Institute of Infectious Diseases (*5*). International partners, including the US military, established a temporary laboratory network to provide Ebola test results within 24 hours to anywhere in the country. By December 2014, real-time reverse transcription PCR testing for Ebola genomic RNA was available at 10 laboratories nationwide. Throughout the outbreak, adequate staffing and rapid transport for specimens, such as by helicopter from remote areas, remained challenging.

Patient Isolation, Case Management, and Epidemic Trends, July–November 2014

The IMS emphasized 4 pillars for interrupting Ebola transmission: 1) early detection, isolation, and treatment of cases; 2) safe transport of patients with suspected cases; 3) safe burial; and 4) infection prevention and control (IPC) in healthcare settings. Isolating persons with Ebola was an immediate, overriding objective. Initially, contact tracing was difficult because of the large number of cases and the urgent need to isolate patients and dispose of cadavers.

By mid-July 2014, only 2 ETUs (20 beds each) were operational, in Foya (Lofa County) and Monrovia (Montserrado County). The principal organizations working with MOHSW to provide care for Ebola patients were Médecins Sans Frontières (MSF) and Samaritan's Purse, but Samaritan's Purse withdrew after several of their staff members became infected with Ebola virus (*6*). By the end of July 2014, the country faced a crisis as the ETUs were filled beyond capacity and Ebola patients were turned away, often dying on hospital grounds, in city streets, or in their homes. In mid-August 2014, MSF opened a tented 120-bed ETU in Monrovia, the largest ever built, with capacity to expand to 400 beds (*15*). The rapid increase in Ebola cases (*5*), extension of the epidemic to all counties, and projections from mathematical modeling (*10*) led the IMS to envision ETUs nationwide. Although a total of 27 ETUs with >2,000 beds were planned, ultimately, 25

were built, of which 3 never opened and many remained underutilized as the epidemic waned.

In early August 2014, intense discussions occurred with MOHSW about the level of care that limited staff could safely provide. Partners including MSF, CDC, and Samaritan's Purse concluded that care should be simplified and that often only oral instead of intravenous rehydration could be safely provided (MSF and CDC, unpub. data; [*5*]). Partners also considered management in the community if patients were unwilling or unable to be evacuated. Lower level community care centers (CCCs) were developed to meet the urgent need for local isolation facilities before sufficient ETUs were constructed (*15*,*16*). However, capacity to build CCCs was also limited and impeded by concern for inferior care or safety. Although >80 CCCs were envisaged, <10 became operational.

The number of available ETU beds initially lagged behind need, but by late September 2014, bed capacities exceeded new cases (Figure 3) (*17*). In retrospect, different data sources suggest that the incidence of disease that had started to increase exponentially in June peaked in early October 2014 and that during July–August, the epicenter shifted from Lofa County to Montserrado County (*5,18*). By November 2014, the epidemic was characterized by low numbers of cases overall, about half in Monrovia and the rest in small clusters in remote locations across the country, frequently initiated by infected travelers from the capital (*19,20*). Lower case counts and increased staff facilitated data reconciliation. Manual matching of laboratory results with ETU and burial data became logistically feasible. Although incomplete, verified laboratory data proved the most useful indicator of epidemic trends (Figure 4). Data from ETUs, although not capturing all cases, provided descriptive characteristics of persons with Ebola.

Management of Cadavers, July–December 2014

The International Federation of Red Cross and Red Crescent Societies and the nongovernmental organization Global Communities led safe collection and disposal of cadavers, a culturally sensitive issue. Starting in September 2014, initial efforts in Lofa and Montserrado Counties were expanded nationwide. Ebola testing of postmortem blood or oral swab samples enabled detection of unrecognized

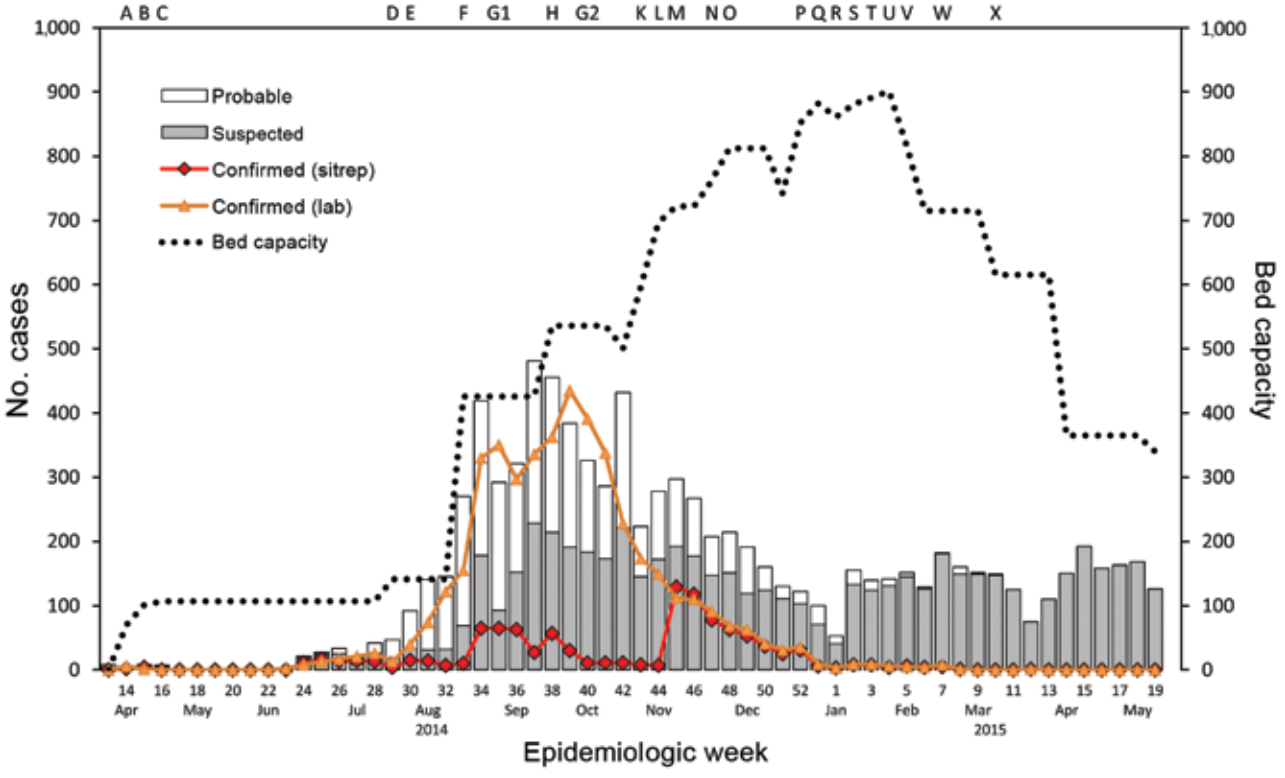

Figure 3. Trends over time for suspected, probable, and confirmed cases of Ebola virus disease from situation reports (sitreps); for confirmed cases from laboratory reports (lab); and for numbers of Ebola treatment unit beds, Liberia 2014–2015. Ebola treatment unit build completion: A, Foya; B, Firestone; C, Eternal Love Winning Africa (ELWA) 1; D, ELWA2; F, ELWA3, John Fitzgerald Kennedy Hospital; H, Bong, Island; K, Unity; L, Ministry of Defense; M, Monrovia Medical Unit; N, Bomi, Kakata; O, China; P, Buchanan; SKD*; Q, Sinje, Ganta, Gbediah; R, Bopulu; S, Tappita, Zwedru; T, Voinjama; U, Zorzor, Greenville*; V, Barclayville; W, Fishtown*; X, Harper.* Other response events: E, Incident Management System implemented; G1–G2, burial teams trained and deployed. *Ebola treatment units built but never opened.

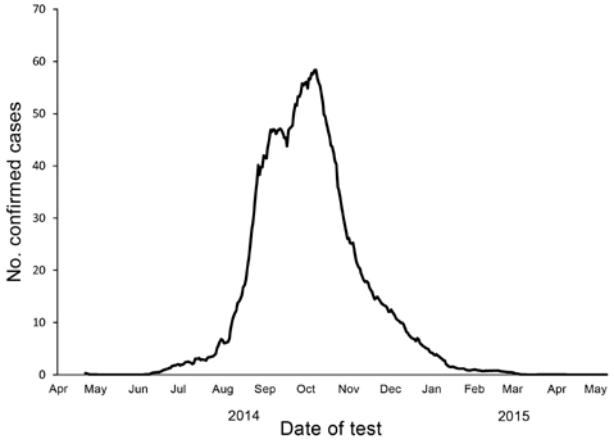

Figure 4. Epidemic curve for laboratory-confirmed cases of Ebola virus disease, Liberia, April 2014–May 2015. Confirmed cases were based on laboratory data per 21-day moving average.

Ebola cases and assessment of excess deaths resulting from Ebola (*21*). In Monrovia, swampy topography and heavy rains in early August 2014 led to resurfacing of recently buried bodies, causing public outrage. The president of Liberia decreed mandatory cremation, a taboo that was accepted reluctantly and incompletely. The decree was lifted in late December 2014 when a public cemetery was opened outside the capital.

Ebola virus–positive cadavers in Montserrado County peaked at 380 during the week of September 15, 2014 (*22*). From October to December, the estimated proportion of Ebola virus–positive cadavers in Montserrado County declined from 35% to 5%. However, the proportion of all bodies collected by burial teams was estimated at <50%, even lower outside of Montserrado County. An algorithm to use cadaver swab sample results to guide contact tracing was extensively discussed but incompletely adopted (Figure 5).

Ebola in Healthcare Workers and IPC, July 2014–May 2015

Early investigations demonstrated greatly increased risk for Ebola among healthcare workers (*5*,*23*), who accounted for 97 (12%) of 810 cases reported by mid-August 2014 (*23*). The greatest proportions of cases were in nurses and nurse aides (34/97; 35%) and physicians and physician assistants (17/97; 18%) (*23*). Most healthcare worker infections were acquired outside ETUs. During July–August 2014, a total of 11 clusters of Ebola involving healthcare workers were investigated; only 1 was in persons working in an ETU (*23*), but even for those in that cluster, exposure in the adjacent hospital was considered probable (*6*). Early estimates were that only ≈25% of Ebola patients received treatment in ETUs (*23*), and the overall proportion of healthcare worker infections thought to have been acquired in ETUs was only 2.4%; the rest were acquired in general hospitals or clinics, including informal venues (MOHSW, unpub. data through December 9, 2014) (Figure 6). Over the course of the epidemic in Liberia, 378 healthcare workers had confirmed cases of Ebola and 192 died (case-fatality rate 50.8%). These numbers represent 12.0% (378/3,157) of all confirmed cases and 4.0% (192/4,808) of all Ebola deaths.

Weak IPC rendered all 657 healthcare facilities in Liberia vulnerable (*5*). Most facilities where Ebola transmission occurred subsequently closed down. A national IPC

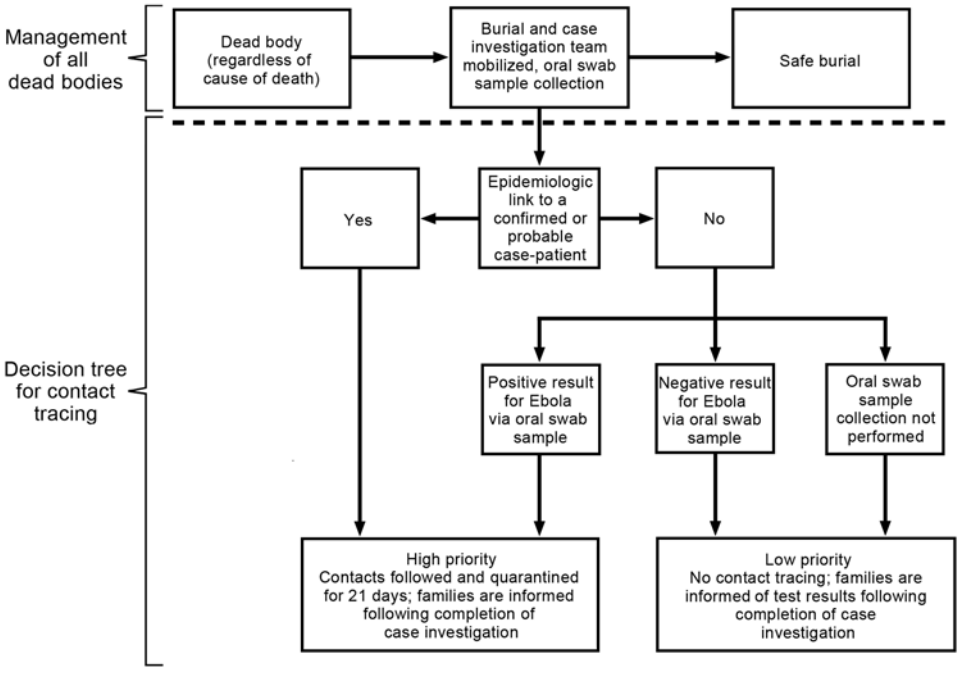

Figure 5. Proposed algorithm for management of dead bodies and associated contact tracing for Ebola virus disease, Liberia, 2014–2015.

task force was established, and an IPC strategy was developed to guide MOHSW, assess facilities and their needs, provide standardized training (Keep Safe, Keep Serving), and conduct investigations. Of the 79 healthcare facilities surveyed before the end of 2014, an estimated 57% lacked protocols for triage and isolation of persons suspected to have Ebola; 43% did not have access to gloves, face shields, or gowns; and 24% lacked running water (MOHSW, unpub. data). IPC committees in healthcare facilities were universally lacking.

By the end of 2014, >4,000 healthcare workers from 350 facilities had received training in basic IPC. A cadre of physicians were trained to serve as technical advisors in the counties. IPC focal points for major hospitals were selected and trained; surveillance and investigative capacity for Ebola in healthcare workers was developed; and personal protective equipment was delivered to major facilities nationwide (gloves and bleach were made as widely available as possible).

The value of surveillance among healthcare staff was highlighted by a single transmission chain in early 2015, in which 166 non-ETU healthcare workers at 10 facilities were exposed to the virus; remarkably, only 1 healthcare worker became infected (*24*). An innovative intervention in response to this cluster was the ring IPC strategy, which provided intensified IPC training and support to healthcare facilities around areas of active transmission (*25*). Despite impressive accomplishments during the epidemic, enormous challenges and deficiencies remain.

Social Mobilization and Public Communication, August 2014–March 2015

MOHSW led comprehensive social mobilization to educate the public on the signs and symptoms of Ebola and provide essential health protection information. Because Ebola was new to Liberia, the first communication strategy comprised messages to counter disbelief (e.g., Ebola is Real). As fear of Ebola and stigma increased, hiding illness became common, prompting messages to encourage help-seeking behavior.

Liberia has a strong tradition of oral communication; therefore, thousands of general community health volunteers were trained to share health messages locally. In October 2014, traditional leaders convened and resolved to support government interventions, opening another trusted channel of health information. During November 2014, traditional and community leaders supported training in all of Liberia's 88 districts. Novel methods were instituted, such as providing traditional chiefs with mobile phones to report suspected cases.

By December 2014, when cases were fewer and response capacity was more robust, a national campaign to reduce Ebola incidence to zero was declared. The evidence-based "Ebola Must Go!" campaign defined 5 essentials in commonly used language: safe burial, rapid isolation of suspected cases, provision of treatment, identification and 21-day monitoring of contacts, and encouragement to speak out against concealment of illness.

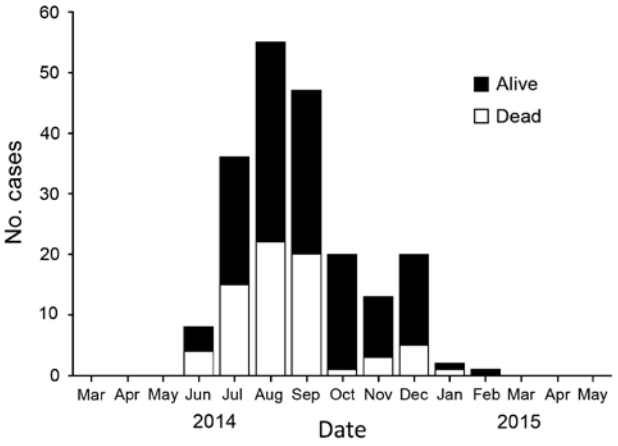

Figure 6. Trends for reported Ebola virus infections among 202 healthcare workers, by status and month, Liberia, March 2014–May 2015. Data source: daily aggregate reports of new cases in healthcare workers in Liberia and Liberia Ministry of Health and Social Welfare situation reports.

Getting to Zero in a Declining Epidemic, Mid-November 2014–May 2015

Starting in mid-November 2014, several events heralded the waning of the epidemic in Liberia. About half of Ebola cases were now part of discrete rural outbreaks often affecting villages so remote that even motorcycles could not reach them. Helicopter airlift was limited by restricted US military air deployments and delays in commercial contracts. Epidemiologic field teams faced harsh living conditions and challenging logistics, often having to walk long distances to and from settlements.

The IMS promoted the Rapid Isolation and Treatment of Ebola (RITE) strategy, which empowered county authorities, with support from partners, to respond quickly to remote hot spots (*22,23*). Essentials were engagement of community leaders, community education, active case finding and contact tracing, quarantine of high-risk contacts, isolation and care for patients, and safe burials. Voluntary quarantine and ad hoc rudimentary clinical facilities were used as needed; emphasis was placed on moving suspected case-patients and contacts from remote locations closer to ETUs. In Bong County, tents were erected in a football stadium to shelter and feed ≥40 contacts during their 21-day monitoring period.

Of 15 remote outbreaks in 2014, detailed analyses were conducted for 12 (in 9 counties) (*20*), of which 9 gave insight into transmission dynamics (*26*). After implementation of the RITE strategy, clusters were recognized earlier, greater proportions of patients were isolated and diagnoses

laboratory confirmed, transmission chains became fewer, durations of outbreaks shortened, and case-fatality proportions declined (*20,26*). The 0–27 secondary cases before RITE declined to 0–4, and the number of secondary cases generated by 1 primary case (basic reproduction number) decreased by 94% (*26*).

Application of fundamental principles—suspect case isolation, rapid diagnosis, and contact tracing—yielded results but required different tactical approaches in varied settings. Ebola had become less widespread, enabling recognition of individual transmission chains in a way not possible earlier. By December 2014, <10 cases were being reported daily, and focus turned to case investigation and contact tracing around laboratory-confirmed cases. In December 2014, Montserrado County set up its own IMS. A sector approach was introduced to decentralize activities in Monrovia, dividing the city into 4 sectors in which partners supported an integrated response for smaller populations.

Considerable resources were invested in containing the last known transmission chains. The Saint Paul's Bridge cluster (22 cases in Monrovia, 15 fatal) was characterized by 3 generations of transmission and challenging social circumstances including resistance, poverty, urban gangs, substance abuse, and extensive exposures in healthcare settings. The last patient in the cluster was isolated on February 18, 2015, and discharged with negative Ebola test results in early March.

Liberia was ready to be declared free of Ebola when on March 20, 2015, an Ebola diagnosis was confirmed for a 44-year-old woman in Monrovia, who died 1 week later (*27*). Her most likely exposure was through sexual intercourse with an Ebola survivor whose illness had occurred >5 months earlier. His semen was positive by PCR for Ebola virus 199 days after onset of his illness, and Ebola genomic material from both partners shared common mutations (*27*). This patient represented the last Ebola case in the second phase of the Liberia epidemic (*12*). On June 29, 2015, a third epidemic phase began with 6 cases in Margibi and Montserrado Counties without further spread; the origin of infection in the index case-patient was uncertain. Liberia was again declared free of Ebola on September 3, 2015 (*13*).

Essentials in Containing Ebola, July 2014–September 2015

No single factor explains Liberia's control of Ebola, and at least 6 issues deserve mention: 1) government leadership and sense of urgency, 2) coordinated international assistance, 3) sound technical work, 4) flexibility guided by epidemiologic data, 5) transparency and communication, and 6) efforts by communities themselves. Instituting the IMS in July 2014 (*14*) was critical for accountability and coordination of multiple partners. The government was always in charge but receptive to external advice channeled through the IMS. The declaration of a state of emergency in August 2014 signaled the gravity of the situation, as did the subsequent closure of land borders with neighboring Sierra Leone and Guinea. Entry and exit screening at airports started in late July 2014, and domestic movement of ill persons was restricted (*28*).

Technical interventions included the early increase in ETU beds in Montserrado County and implementation of burial teams. In Monrovia, isolation of large numbers of patients in late September 2014 and prompt removal of infectious cadavers from the community preceded the documented decrease in cases in the county. Flexibility in response to the changing epidemic was illustrated by the shift in focus from ETUs to CCCs and then to implementation of the RITE strategy, establishment of an IMS for Montserrado County, development of the ring IPC approach, and prioritization of laboratory-confirmed data for guiding interventions.

Community engagement resulted in remarkable behavior change. Physical contact with others ceased; chlorinated handwashing stations sprang up everywhere; and in-country movement reduced. The presidential order for cremation of cadavers in Monrovia was generally respected. By contrast, forcible isolation of case-patients and quarantine of a slum community in Monrovia in August 2014 led to violence, to which the government responded, commendably, with dialogue. Subsequently, voluntary quarantine was instituted only with community agreement and appropriate support, especially provision of adequate food. Many affected communities, some very remote, initiated or supported investigation, contact tracing, and other control efforts with great resilience (*29,30*).

Despite selective reporting suggesting discord (*31*), international partners collaborated well with government and the media. The deployment of the US military provided a logistic and psychological boost. Although some individuals and commercial entities left Liberia early and many airlines ceased operations, major organizations including United Nations agencies stayed and Liberia did not feel abandoned. The president and government communicated clearly and honestly. The "Ebola is Real" and "Ebola must Go!" campaigns transmitted critical information and may have contributed to community resistance being less extensive there than elsewhere.

In retrospect, the response would have been enhanced by much greater investment early on in all aspects of data management, including selection of the most appropriate database. Greater efficiency might have been realized with more support for administrative systems such as personnel, payroll, procurement, and logistics though the IMS. The amount of research conducted was limited, reflecting competing demands but also a missed opportunity.

Staying at Zero and Beyond, May 2015–September 2015

After the Ebola epidemic, the 2 priorities in Liberia are ensuring rapid recognition and containment of resurgent disease and restoring health services to prevent loss of life from traditional concerns such as vaccine-preventable diseases (*32*) or malaria (*33*). The most likely sources of new cases will be importations from Sierra Leone or Guinea, unrecognized transmission chains within Liberia, or sexual transmission from survivors. Reintroduction of the virus from its natural habitat is theoretically possible, and Ebola becoming endemic is a concern.

Border screening and community event–based surveillance in counties bordering Sierra Leone and Guinea have been instituted (*34*). Monrovia is a favored destination for travelers, and tracking of visitors from affected countries has been proposed but may be difficult to implement. Heightened surveillance is indicated in the immediate post-Ebola period. Testing of oral swab samples from cadavers throughout the country should continue until the region is free from Ebola; the value of this practice was demonstrated by recognition of the index case in the third epidemic wave.

Healthcare facilities should maintain clinical suspicion for Ebola and surveillance among healthcare workers, a sentinel population. Simplifying and expanding Ebola testing without all tests triggering ETU admission and contact tracing will be necessary. After approval, Ebola rapid tests could profoundly change clinical practice. Laboratory capacity to distinguish differential diagnoses such as malaria, Lassa fever, yellow fever, and dengue is needed. Enhancement of IPC nationally must continue.

Investment is needed in surveillance, laboratory strengthening, emergency operations center support, epidemiology expertise, outbreak response capacity (including risk communication and health promotion), and the ability to base decisions on data (*35,36*). In retrospect, it was lack of such public health systems that enabled the Ebola epidemic to grow in West Africa with such devastating consequences (*36*). A recently evaluated Ebola vaccine may have a role in containing future outbreaks (*37*); priority populations will include high-risk contacts and healthcare workers.

Preliminary observations suggest that about one quarter of Ebola survivors report visual disturbances, the most severe cause being uveitis (*38*), and about half report severe fatigue and joint pains. The medical, psychological, and social sequelae of Ebola should be assessed, including the number and needs of orphans; and medical, psychosocial, and material support provided for survivors. Evidence of sexual transmission (*27*) and prolonged Ebola persistence in semen (*39*) demand study of postrecovery infectiousness for formulation of public health advice.

Ebola survivors in West Africa, who number in the thousands, have suffered stigma and discrimination, now exacerbated by the possibility of sexual transmission. Many Ebola infections resulted from acts of compassion, such as assisting the sick or participating in funerals. Ostracism of survivors would be an unacceptable conclusion to this unique event in global health, the response to which has been a credit to the government and people of Liberia.

Note Added in Proof

Since acceptance and publication on-line of this report, a 15-year-old-boy in Montserrado county tested positive for Ebola on November 22, 2015 and died the next day. Two other family members subsequently tested positive and survived. Rapid response and containment were achieved using the containment strategies and procedures outlined in this report. The source of the cluster was believed to be viral re-emergence in a persistently infected survivor. Liberia was again declared Ebola-free on January 14, 2016.

Acknowledgments

We gratefully acknowledge the contributions of many diverse staff members from different organizations, international and Liberian, who contributed to the public health response described here. Special thanks go to colleagues from CDC, MSF, the US Agency for International Development, and WHO, with whom we worked most closely.

Mr. Nyenswah is a lawyer who has led Liberia's Incident Management System for Ebola since August 2014. He was appointed by President Sirleaf as Deputy Minister of Health for Public Health Emergencies.

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SYNOPSIS

Epidemiology of Epidemic Ebola Virus Disease in Conakry and Surrounding Prefectures, Guinea, 2014–2015

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In 2014, Ebola virus disease (EVD) in West Africa was first reported during March in 3 southeastern prefectures in Guinea; from there, the disease rapidly spread across West Africa. We describe the epidemiology of EVD cases reported in Guinea's capital, Conakry, and 4 surrounding prefectures (Coyah, Dubreka, Forecariah, and Kindia), encompassing a full year of the epidemic. A total of 1,355 EVD cases, representing ≈40% of cases reported in Guinea, originated from these areas. Overall, Forecariah had the highest cumulative incidence (4× higher than that in Conakry). Case-fatality percentage ranged from 40% in Conakry to 60% in Kindia. Cumulative incidence was slightly higher among male than female residents, although incidences by prefecture and commune differed by sex. Over the course of the year, Conakry and neighboring prefectures became the EVD epicenter in Guinea.

Ebola virus disease (EVD) in West Africa was first reported during early March 2014 in Guinea's 3 southeastern prefectures (Gueckedou, Macenta, and Kissidougou), which border Liberia and Sierra Leone; however, retrospective investigations indicate Ebola virus (EBOV) transmission might have occurred in Guinea since December 2013 (*1*–*4*). On March 27, 2014, EVD was reported in Conakry (population 1,667,864), the capital of and largest city in Guinea (*1*,*5*). EBOV rapidly spread through much of Guinea, where it was reported in 32 of 34 prefectures, and to Liberia and Sierra Leone, causing the largest EVD epidemic since EBOV was discovered in 1976 (*2*,*3*,*6*). As of November 1, 2015, West Africa reported >28,000 EVD cases, of which >3,800 (including >2,500 deaths) were reported from Guinea; (*7*). The presence of EVD in Conakry led the Guinea Ministry of Health (MoH) to request assistance from the US Centers for Disease Control and Prevention, the World Health Organization (WHO), Médecins Sans Frontières, and other partners to establish a systematic disease-surveillance process and to implement control measures nationwide. Here we characterize EVD cases in Conakry and the 4 surrounding prefectures, which together became the epicenter of the EVD epidemic in Guinea.

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DOI: http://dx.doi.org/10.3201/eid2202.151304

Methods

We conducted a descriptive analysis of data reported in the Epi Info Viral Hemorrhagic Fever Application (Epi Info VHF, http://epiinfovhf.codeplex.com/), software used to maintain the Guinea MoH national case database. Patient-specific data (i.e., demographic, clinical, epidemiologic, and laboratory information) were collected by using a standardized EVD case notification form. Demographic information (age, sex, and residence) was obtained from the standardized notification forms completed during patient admission to an Ebola treatment center (ETC) or at the corpse collection for persons who died outside of an ETC or hospital (community deaths). Final vital outcome status for patients admitted to an ETC was obtained from ETC line listing data; all data were updated in Epi Info VHF.

EVD cases were classified into 1 of 3 WHO case definitions: suspected, probable, or laboratory-confirmed cases. A suspected case was defined as disease in a living person with 1) a history of contact with a person who had laboratory-confirmed or probable EVD and 2) who had unexplained bleeding or sudden onset of high fever or ≥3 of the following signs and symptoms: headache, anorexia/loss of appetite, lethargy, aching muscles or joints, breathing difficulties, vomiting, diarrhea, stomach pain, difficulty

Table 1. Ebola virus disease cases by prefecture and sex in Conakry, the capital city, and surrounding prefectures, Guinea, January 1, 2014–March 29, 2015*

Location	No. (%) cases by classification		No. (%) cases, no. cases/100,000 persons			Median age, y (Q1, Q3)	% ETC case-fatality, (95% CI)†	No. (%) community deaths‡
	Laboratory confirmed	Probable	Total	Men	Women			
Conakry	519 (42)	34 (26)	553 (41), 33.2	307 (45), 36.6	246 (37), 29.7	30 (22, 44)	40 (35–45)	93 (18)
Coyah	229 (19)	7 (6)	236 (17), 89.3	112 (16), 85.5	124 (18), 93.1	30 (20, 43)	47 (40–55)	44 (19)
Dubreka	115 (9)	8 (6)	123 (9), 37.5	65 (9), 40.3	58 (9), 34.7	30 (18, 40)	46 (35–58)	32 (28)
Forecariah	290 (24)	45 (35)	335 (25), 136.9	155 (23), 132.2	180 (27), 141.3	30 (18, 45)	53 (46–60)	78 (27)
Kindia	73 (6)	35 (27)	108 (8), 24.6	45 (7), 21.2	63 (9), 27.8	35 (22, 50)	60 (47–73)	12 (16)
Total	1,226 (100)	129 (100)	1,355 (100), 46.0	684 (100), 46.8	671 (100), 45.3	30 (20, 45)	46 (43–49)	259 (21)

*Data were obtained from the Guinea Ministry of Health national case database (Epi Info Viral Hemorrhagic Fever Application, http://epiinfovhf.codeplex.com/). ETC, Ebola treatment center; Q1 and Q3, lower and upper quartiles, respectively.
†Case fatality = ETC deaths/(laboratory-confirmed cases with final status known – community deaths); see Table 2.
‡Percent community deaths = community deaths/laboratory-confirmed cases; see Table 2.

swallowing, and hiccups. Probable cases were defined as disease in deceased persons who had an epidemiologic association with EVD but no laboratory testing. Laboratory-confirmed cases were defined as cases in any persons, dead or alive, who had laboratory-confirmed EVD (*8*,*9*). Laboratory confirmation of EVD cases was made on the basis of positive real-time reverse transcription PCR results or, for samples tested >10 days after symptom onset and for PCR-negative samples, on the basis of positive serologic testing results. Only laboratory-confirmed and probable cases are described in this report because suspected cases had already been reclassified at the time of this analysis.

Our analysis included cases reported in Epi Info VHF during January 1, 2014–March 29, 2015 (epidemiologic week 1, 2014, to epidemiologic week 13, 2015). All persons whose place of residence was listed as Conakry, including its 5 communes (Dixinn, Kaloum, Matam, Matoto, and Ratoma), or as 1 of the 4 surrounding prefectures (Coyah, Dubreka, Forecariah, and Kindia) were included in the analysis. Epidemiologic weeks were in accordance with those designated by in-country situation reports. For numerators for cumulative incidence, we used all laboratory-confirmed and probable cases, by commune and prefecture. For denominators, we used preliminary 2014 population data obtained from the Guinea National Statistics Institute, Ministry of Planning (*10*). To remain consistent with WHO reporting, we calculated the case-fatality percentage by using the number of laboratory-confirmed deaths in ETCs as the numerator and the number of laboratory-confirmed cases for which final status was known, excluding community deaths, as the denominator. The percentage of laboratory-confirmed community deaths was determined by dividing the number of laboratory-confirmed community deaths by the total number of laboratory-confirmed cases. These surveillance data were collected and used for public health practice purposes to control the epidemic, not for human subject research.

Results

During January 1, 2014–March 29, 2015, a total of 553 EVD cases were reported in Conakry, and an additional 802 were reported in the 4 surrounding prefectures. Among these 1,355 cases, a total of 1,226 (90%) were laboratory-confirmed and 129 (10%) were probable cases. The median

Table 2. Ebola virus disease cases and associated deaths Conakry, the capital city, and surrounding prefectures, Guinea, January 1, 2014–March 29, 2015*

Location	No. cases by classification				No. (%) deaths		
	Total	Probable	Laboratory-confirmed	Laboratory confirmed with known final status	ETC	Community	Total†
Prefectures							
Conakry	553	34	519	513	168	93	295 (53)
Coyah	236	7	229	221	84	44	135 (57)
Dubreka	123	8	115	110	36	32	76 (62)
Forecariah	335	45	290	277	106	78	229 (68)
Kindia	108	35	73	70	35	12	82 (76)
Total	1,355	129	1,226	1,191	429	259	817(60)
Conakry commune‡							
Dixinn	59	2	57	56	15	11	28 (47)
Kaloum	55	7	48	48	9	8	24 (44)
Matam	61	6	55	53	19	9	34 (56)
Matoto	198	13	185	185	62	43	118 (60)
Ratoma	145	4	141	140	51	20	75 (52)

*Data were obtained from the Guinea Ministry of Health national case database (Epi Info Viral Hemorrhagic Fever Application, http://epiinfovhf.codeplex.com/). ETC, Ebola treatment center.
†Probable plus ETC plus community deaths divided by all cases.
‡Counts do not sum because 35 cases were missing commune information.

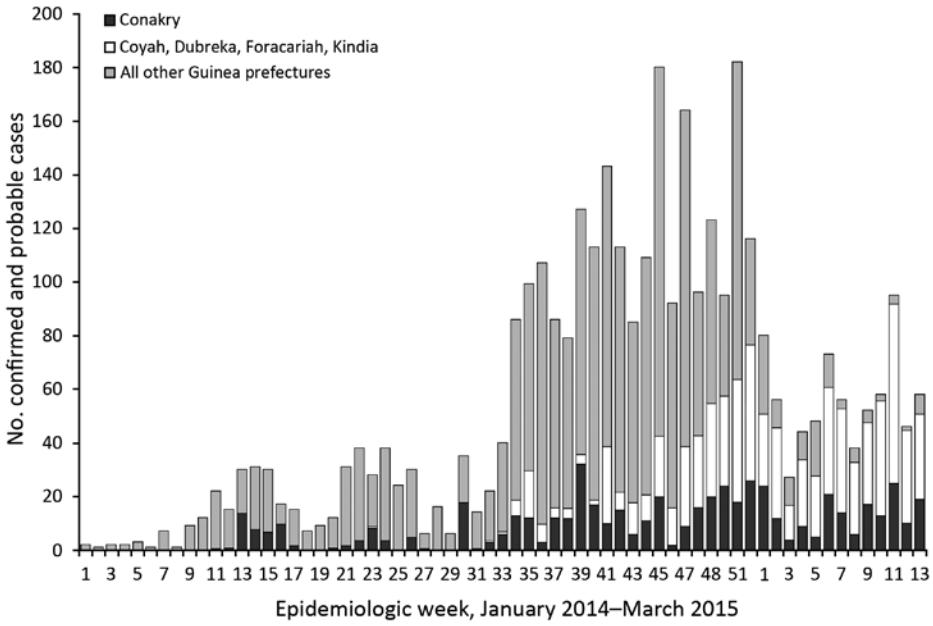

Figure 1. Ebola virus disease cases in Conakry, the capital city; 4 surrounding prefectures; and all remaining prefectures, Guinea, January 1, 2014–March 29, 2015. Data were obtained from the Guinea Ministry of Health national case database (Epi Info Viral Hemorrhagic Fever Application). Epidemiologic week 52 ended on December 27, 2014.

age of persons with EVD was 30 years (lower and upper quartiles 20 and 45 years, respectively); 283 (21%) infected persons were ≤18 years of age, and 671 (50%) were female. The most commonly reported signs and symptoms during the first visit to an ETC were fever (96%), fatigue (96%), and anorexia (86%). Records indicated that 118 (9%) EVD cases were in healthcare workers. A total of 817 (60%) infected persons died; of these, 259 (21%) died in the community (Tables 1, 2). The number of community deaths per epidemiologic week fluctuated from 0 to 27.

The first laboratory-confirmed EVD case in Conakry was reported in late March 2014 (epidemiologic week 11), approximately 3 months after cases were identified in Guinea (Figure 1). During March–September 2014, the number of weekly EVD cases reported in Conakry ranged from 0 to 18 (Figure 1). During early October (epidemiologic week 39), the number of weekly reported cases in Conakry peaked at 32. Beginning mid-January 2015 (epidemiologic week 3, 2015), weekly EVD cases for all Guinea prefectures briefly declined, and most of the weekly cases

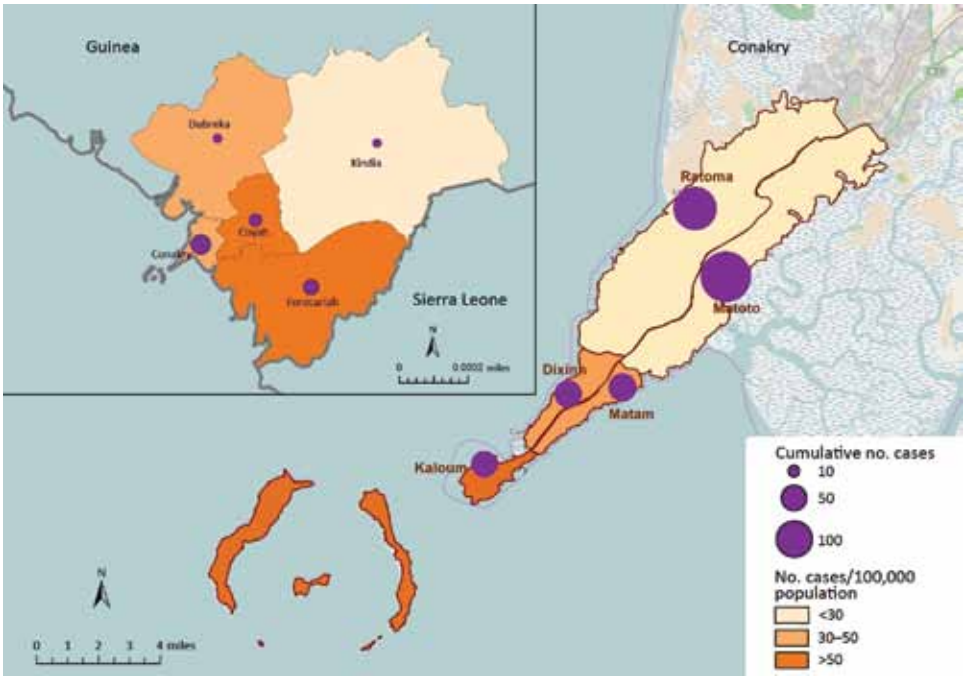

Figure 2. Cumulative incidence of Ebola virus disease cases in Conakry, the capital city, and 4 surrounding prefectures, Guinea, January 1, 2015–March 29, 2015. Data were obtained from the Guinea Ministry of Health national case database (Epi Info Viral Hemorrhagic Fever Application, http://epiinfovhf.codeplex.com/). A total of 35 cases were missing commune information and are not represented in the figure. Inset shows locations of prefectures in Guinea; larger map shows locations of communes in Conakry.

reported at that time and up until March 2015 were among Conakry residents and persons residing in the 4 neighboring prefectures (Figure 1).

During the study period, the overall number of EVD cases per 100,000 persons was 33.2 in Conakry, 89.3 in Coyah, 37.5 in Dubreka, 136.9 in Forecariah, and 24.6 in Kindia (Table 1; Figure 2). Cumulative incidence was slightly higher among male (46.8 cases/100,000 persons) than female (45.3 cases/100,000 persons) residents. Furthermore, incidence varied by sex in prefectures; incidence was higher among female residents in Coyah, Forecariah, and Kindia (Table 1). Excluding community deaths, the case-fatality percentage among EVD-infected persons ranged from 40% (95% CI 35%–45%) in Conakry to 60% (95% CI 47%–73%) in Kindia. Among all cases, community deaths were highest for residents of Dubreka (28%) and Forecariah (27%).

Among the 5 communes in Conakry, Kaloum, the smallest by population, had the highest overall incidence (87.8 cases/100,000 persons) but the lowest case-fatality percentage (23%) (Table 3). Ratoma, the second most populated commune, had the lowest overall incidence (22.2 cases/100,000 persons) but a case-fatality percentage of 43%. Kaloum was the only commune that had a higher incidence of EVD among female (97.9 cases/100,000 persons) than male (77.4 cases/100,000 persons) residents. The percentage of EVD community deaths was highest in Matoto (23%).

Discussion

EVD transmission first occurred during March 2014 in Conakry, the capital and largest city in Guinea. During the weeks that followed, reported EVD cases in the capital remained low, but virus transmission continued in the city. Sustained transmission was attributed to the continued refusal by a limited number of families to accept clinical intervention and isolation (*11*). However, in December 2014 (epidemiologic week 52), the epidemic peaked in Conakry and the 4 surrounding prefectures (Figure 1); at that time, EVD cases in Conakry, Coyah, Dubreka, Kindia, and Forecariah represented most cases in Guinea. This shift of the EVD outbreak from other parts of Guinea to Conakry, with its population of 1.7 million persons, was a landmark event during the epidemic, and implementation of targeted control measures fortunately prevented substantial outbreak amplification (*11*). Cenciarelli et al. (*12*) suggest that Guinea had better EVD management, treatment, and laboratory support than the other affected countries, resulting in Guinea having a slower rise in cases. Conakry and the 4 surrounding prefectures remained a principal focus of the outbreak in Guinea. From the beginning of the epidemic in the capital, a total of >550 EVD cases were reported from Conakry and >800 were reported from the 4 surrounding prefectures. Together, these areas accounted for ≈40% of the total number of cases in Guinea during the study period.

The overall incidence of EVD cases varied by prefecture; Forecariah, a prefecture bordering Sierra Leone, had an incidence 4 times higher than that of Conakry. Kaloum, the smallest (by population size) commune in Conakry, had the highest overall incidence and highest incidence among female residents. Overall, the cumulative incidence of EVD cases was slightly higher for male than female residents; however, the incidence among female residents was higher than that among male residents in 3 prefectures (Coyah, Forecariah, and Kindia) and 1 commune (Kaloum). A United Nations report suggests that this difference in incidence by sex may be attributed to the role of women as primary frontline caregivers for sick persons, putting them at a higher risk for exposure to EBOV (*13*).

Continued EVD transmission in the Conakry area is attributed to multiple factors, including community and family transmission, high mobility of EVD patients to and from Conakry and neighboring prefectures, and localized resistance to EVD interventions (*11*). Case investigations have shown that residents of Conakry often have relatives in other prefectures whom they visit (and vice versa) (*11*). In addition, patients with EVD-like symptoms travel to Conakry to seek treatment and improved healthcare services (*6*). On arrival at ETCs, these patients, compared with those who do not have to travel for care, can be further along in the disease course and have high virus loads, increasing the risk for exposure of healthcare personnel, relatives, and other community members before hospitalization and isolation. In addition, certain patients might have been hospitalized or otherwise cared for outside of ETCs, causing a potential for outbreak amplification and continuation of the EVD transmission chain in the community, as demonstrated in previously reported cases (*2*). We showed that the case-fatality percentage in Conakry was lower than that in the surrounding prefectures, possibly reflecting differences in resources and case management. Conakry has many public and private hospitals and clinics, including Donka Hospital, the site of Conakry's first ETC (*2*). Additional challenges to reducing disease transmission in the capital area included initial limited awareness and acceptance of the disease, fear and mistrust, and stigma associated with the disease (*6*,*14*).

Although EVD surveillance via Epi Info VHF in Conakry and throughout Guinea was constantly updated to accurately capture correct case information, the database captures only what is reported. For example, certain variables (e.g., clinical data) are often incomplete. Furthermore, a study from Barry et al. (*6*) indicated a general underreporting of EVD cases because certain patients never seek medical care at an ETC. Therefore, EVD incidence in Conakry and the 4 surrounding prefectures is probably higher than we

Table 3. Ebola virus disease cases by Conakry, the capital city, and its 5 communes, Guinea, January 1, 2014– March 29, 2015*

Location, commune	No. (%) cases by classification		No. (%) cases, no. cases/100,000 persons			Median age, y (Q1, Q3)	% ETC case-fatality (95% CI)†	No.(%) community deaths‡
	Laboratory confirmed	Probable	Total	Men	Women			
Conakry§	519 (100)	34 (100)	553 (100) 33.2	307 (100) 36.6	246 (100) 29.7	30 (22,44)	40 (35–45)	93 (18)
Dixinn	57 (12)	2 (6)	59 (11) 42.9	35 (12) 50.2	24 (10) 35.5	29 (18,44)	33 (20–49)	11 (19)
Kaloum	48 (10)	7 (22)	55 (11) 87.8	24 (9) 77.4	31 (13) 97.9	30 (19,41)	23 (11–38)	8 (17)
Matam	55 (11)	6 (19)	61 (12) 42.5	31 (11) 43.4	30 (13) 41.5	35 (26,45)	43 (28–59)	9 (16)
Matoto	185 (38)	13 (41)	198 (38) 29.5	115 (40) 34.1	83 (36) 24.9	32 (22,45)	44 (35–52)	43 (23)
Ratoma	141 (29)	4 (12)	145 (28) 22.2	79 (28) 23.9	66 (28) 20.4	30 (23,40)	43 (34–52)	20 (14)

*Data were obtained from the Guinea Ministry of Health national case database (Epi Info Viral Hemorrhagic Fever Application, http://epiinfovhf.codeplex.com/). ETC, Ebola treatment center; Q1 and Q3, lower and upper quartiles, respectively.
†Case fatality = ETC deaths/(laboratory-confirmed cases with final status known – community deaths); see Table 2.
‡Percent community deaths = community deaths/laboratory-confirmed cases; see Table 2.
§Counts do not sum because 35 cases were missing commune information.

report (*6*). Enhancements to the primary data collection systems at the national and prefecture levels have been ongoing in Guinea. Starting in early 2015, weekly situation reports included detailed prefecture assessments with commune-level analyses, indicators to measure the number and severity of all security incidents (e.g., violent threats toward public health professionals), and situations of refusal to cooperate (e.g., failure to disclose names of contacts). The integration of transmission data chains with primary case notification records enables the identification of new cases from known contacts and unknown chains of transmission.

Our findings are subject to limitations. First, notable underreporting of probable cases is indicated by a higher number of investigated burials included in aggregated country daily reports produced by the Guinea MoH with the assistance of WHO and other partners. Underreporting of probable cases might be unevenly distributed across the country and thus affect comparisons with cumulative case numbers across prefectures. Efforts to retrospectively complete case-based notification of probable cases were strengthened starting at the end of 2014. Second, the information (e.g., ascertainment of professions, including healthcare workers) across these settings is incomplete and may involve information bias. Ongoing data quality assessments are in place and focus on key variables, including case definition category, final vital status, and prefecture of residence. Despite these limitations, comparisons with aggregated figures from situation reports and case-based data from the national database indicate that Guinea's Epi Info VHF data represent the history of this EVD epidemic (*7*).

In summary, in late December 2014, during the first year of the Ebola epidemic, weekly cases in Conakry and the 4 surrounding prefectures surpassed reported cases from all other Guinea prefectures. To date, these areas have remained a focal point of disease transmission. High mobility within Conakry and surrounding prefectures is common, and, thus, contact tracing and transmission chain tracking are challenging. Because of these factors, the daily information exchange regarding cases and contacts among epidemiologists and contact tracers working in Conakry and the surrounding prefectures is essential. Finally, improving surveillance efforts at the commune level to identify where more prevention and effective communication measures are needed is critical.

Acknowledgments

We acknowledge the Guinea Ministry of Health and Direction Préfectorales/Communales de la Santé, Guinea, for surveillance activities.

Support for this project was provided by the Guinea Ministry of Health, World Health Organization/Global Outbreak Alert and Response Network, and the Global Guinea Ebola Response Team, US Centers for Disease Control and Prevention.

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Hospital Preparations for Viral Hemorrhagic Fever Patients and Experience Gained from Admission of an Ebola Patient

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The Major Incident Hospital of the University Medical Centre of Utrecht has a longstanding history of preparing for the management of highly pathogenic and infectious organisms. An assessment of the hospital's preparations for an outbreak of viral hemorrhagic fever and its experience during admission of a patient with Ebola virus disease showed that the use of the buddy system, frequent training, and information sessions for staff and their relatives greatly increased the sense of safety and motivation among staff. Differing procedures among ambulance services limited the number of services used for transporting patients. Waste management was the greatest concern, and destruction of waste had to be outsourced. The admission of an Ebola patient proceeded without incident but led to considerable demands on staff. The maximum time allowed for wearing personal protective equipment was 45 minutes to ensure safety, and an additional 20 minutes was needed for recovery.

The Ebola virus disease (EVD) epidemic during 2014–2015 led hospitals worldwide to prepare for the triage and admission of Ebola virus (EBOV)–infected patients (*1*). During the fall of 2014, the Ministry of Health, Welfare, and Sport of the Netherlands requested that the Major Incident Hospital (MIH) provide 4 beds for the admission of EBOV-infected international healthcare workers and military personnel. The MIH is a government-funded, standby facility in the basement of the University Medical Centre of Utrecht (UMC Utrecht) that provides 200 beds to ensure capacity and optimal infrastructure for the triage and care of victims of large-scale trauma, nuclear, chemical, or biological incidents (*2*). The MIH benefits from a substantial amount of resources (e.g., materials and personnel) shared with UMC Utrecht and the adjoining Central Military Hospital.

The MIH contains an isolation facility separate from other hospital infrastructure and air systems for the care of patients infected with highly pathogenic and infectious organisms (those designated as Biosafety Levels 3 and 4). This facility contains 4 isolation rooms equipped with a negative air pressure system and double air filtering. For the past 14 years, the MIH has been training staff to care for patients with viruses with aerosol transmission, and the MIH is the only center in the Netherlands designated to treat smallpox. Following the request of the Ministry of Health, Welfare, and Sport to prepare for the admission of EBOV-infected patients, all previously developed procedures were revised for the treatment of patients with viral hemorrhagic fever (VHF). We present an overview of the preparations made at the MIH in the fall of 2014, pending a possible VHF outbreak, and the experience gained from the admission of an EBOV-infected patient.

Preparation Phase

A task group, which consisted of infection prevention experts and specialists in infectious diseases, virology, acute medicine, intensive care, pediatrics, and occupational medicine, prepared for all procedures involved in handling VHF. Other members included team leader nurses; officers for communication, security, waste management, and support services; and management representatives.

Command

The chain of command was demarcated in 4 levels (Figure 1). First, the crisis management team would be responsible for external communication and coordination with the adjoining hospitals. Second, the crisis coordination team would operate between the crisis management team and the command team to ascertain continuity of care at UMC Utrecht. Third, the command team would oversee the admission of a patient to the MIH. Finally, the treatment team would provide medical treatment for the patient.

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DOI: http://dx.doi.org/10.3201/eid2202.151393

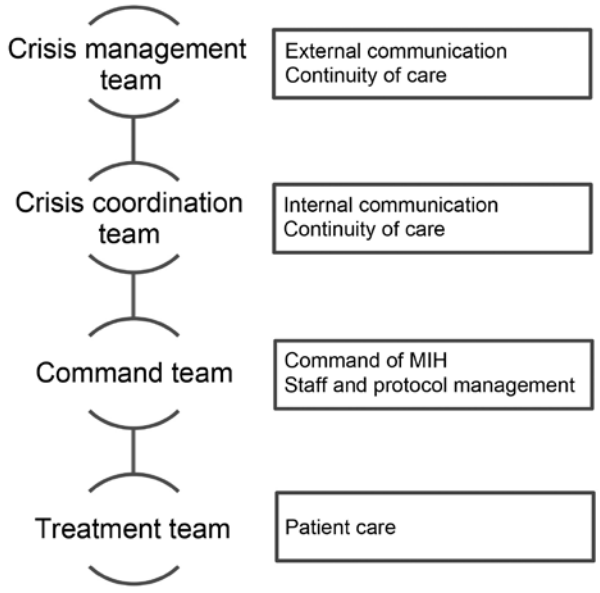

Figure 1. Planned command structure for potential admission of a patient with viral hemorrhagic fever, Major Incident Hospital (MIH), University Medical Centre of Utrecht, the Netherlands, 2014.

Scenario Description and Routing

Flowcharts were developed that described 3 scenarios for routing a patient with suspected or confirmed VHF: 1) self-referral, 2) external referral, and 3) in-hospital referral from another ward. Security staff and a nurse would guide the patient to the MIH via a designated cleared route. Meanwhile, an emergency department nurse would open the MIH, activate the negative pressure system, and alert a team of trained nurses and an infectious disease specialist, all of whom would perform triage and assess the need to scale up the response.

VHF patients arriving by ambulance would enter the MIH through a separate entrance in the MIH. At this entrance, a 3-zone area was drawn on the floor to indicate the safe zone and potentially contaminated zones and to delineate doffing zones (where potentially contaminated clothing and gear are removed) for ambulance and disinfection personnel (Figure 2). Personnel from the appointed ambulance services also were trained in accordance with the revised protocols.

Infection Prevention Measures

Personal Protective Equipment

The selection of personal protective equipment (PPE) was based on national and international guidelines and tested for workability and comfort (*3*,*4*). A protective overall with a fluid-protected surgical gown combined with an FFP2 respirator (which filters >94% of airborne particles), a face shield, double-layer gloves, and double-layer foot protection would be worn over the standard surgery scrubs and clogs. Full-face masks conferring FFP3-level protection (i.e., filtering >99% of airborne particles) would be available for those performing high-risk procedures. PPE would be stocked to the extent that 1 patient could be treated for up to 14 days, and a list for backup suppliers and materials would be set up.

Figure 2. Entrance of isolation unit with demarcated zones, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014. Markings on the floor indicate a safe zone and potentially contaminated zones and delineate doffing zones (where potentially contaminated clothing and gear are removed) for ambulance and disinfection personnel.

Medical and Other Equipment

In the patient's room, disposable equipment would be used, such as cardboard pots with fluid-absorption granules for urine and feces, eating utensils, and other accessories. Exceptions would only be possible for items that could withstand final disinfection procedures after discharge.

Working Procedures

To ensure the safety of personnel, the "buddy system," an extended version of the trainer-observer method (*4*), was

introduced. In this system, a specialized nurse (buddy) guides and monitors all activities of the staff who are wearing PPE, starting with the donning of clothing and gear and ending with discarding all PPE. The buddy would be seated outside the unit in front of the glass window looking into an isolation room (Figure 3) and would guide the care provider in the room by reading aloud every step of the protocol being used, ensuring the minimization of risk behaviors arising from haste, stress, and the uncomfortably warm conditions felt while wearing PPE. The buddy and care provider would communicate by speakerphone inside the room connected to a mobile telephone. The glass windows would also facilitate monitoring of and communication with the patient. The maximum number of medical personnel present in an isolation unit was set to 1 at a time to ensure maximum safety. The maximum time spent in PPE was set at 45 minutes to minimize the loss of concentration caused by discomfort.

While a patient is in an isolation room, the nurses would work 8-hour shifts in teams of 3 persons (i.e., a bedside nurse, a buddy nurse, and a coordinating nurse). All procedures were summarized in task cards that would be used by the buddy to guide the bedside nurse. All protocols and task cards were made available through the hospital intranet.

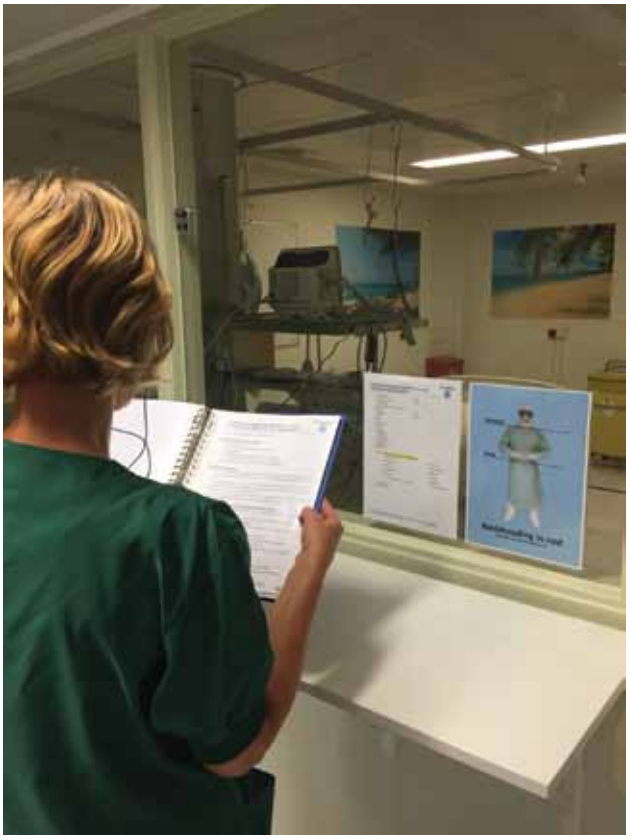

Figure 3. A buddy nurse demonstrates reading instructions in front of the isolation unit glass window for healthcare personnel working inside the unit, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014.

Waste Management

Preparations for waste management were a major concern given the expected amount of waste and the time-consuming procedures involved (replacing a single waste container in the isolation unit can take as long as 20 minutes). Designated, sealable, 60-L waste containers would be used for waste storage, and waste management procedures were strictly protocolled and repeatedly conveyed through training.

In-hospital autoclave capacity appeared insufficient; therefore, waste destruction would be outsourced to an external facility. In accordance with transportation laws, one specific 20-L container had been approved for transport by public road (*5*). However, these containers were too small, and opening and closing them presented a safety risk. Therefore, category A medical waste (UN2814) containers were chosen; these were to be packed in a large plastic drum and the waste stored in a guarded and certified cooled sea container outside the hospital before transport.

Cleaning Procedures

The nursing staff were trained to perform the daily cleaning in the isolation unit. A limited number of cleaning staff were trained to perform the first disinfection after a patient transfer. For the final cleaning of the unit, an external company was contracted to perform disinfection with hydrogen peroxide treatment.

Personnel

The required number of personnel was calculated for the admission of multiple patients to guarantee successful upscaling. During preparations, it proved necessary to activate the crisis coordination team to guarantee the availability of personnel from the hospital for frequent training sessions to ensure maximum availability during the admission of a patient. Flowcharts directed the alerting of in-house staff by team leaders during the acute phase; as necessary, a computerized alarm system would be activated to warn personnel by telephone.

Some personnel were excluded from participation because of certain conditions (e.g., claustrophobia). Personnel were repeatedly trained in sessions of 1.5 hours, during which the donning and doffing of PPE, the buddy system, and other procedures were rehearsed (e.g., waste management, cleaning, and diagnostic procedures). These sessions were repeated every 10 weeks and resulted in a noticeable increase in the quality and safety of working conditions. The nursing staff of the MIH were prepared to fulfill the roles of buddy and trainer. A total of 126 staff members were trained (Table 1). After 2 training sessions, a survey

Table 1. Personnel trained in preparation for admission of a patient with viral hemorrhagic fever, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014

Specialty/title	No. trained
Anesthetics specialist	1
Nurse trainer	13
Infectious disease specialist	8
Intensive care specialist	4
Internal medicine specialist	6
Nurse, emergency department	33
Nurse, intensive care unit	18
Nurse, infectious diseases	13
Pediatric infectious disease specialist	3
Pediatric intensive care specialist	5
Nurse, other department	11
Nurse, pediatric intensive care	5
Nurse, pediatrics	4
Resident infectious disease specialist	2
Total	126

conducted among personnel indicated that they felt sufficiently prepared (Figure 4).

Occupational health and safety service guidelines were developed for staff. All employees whose work involved contact with a VHF patient under protected conditions would be registered and required to monitor their temperature. In the event of unprotected contact with a VHF patient, personnel would be excluded from activities in the hospital and closely monitored by the public health service. If onset of VHF-associated symptoms occurred, personnel would be requested to contact the hospital's occupational health and safety service and would be admitted to the isolation unit.

Diagnostics

PCR testing for VHF was to be performed in 2 reference centers (the Erasmus Medical Center, Rotterdam, the Netherlands, and the Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany), which were appointed in accordance with national regulations and are in compliance with safety protocols (*6*,*7*). PCR testing was to be performed for EBOV, Marburg virus, Lassa virus, Crimean-Congo hemorrhagic fever virus, HIV, *Plasmodium* spp., and *Leptospira* spp. All materials were to be stored in plastic safety bags, placed in plastic containers, and then placed in cardboard shipping boxes (i.e., the "box-in-box" method).

Point-of-care laboratory tests were to be performed in the isolation unit by using I-STAT portable clinical analyzer (Abbott Point of Care, Inc., Princeton, NJ, USA). More extensive testing would be possible in one of the appointed external diagnostic centers.

A stethoscope equipped with a Bluetooth connection was acquired for auscultation of patients without physical contact. The radiology department was consulted to explore the possibilities of imaging in certain circumstances, such as the localization of a central venous catheter. However, these possibilities were limited by the confined space in the isolation rooms and by the need to decontaminate the equipment. It was then decided that the use of conventional radiography would not be possible. The option of a small portable ultrasound device was explored; however, the quality of the imaging was insufficient.

Isolation Department and Adaptations

Only small adaptations to the isolation units were necessary. To prevent spread of the virus, running water taps were shut off, and sinks were disconnected from the sewage system. To ensure safety in cases of patient delirium and to enforce involuntary quarantine, the units were equipped with locks and safety glass. A schematic overview of the isolation department is shown in Figure 5.

Medical Treatment Protocol

A medical protocol was developed to describe the standard diagnostic procedures and medical treatment according to the available guidelines. The protocol included preemptive treatment for malaria and administration of antimicrobial drugs for possible bacterial sepsis.

Communication and Information

Flyers and banners were posted in the outpatient departments and in hospital entryways, requesting that patients, visitors, and personnel who had recently traveled to a high-risk area for EBOV infection inform hospital staff. Additionally, information was broadcast on a television screen in the emergency department entryway and waiting room. Staff at the emergency department and hospital wards were instructed to enquire actively about risk factors for VHF for patients with fever.

Local hospitals and other external partners were informed and advised about VHF procedures and referral to the MIH. Meetings to inform noninvolved hospital staff

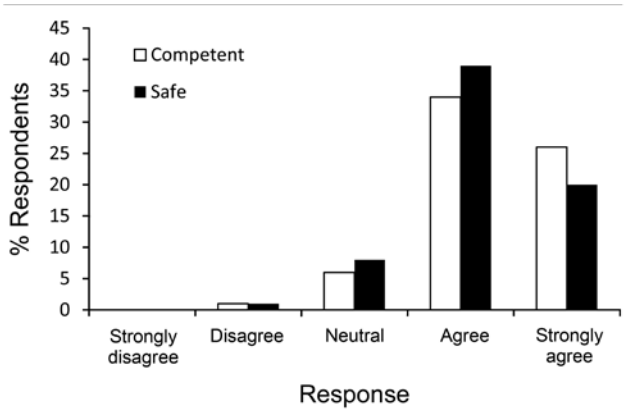

Figure 4. Results of a post-training survey conducted among staff indicating whether they felt competent and safe in caring for patients with Ebola, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014.

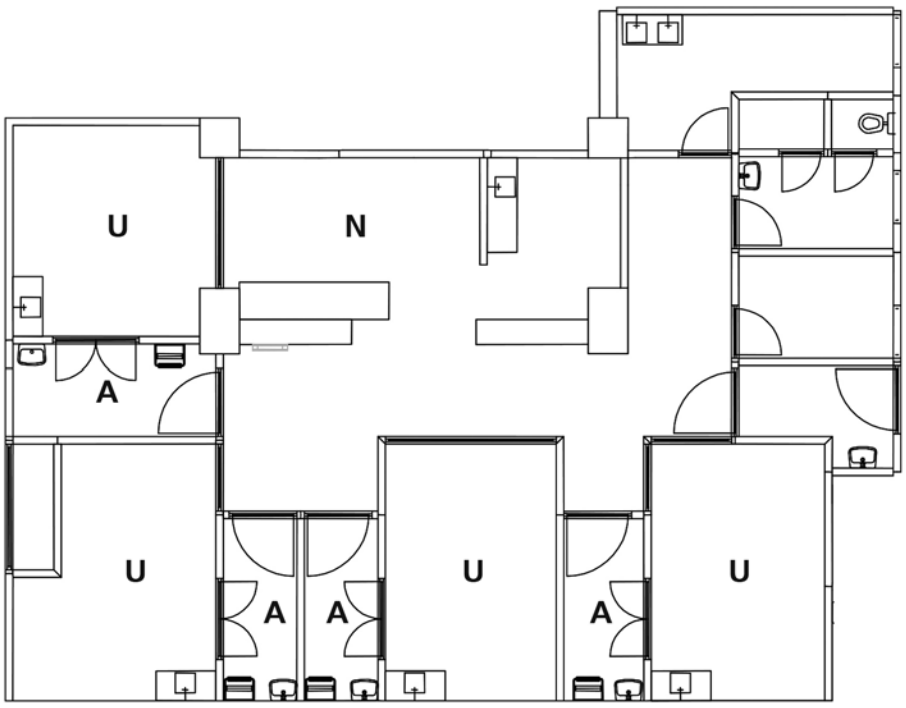

Figure 5. Schematic overview of the isolation department, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014. U, isolation unit; N, nursing station; A, access valve.

about EVD and the precautions taken by the hospital were organized and, before the arrival of an actual patient, repeated for the relatives of involved staff.

Experiences during the Admission of an Ebola Patient

On December 4, 2014, the Ministry of Health, Welfare, and Sport requested that the MIH admit an EBOV-infected soldier who was residing in a hospital in Liberia. Arrival was planned for 2 days later. Daily updates about the health status of the patient and his transport allowed optimal preparation. The command structure functioned as outlined. Additional informational meetings were held for staff and relatives and were especially appreciated by the families of personnel, whose anxiety had been amplified by the increased media coverage of Ebola.

Transfer and Admission

Before arrival of the patient, the transfer procedures were rehearsed twice with the involved ambulance and MIH personnel. Some changes had to be made to the infrastructure of the hospital entrance because of the differing PPE doffing procedures used by the designated ambulance services. It was then decided to involve no more than 2 ambulance services for future transport of VHF patients to the MIH.

At the time of patient admission, the command team, 4 trained nurses, and an infectious disease specialist were present, and intensive care unit (ICU) personnel and a cleaning team were on standby. Security personnel were present to prevent unauthorized persons from entering the unit and to prevent members of the news media from entering the hospital grounds.

The command team was continuously updated about the progress of the ambulance en route to the hospital. At 15 minutes before expected arrival, the first team of nurses started PPE donning procedures. The transfer of the patient at the hospital entrance was time-consuming because the patient was enveloped in a body bag, which resulted in more time required for triage by the first crew in PPE. The initial triage indicated no need for ICU admission. The initial diagnostics and medical treatment proved to be more time-consuming than expected. Cleaning and disinfection of the hospital entrance took >1 hour instead of the planned 45 minutes.

Treatment

All necessary medical and nonmedical activities (e.g., food delivery) were bundled to minimize the number of entries into the isolation room, resulting in 2–3 entries per 8-hour shift. Diagnostic blood samples were collected from an intravenous line to avoid high-risk procedures. Microbiological diagnostic procedures and sample transport to the diagnostic centers were supervised by the attending virologist and proceeded without incident. The results became available the same day. Coagulation tests were not available at the time; however, they were not required in this particular case.

Daily physician visits, except for replacement of the intravenous tube, were primarily conducted through the

glass window by telephone. Spiritual counseling was provided at the request of the patient, and a tablet computer was provided for distraction and contact with family. The continuous presence of 3 nurses proved necessary during all 3 daily shifts. The availability of a coordinating nurse ensured that the interaction between the buddy and the bedside nurse was never disturbed. No safety incidents occurred. The limited working time of 45 minutes in PPE proved to be appropriate; however, an additional 20 minutes for recovery seemed to be warranted. PPE stock levels were always adequate.

Waste production was lower than expected because of the relatively stable condition of the patient. The maximum number of 60-L barrels used was 8 on the first day and 3 on every following day. Guided transport of the barrels to the external facility was necessary only twice weekly. A complicating factor was the difficulty of appropriately closing the barrels in 5 instances, which necessitated the resealing of boxes inside other boxes before further transport.

Discharge

After 6–7 days, all signs and symptoms of EVD in the patient had disappeared; however, the patient was dismissed from isolated treatment another 7 days later, after 2 PCR blood test results were negative for EBOV. The discharge procedure had been described only minimally in protocols and was developed in the days before dismissal. The isolation unit was sealed awaiting decontamination of the room by hydrogen peroxide gassing, which was performed 1 day after discharge. The isolation room was not made available until 14 days later because of the mandatory incubation period, the time required to interpret biostrips used to monitor the space, and the unavailability of staff from the external company during the end-of-year holidays. The entire isolation unit was unavailable for the admission of patients on the day of gassing because of interconnected air systems.

Evaluation

All involved personnel were monitored daily for 3 weeks after their last shift, and none experienced onset of symptoms. The experiences of the admission were shared with other medical centers and the National Institute for Public Health. Revisions to the design of the isolation unit are under way and include the installation of automatic sliding doors and improvement of the communication equipment.

Discussion

The EVD epidemic in West Africa during 2014–2015 underscored the need for hospitals worldwide to prepare for outbreaks of disease caused highly pathogenic and infectious organisms (*8*,*9*). During the fall of 2014, UMC Utrecht, which was already equipped with the MIH, intensified its preparations for the admission of VHF patients. These preparations proved to be time-consuming for all key players. In addition, the frequent training of staff led to scheduling complications; however, after activation of the crisis coordination team during the preparation phase, the sense of urgency increased, and departments were more motivated to provide staff.

Tracing procedures in the hospital resulted in increased alertness for VHF in patients with fever. Simulation exercises confirmed the value of protocols for triage and care and led to improvements of the procedures. Regular repetition was needed to sustain the level of alertness and knowledge of procedures. The value of protocols has been confirmed by the experience of other Western hospitals that have cared for patients with suspected or confirmed EBOV infection (*10*,*11*). However, data about preparedness and infection prevention measures are scarce; a single report about hospital preparations indicates that the trainer-observer method was used during PPE doffing and donning and that autoclaving took place at the hospital, but other procedures were not described (*12*).

The admission of an EBOV-infected patient was an opportunity to test all developed procedures. The initial transfer proved time-consuming and warrants further training with ambulance services. Moreover, because every regional ambulance service has slightly different PPE procedures, it proved appropriate to restrict the number of involved ambulance services for a single hospital.

The treatment of 1 patient was demanding on staff resources. Because the isolation unit was located outside the regular hospital wards, additional personnel were needed to staff the front office and to secure the cooling container on the premises. PPE use occurred without incident, but the discomfort caused by PPE was the largest complicating factor and warranted limiting the time spent in PPE to 45 minutes, with a 20-minute recovery period.

Minimal requirements for the selection of PPE had already been determined by national authorities on infection prevention. However, the selection of PPE differed among the UMC Utrecht and other hospitals in the Netherlands appointed for admission of Ebola patients; for example, some centers used rubber boots (instead of clogs), a hood with a powered air supply, or cooling vests. Although some of these options might provide more comfort, they are more costly and do not always provide additional safety. The buddy system was based on the trainer-observer method of donning and doffing PPE (*4*) but was extended to all high-risk procedures conducted in the isolation unit and ensured the safety and confidence of personnel. However, it was determined that the admission of ≥4 patients simultaneously should be avoided because the presence of multiple buddies in the isolation unit would compromise the audibility of instructions.

Table 2. Key lessons learned from admission of an Ebola patient, Major Incident Hospital, University Medical Centre of Utrecht, the Netherlands, 2014

Considerations for the future
• Protocols should be in place for all procedures.
• Limit the number of ambulance services eligible for patient transfer.
• The buddy system as extension from the trainer-observer role is invaluable in care for patients with viral hemorrhagic fever.
• Regular repetition of training is necessary.
• Time in personal protective equipment should be limited to 45 minutes, with an additional 20 minutes for recovery.
• The volume of biologic waste will be more than expected, and procedures for waste management need to be explored at an early stage.
• Remote, noncontact, sensors should be explored as possible tools in diagnostics.
• Specific engineering solutions are needed for every different infection scenario.

Waste destruction had to be outsourced because of the lack of autoclave capacity at the MIH, which led to additional costs and workload. Apart from the incidental failure to close a waste container, no safety incidents occurred.

Although the patient did not need care in the ICU, the admission increased awareness that the number of trained ICU staff would be insufficient to treat a patient in need of mechanical ventilation or hemodialysis for any extended period (*13*). The staffing required for these procedures would certainly lead to a restriction of available ICU beds for other patients. An intensified training program for ICU staff was devised, and more detailed ICU protocols are in development.

Although the absence of coagulation tests did not lead to a problem in this case, it might have in the case of a severely ill patient. Limited coagulation tests became available later. Also, the use of conventional radiography was deemed impossible; portable ultrasound devices are now being tested. A recent report underscores the need for advanced protocols to perform radiologic imaging in these circumstances (*14*).

During admission, protocols for discharge were still under development, leading to last-minute changes and some agitation among staff; however, discharge itself went well. The final cleaning procedure took longer than expected; on the day the patient was discharged, all 4 rooms were unavailable because the air treatment systems were interconnected. The system will therefore be adjusted (bifurcated) in the future.

Communication was of utmost importance; not only did the hospital staff need information, but so did their relatives, who were concerned about the risks of working with Ebola patients. Also, the demand for extra security personnel was high because of the need to secure the stored waste and limit access to the MIH.

Although the isolation units in UMC Utrecht are located in a separate facility (the MIH), our experiences might be useful in other hospital settings (Table 2). Existing international and national protocols describe only the minimum requirements and therefore are not suitable for comparison. The preparations made and the lessons learned during the admission of an Ebola patient confirm the necessity of clear and practical protocols, a buddy system, and intensive staff training, all of which increase the safety of healthcare workers. Because of these measures, we experienced virtually no reluctance of personnel to be involved in the care of VHF patients. The demand on resources to treat VHF patients is high and can lead to understaffing at other departments at the expense of other patients. The availability of a dedicated major incident hospital has greatly increased the resources and preparedness of our center.

Mr. Haverkort is a medical doctor working with the MIH at UMC Utrecht. He conducts research in the field of disaster medicine and has a special interest in optimization of preparedness for disaster scenarios.

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Trematode Fluke *Procerovum varium* as Cause of Ocular Inflammation in Children, South India

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Trematodes are recognized as a group of emerging parasites in tropical countries. We identified a trematode as a cause of ocular granulomas that developed in children who bathed in ponds or rivers in South India. DNA was isolated from patients' surgically excised granulomas and from the trematode cercariae (larvae) released by the snail *Melanoides tuberculata* in water in which the children bathed. Real-time and conventional PCRs were performed that targeted ribosomal DNA regions spanning the internal transcribed spacer 2 and 28S sequences of this trematode. The PCR-amplified products were subjected to bidirectional sequencing. Analysis of sequences for the granuloma samples and the trematode cercariae showed maximum sequence similarity with *Procerovum varium* (family Heterophyidae). Our results confirmed the etiology of the ocular infection, implicating snail vectors as environmental risk factors for ocular parasitosis.

Diseases caused by helminths (e.g., nematodes, cestodes, and trematodes) are a major public health concern worldwide, particularly in developing countries because of poor hygiene, lack of public health education, and limited medical resources (*1,2*). The people of Southeast Asia are especially at risk for exposure to at least 70 species of foodborne and waterborne trematodes, including blood flukes, intestinal flukes, liver flukes, and lung flukes (*3*). However, epidemiologic data on parasitic diseases of trematode origin in the Indian subcontinent are scarce because of lack of screening programs. The diagnosis of parasitic diseases is much more difficult when the patient is not a definite host but is instead an intermediate or accidental host; in such cases, fecal egg identification is of no use. Serum samples are also unreliable mainly because of cross-reactive antigens (*4,5*). Molecular diagnostics can play a vital role in overcoming these obstacles and may lead to a precise diagnosis (*6,7*).

Ocular infections caused by helminths in human are rare. Among helminths that affect the eye, few have a natural predilection for the eye as their habitat; consequently, ocular invasion may occur by accident but results in eye disease (*8*). Several sporadic reports from various parts of the world have identified trematodes (i.e., *Philophthalmus* spp., *Fasciola hepatica*, and schistosomes) in the conjunctival sac and anterior chamber of the eye (*9–16*). On the basis of histopathologic work-up, researchers from our hospital previously reported possible trematode infection in a large group of children with ocular inflammation (*17,18*). We later reported molecular evidence that established the trematode *Procerovum varium* as the source of the ocular granuloma in a single patient from the same region as that of the children in the previous study (*19*).

Members of the genus *Procerovum* (class Trematoda, subclass Digenea, family Heterophyidae, subfamily Haplorchiinae) parasitize predominantly fish-eating birds, which are the definitive hosts. In the definitive host, the cercariae mature to adults and lay eggs that are discharged with the host feces into the environment and surrounding bodies of water. The eggs hatch to release free-swimming miracidia, which infect snails, the first intermediate hosts. The cercariae released from the snails use various freshwater and marine fish as the second intermediate hosts (*20,21*). Humans become accidental hosts when they are exposed to these cercariae and become infected by eating infected, uncooked fish. *Procerovum* spp. are known to inhabit China, Japan, the Philippines, Australia, Taiwan, India, Vietnam, and Thailand (*20–24*). Ocular parasitosis caused by *Procerovum* spp. was first reported in fish in the Philippines (*22*). In South India, infection with the trematode *P. varium* was reported in the pond heron *Ardeola grayii*, and heavy infections with metacercariae were also found in the fish *Oryzias melastigma* (*21*).

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DOI: http://dx.doi.org/10.3201/eid2202.150051

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Identifying the exact species of trematode in granulomatous tissue is morphologically and histopathologically difficult for various reasons. First, when the granuloma is aspirated with a fine needle, the parasite comes out in pieces and loses its morphology. Second, the parasite degenerates rapidly because of an immunologic reaction in the host. Recent development of molecular methodologies like real-time PCR, sequencing, and BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) offer opportunities for identifying the parasite at the species level (*25*–*28*).

Our previous study involving a single patient with ocular granuloma pointed to infection by the trematode *P. varium* (*19*). During March 2010–February 2013, ocular complications were being detected in children in South India in whom ocular granulomas developed after they were exposed to snail-infested water in ponds or rivers. We sought to confirm the etiology of the granulomatous eye disease. To discover and ascertain the causative agent of the disease, we performed DNA-based molecular analysis, targeting trematode larvae as they developed and were subsequently released by the vector snails.

Materials and Methods

Patients' Granuloma Sample Analysis

The study protocol was approved by the Institutional Review Board of the Aravind Eye Care System, Madurai, India. All procedures adhered to the tenets of the Helsinki Declaration. Informed consent was obtained from patients or their parents after they received a detailed explanation of the study. The study included 35 boys and 7 girls, 6–17 years of age, who had visited Aravind Eye Hospital in Madurai during March 2010–February 2013 with a history of redness, itching, and swelling of the eye. Of the 42 children, 28 had anterior chamber granuloma (≈2–3 mm diameter); 14 had subconjunctival granuloma (≈5 mm diameter). The patients were from 33 different villages in the states of Tamil Nadu and Kerala in South India.

All patients underwent a complete ocular examination with a slit lamp and indirect ophthalmoscopy, and each also had a complete physical examination. Common causes of eye disease, such as tuberculosis, sarcoidosis, and fungal granuloma, were ruled out by clinical, radiologic, serologic, and histopathologic examinations. Patients' granuloma samples were tested with nested PCR that targeted the MPB64 and 28S rDNA genes to rule out the possibility of tuberculosis and fungal infections, respectively. All 42 granuloma samples were subjected to assays (Power SYBR Green PCR Master Mix; Applied Biosystems, Warrington, UK) targeting rDNA spanning the internal transcribed spacer (ITS) 2 sequence of the trematode with custom-designed primers. The real-time PCR–amplified products were analyzed by using bidirectional sequencing and BLAST analysis to identify the trematode at the species level.

Excision of the Granuloma and DNA Isolation

Twenty-eight anterior chamber granulomas were aspirated from children under general anesthesia by using aseptic precautions and a 25-gauge needle passed through the limbus (*18*). Fourteen subconjunctival granulomas were surgically excised from children under general anesthesia. Samples were stored at –80°C. Total genomic DNA was extracted and purified from the 42 biopsied specimens by using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany), according to the manufacturer's instructions (*28*). The granulomas were first immersed in 180 μL of ATL buffer (QIAGEN) with 20 μL proteinase K at 56°C for 2 h. Granulomatous tissue was lysed by using the QIAamp DNA Mini Kit tissue lysis procedure. DNA was extracted with a final elution volume of 100 μL and stored at –20°C.

Real-Time PCR Assay

The rDNA sequence spanning the ITS2 region was amplified from DNA obtained from the biopsied specimens. Real-time PCR was performed according to standard protocol (*25*–*27*). To identify the trematode by real-time PCR, a new set of primers was designed in our laboratory on the basis of conserved ITS2 sequences of the digenean trematodes, including *P. varium*, *P. cheni*, *Haplorchis pumilio*, and others belonging to the Heterophyidae sequences available in GenBank (Table 1). Real-time PCR was conducted in a 25-μL reaction mixture containing 12.5 μL

Table 1. Primers used for amplification of the DNA region ITS2 and the 28S rDNA of trematode found as the source of ocular granulomas in children, South India*

DNA partition	Primer name	Primer sequence, 5′→ 3′	DNA amplicon size, bp	Application	Reference
ITS2	3SF	GGTACCGGTGGATCACTCGGCTCGTG	539	PCR and sequencing	(*29*)
	BD2R	GGGATCCTGGTTAGTTTCTTTTCCTCCGC			
ITS2	AP101F	ATGAATGGCGCAGCTTTGACATCG	156	Real-time PCR	This study
	AP101R	AAAGCACAAGGAAATCACGCCAGC			
ITS2	AP102 F	AGAGCGCAGCCAACTGTGTGA	369	Real-time PCR and sequencing	This study
	AP102 R	TGCCACGTCCTAGCATCAGCC			
28S rDNA	AP103 F	AGAGCGCAGCCAACTGTGTGA	715	PCR and sequencing	This study
	AP103 R	TGCCACGTCCTAGCATCAGCC			

*ITS, internal transcribed spacer.

(× 2) of Power SYBR Green PCR Master Mix solution, 2.0 μL of double-distilled DNase-free water (Affymetrix, Cleveland, OH, USA), 100 nmol/L of each primer, and 10 μL of the DNA extracted from each granuloma sample. PCR was performed on an ABI 7900HT Fast Real-Time PCR System (Applied Biosystems) with the following cycling conditions: 50°C at 2 min, 95°C for 10 min, and 40 cycles of amplification (95°C for 15 s and 60°C for 1 min). No template controls (i.e., nuclease-free water) were included. Quantitative standards (i.e., DNA of *F. gigantica* recovered from cow liver) were included each time, and PCR was undertaken to detect false-positive results that could occur because of contamination and to construct a standard curve. Products amplified with real-time PCR were further analyzed by agarose gel electrophoresis. All assays were performed in triplicate.

DNA Sequencing and BLAST Analysis

The real-time PCR amplicons were analyzed on 2% agarose gel stained with ethidium bromide. Confirmed samples were further subjected to bidirectional sequencing. The amplified products were loaded on the gel and purified by using the DNA Purification Kit (Promega, Madison, WI, USA). The cyclic sequencing reaction was performed with Big-Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Samples were denatured at 96°C for 2 min and then cycled 28 times at 96°C for 10 s, 52°C for 10 s, and 60°C for 4 min. Unincorporated nucleotides were removed by using sodium carbonate, 125 mmol/L EDTA, and absolute alcohol. HI-Di Formamide (Applied Biosystems) was used to stabilize the single-strand template before sequencing was performed in the ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were analyzed by using BLAST for species-level identification.

Environmental Sample Analysis

Sampling of Snails and Harvesting of Trematode Cercariae

Village ponds and rivers were selected on the basis of patients' history of frequent bathing in them before symptoms developed. Village maps were obtained from village authorities, and ponds and rivers were surveyed for snail collection. We visited 68 villages in 11 districts in Tamil Nadu to collect snails (Figure 1) during a 3-year period (March 2011–February 2014).

Snails were collected by hand picking them from the muddy ponds and rivers of the disease-affected localities. We collected ≈7,200 snails comprising 7 species. Each site was sampled twice during the study period. Snails were placed in plastic containers filled with water from the same habitat and transported alive to the laboratory. The snails were cleaned by using pond water to reduce the debris and placed separately in a small plastic petri dish containing 50 mL of filtered pond water. The snails were exposed to sunlight for 1.5–2 h to induce shedding of cercariae. Each snail was carefully observed under a dissecting microscope every day after the exposure of sunlight; when the shedding was complete, the cercariae were separated. The snails were maintained in the laboratory at room temperature for up to 2 weeks. The filtered pond water was replenished daily to avoid pH changes (*30–32*).

The procedure was repeated until several cercariae were obtained from each species of snail. The released cercariae were collected individually and placed in 95% ethanol for DNA extraction and 10% formalin for morphologic identification by using a borax-carmine procedure, as described (*33*). After complete cercarial examination, snails were treated with 10% sodium hypochlorite and dried in sunlight. The dry shells of snails were sent to the Zoological Survey of India in Kolkata for species identification.

DNA Isolation from Trematode Cercariae

Cercariae preserved in 95% ethanol were centrifuged at 12,000 rpm for 10 min, after which the supernatant was

Figure 1. Eleven district sites (gray shading) where snails were collected in the state of Tamil Nadu, India, for testing as part of a study of ocular granulomas in children. Inset shows location of Tamil Nadu in India.

discarded and pellets were air-dried. Genomic DNA extraction from cercariae was performed by using the QIAamp DNA Mini Kit, according to the manufacturer's instructions (*28*). DNA was eluted with 100 µL of buffer AE from the DNeasy spin column (QIAGEN). The DNA concentration was measured by using the NanoDrop spectrophotometer (Thermo Fisher Scientific, Grand Island, NY, USA) and was stored at –20°C.

PCR Assay

Unlike the clinical samples, which used real-time PCR to increase sensitivity because of limited amounts of DNA, conventional PCR was used for the environmental samples because of availability of adequate amounts of DNA from freshwater snails. The ITS2 and 28S rDNA regions were amplified from the trematode cercariae DNA by using standard protocol (*29,34–36*). For species-level identification of the trematode, we used universal primers created on the basis of conserved ITS sequences of the *Schistosoma* species of trematodes (*29*). We also used custom-designed primers targeting the conserved ITS2 and 28S rDNA sequence of the digenean trematodes, including *P. varium*, *P. cheni*, *H. pumilio*, and others belonging to the family Heterophyidae (reported in GenBank; Table 1). PCR was carried out in a 20-µL reaction mixture containing 10X PCR buffer, 10 mmol/L deoxynucleotide triphosphates, 25 mmol/L magnesium chloride, 3 U/µL Taq DNA polymerase (these 4 reagents from Bangalore Genei, Bengaluru, India), 1X Q-solution (Qiagen), 7 pmol of each primer, and 5 µL of DNA template (cercariae DNA). PCR conditions for ITS2 (Thermal Cycler PTC-200; Bio-Rad, Hercules, CA, USA) were as follows: 5 min at 94°C for initial denaturation, followed by 35 cycles (30 s at 94°C for denaturation, 38 s at 57°C for primer annealing, and 72°C for 42 s for extension) for adequate amplification, and a final extension at 72°C for 7 min. The PCR conditions for 28S rDNA were similar to ITS2 except that we used 56°C for primer annealing and 1 min for the initial extension. After amplification, electrophoretic separation of PCR products was performed on 1.5% agarose gel prestained with ethidium bromide and visualized by ultraviolet illumination.

Molecular Sequencing of Environmental Trematode DNA

The amplified PCR products from the DNA extracted from the environmental trematode cercariae were subjected to bidirectional sequencing in the ABI 3130 Genetic Analyzer, and the sequences were deposited in GenBank (accession nos. KM226892–KM226899). BLAST analysis of the sequences was performed for species-level identification.

Results

Patient Demographics and Clinical Findings

Forty-two children with ocular granulomatous inflammation who sought care at our hospital were all exposed to pond or river water. None had a history of consumption of raw or undercooked fish. Fourteen of the 42 children had subconjunctival granuloma (Figure 2, panel A); 28 had anterior chamber granuloma (Figure 2, panel B). Results of general physical examinations were unremarkable. None of the patients had chronic systemic granulomatous disease, which is known to be associated with uveitis and includes tuberculosis and sarcoidosis.

Molecular Analysis of the Patient Samples

Results of testing for 13 of the 42 granuloma samples analyzed were positive for trematode DNA by using SYBR Green quantitative real-time PCR. Of the 13 patients with positive samples (Table 2), 8 had subconjunctival granulomas, and 5 had anterior chamber granulomas. Real-time PCR was performed on 2% agarose gel, and BLAST analysis of amplified sequences revealed the closest identity with the trematode *Procerovum* spp. (family Heterophyidae) (GenBank accession no. KM226891; Figure 3, panels A–C). All samples were found by nested PCR to be negative for *Mycobacterium tuberculosis* and fungal infection.

Environmental Sample Analysis

A total of 7 species of snails were collected from 68 village ponds and rivers. On the basis of shell morphology, snails were identified by the Zoological Survey of India as *Bellamya dissimilis, Pila virens, Melanoides tuberculata, Lamellidens marginalis*, *Paludomus transchauricus*, *Indoplanorbis exustus*, and *Thira scraba*. Among these snail species,

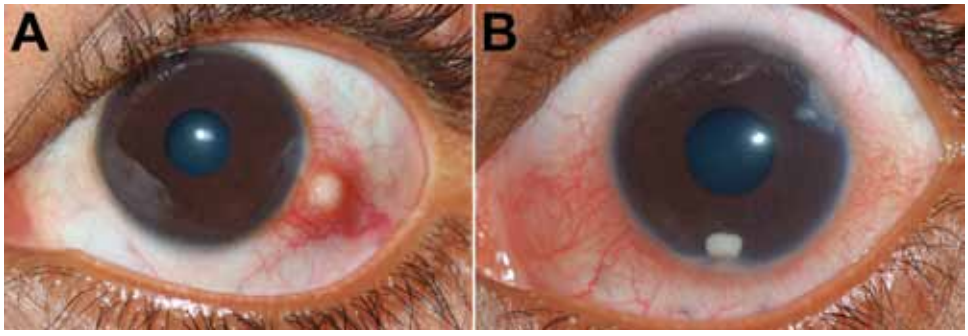

Figure 2. Clinical photographs of patients' eyes in study of ocular granulomas in children, South India. A) Left eye of a 14-year-old boy with a distinct subconjunctival granuloma; B) left eye of a 7-year-old boy with distinct grayish-white granuloma in the eye's anterior chamber.

Table 2. Summary of clinical features of 13 patients whose samples were positive for trematode DNA in study of ocular granulomas in children, South India*

Sample no.	Type of granuloma	Patient age, y/sex	Duration of granuloma, ≈d
1	SCG	9/M	30
2	ACG	9/F	60
3	ACG	11/M	30
4	SCG	11/F	7
5	SCG	15/M	35
6	ACG	9/M	60
7	SCG	11/M	30
8	SCG	11/M	15
9	SCG	18/M	30
10	ACG	7/M	90
11	ACG	14/M	15
12	SCG	6/M	15
13	SCG	16/M	10

*Testing performed with Power SYBR Green Real-Time PCR (Applied Biosystems, Warrington, UK). SCG, subconjunctival granuloma; ACG, anterior chamber granuloma. All patients reported bathing in a pond or river, and all trematodes were determined to be *Procerovum* spp.

only *M. tuberculata, I. exustus*, and *T. scraba* were found to be infected with trematode larvae and released cercariae in the laboratory, although all snail types were exposed to the same environmental conditions. The snail *M. tuberculata* was found in 57 of the 68 water bodies surveyed and comprised on average 20% (range 0%–40%) of all snails collected from the sites. *M. tuberculata* snails released the trematode cercaria identified as *P. varium* (Figure 4, panels A, B). Figure 5 (panels A–C) shows the PCR-amplified product of trematode cercaria DNA that was performed on 1.5% agarose gel. Molecular sequencing and BLAST analysis of the PCR amplicon sequence confirmed maximum sequence similarity with *P. varium* (GenBank accession nos. KM226892 and KM226894) (Figure 6, panels A, B). Besides releasing *P. varium,* the *M. tuberculata* snails also released cercariae of 3 other species, represented by *Haplorchis pumilio* (GenBank accession no. KM226895; family Heterophyidae), *Giganthobilharzia melanoidis* (GenBank accession no. KM226896; family Schistosomatidae), and an unidentified species of the Renicolidae family (GenBank accession no. KM226897). Cercariae released by snails of the other 2 species, *T. scraba and I. exustus*, were identified as *Acanthostomum burminis* (GenBank accession no. KM226898) and *Isthmiophora hortensis* (GenBank accession no. KM226899), respectively.

Discussion

In this study, pediatric granulomatous eye disease developed in a group of 42 children with a history of exposure to village pond and river water in sites we surveyed in South India. Common causes of granulomas (e.g., tuberculosis, sarcoidosis, and fungi) were ruled out by various diagnostic (i.e., clinical, radiologic, serologic, and histopathologic) techniques. Our study results, determined by using molecular techniques, confirmed that the cercaria stage of a digenetic trematode, *P. varium* (family

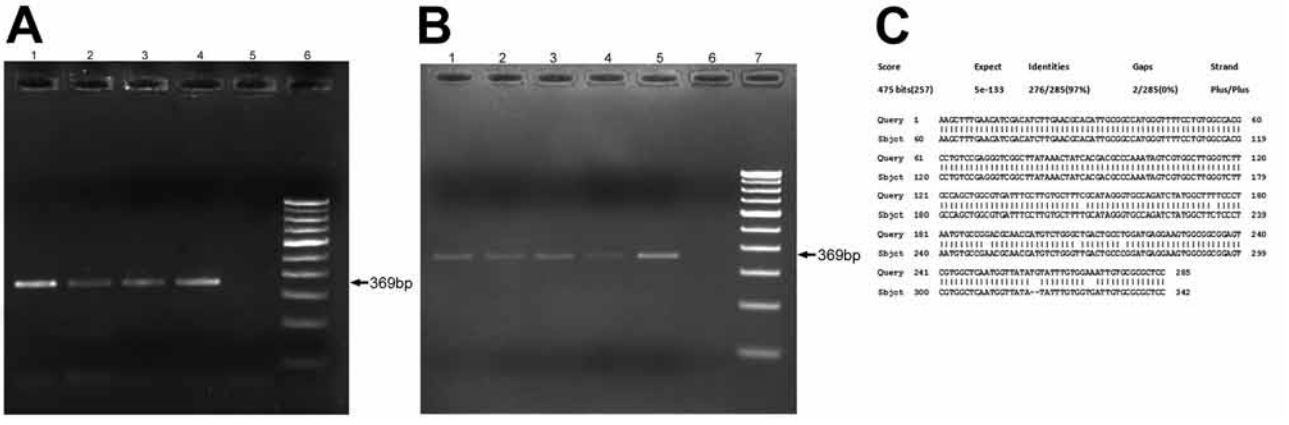

Figure 3. Real-time PCR amplification of ocular granuloma DNA obtained from patients infected with trematodes, South India. Gel electrophoresis was performed on 2% agarose gel by using Power SYBR Green Real-Time PCR (Applied Biosystems, Warrington, UK). A) Lanes 1–4 show subconjunctival granuloma DNA; lane 5, negative control; lane 6, 100-bp DNA marker. Arrow indicates 369-bp amplified DNA product. B) Lanes 1–5 show anterior chamber granuloma DNA; lane 6, negative control; lane 7, 100-bp DNA marker. Arrow indicates 369-bp amplified DNA product. C) BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis output of patient granuloma DNA sequence showing maximum identity with internal transcribed spacer 2 region gene sequence of the *Procerovum* species resembling GenBank reported sequence EU826639.1 from Vietnam.

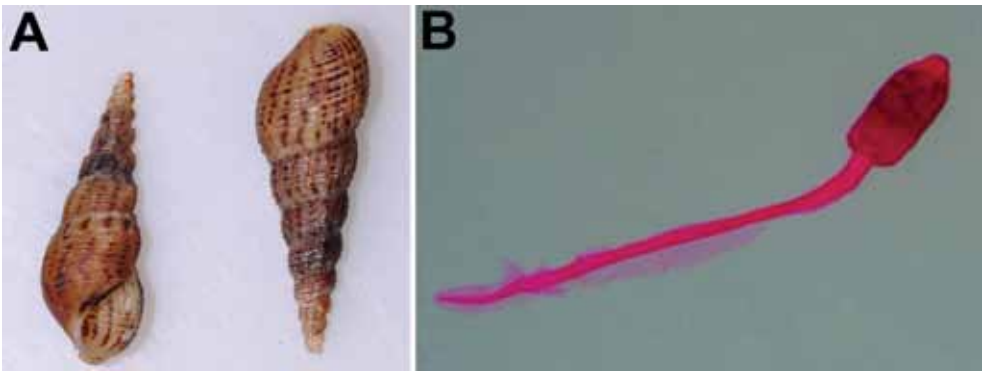

Figure 4. Snail and trematode cercaria from study of ocular inflammation in children, South India. A) *Melanoides tuberculata* snails collected from a pond that was the focus of the infection. B) Staining and light microscopy image of the cercaria larval stage recovered from the snails (original magnification ×200).

Heterophyidae), was implicated in human granuloma tissue. The cercariae are environmental pollutants in snail-infested waters.

Heterophyid flukes, including *Procerovum* spp., need 1 definitive host and 2 intermediate hosts to complete their life cycle. The fluke releases embryonated eggs in the host's feces. The cercaria larvae develop in snails, the first intermediate host; the cercariae encyst as metacercariae in the tissues of a suitable fish, which is the second intermediate host for the parasite. The definitive host is infected by ingesting raw or undercooked fish containing metacercariae; after ingestion, the metacercariae excyst (i.e., attach to the mucosa of the small intestine) and mature into adults. Haplorchid metacercariae were abundantly reported in many freshwater fish in Taiwan and caused cercarial infection in the eye of eel; histopathologic sections showed numerous metacercariae in the muscle tissues, subcutaneous tissue, and cartilage, and edema and hemorrhage were seen in the eye (*20,22*).

In India, heavy infections of metacercariae were reported in the freshwater fish *O. melastigma* (*21*). Morphologically, these metacercariae were identified as the cysted stage of *P. varium*. Natural infections of *P. varium* were found in birds (e.g., the pond heron *A. grayii*) in the same geographic area. Adult flukes of these parasites were successfully raised from metacercariae in chicks, ducklings, and mice. Laboratory and field studies confirmed that the snail *Thiara tuberculata* acts as the first intermediate host (*21*).

In our environmental analysis, of 7 species of snails identified, *M. tuberculata* and 2 other species released cercariae in the laboratory. *M. tuberculata* snails were found predominantly in most village ponds and rivers tested. The DNA sequence of the cercarial larva isolated from the snail was identical to that of *P. varium* seen in the patients' granuloma samples. Besides *P. varium, M. tuberculata* snails also released cercariae of 3 other trematode species: *H. pumilio, Giganthobilharzia melanoidis*, and a representative of the family Renicolidae. *Haplorchis* is a fish-borne intestinal fluke that is highly prevalent in Southeast Asia, but little is known about the infection dynamics and

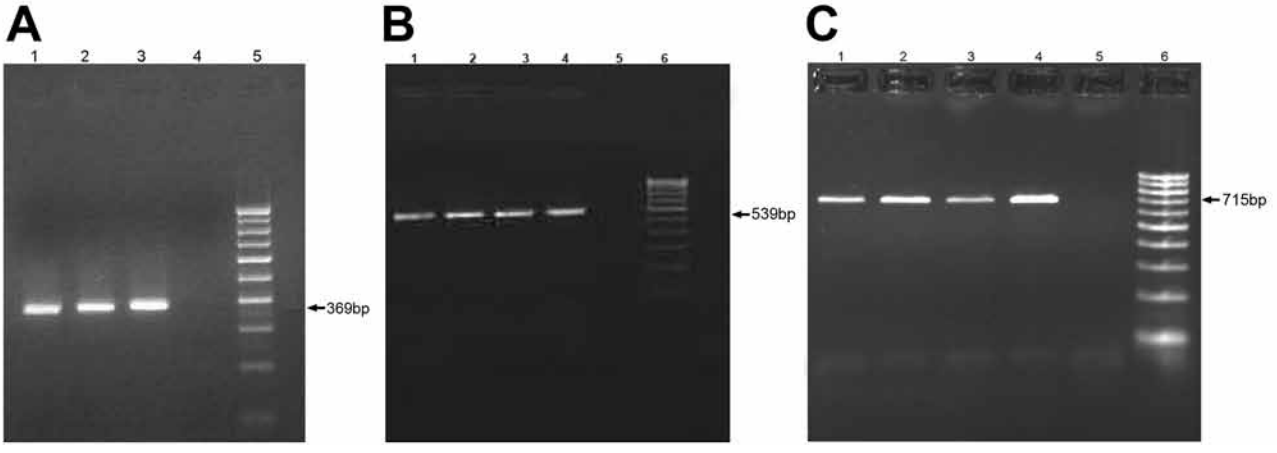

Figure 5. PCR amplification of trematode cercaria DNA obtained from *Melanoides tuberculata* snails in study of ocular inflammation in children, South India. Gel electrophoresis was performed on 1.5% agarose gel. A) Internal transcribed spacer 2 region; arrow indicates 369-bp amplified DNA product. Lanes 1–3, trematode cercariae DNA; lane 4, negative control; lane 5, 100-bp DNA marker. B) Internal transcribed spacer 2 region; arrow indicates 539-bp–amplified DNA product. Lanes 1–4, trematode cercariae DNA; lane 5, negative control; lane 6, 100-bp DNA marker. C) 28S rDNA region; arrow indicates 715-bp amplified DNA product. Lanes 1–4, trematode cercariae DNA; lane 5, negative control; lane 6, 100-bp DNA marker.

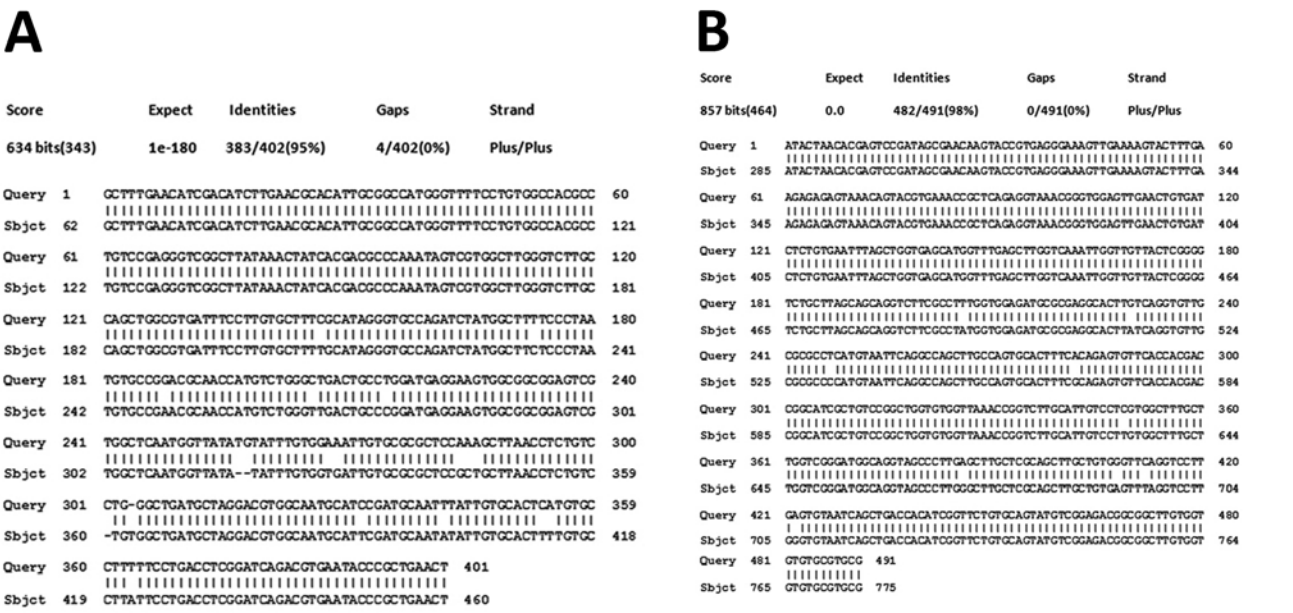

Figure 6. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) analysis output of environmental trematode cercaria DNA sequences from South India. A) Internal transcribed spacer 2 DNA sequence shows maximum identity with *Procerovum* species and resembles GenBank reported sequence EU826639.1 from Vietnam. B) 28S rDNA sequence shows maximum identity with *Procerovum varium* and resembles GenBank reported sequence HM004184.1 from Thailand.

clinical symptoms in hosts, including humans. However, none of these 3 cercarial types were found in our patients' ocular granulomas.

In our patients' sample analysis, 13 (31%) of 42 samples were positive for *P. varium*. Why the remaining patients' samples were negative for trematode DNA is unclear. It is possible that the parasite structures disintegrated rapidly as a result of the localized intense inflammatory response of the host or because of necrosis. Alternatively, different species could have caused the illness. When tested by nested PCR, all samples were negative for *Mycobacterium tuberculosis* and fungus. Potentially blinding granulomatous eye disease in children was previously misdiagnosed as tuberculosis; for several decades, these children were receiving antituberculosis treatment, which was ineffective in controlling the inflammation (*37*). In Brazil, similar types of ocular infection were reported as adiaspiromycosis caused by nonbudding, thick-walled adiaconidia of the Emmonsia spp. fungus (*38*). However, none of our samples showed evidence of fungus or tuberculosis by histopathology or molecular methods.

Although parasitic diseases are a major public health problem in developing countries, they are grouped under "neglected tropical diseases" (*39*). We isolated 5 more trematode species from snails in the same region in addition to *P. varium*, but little is known about the infection dynamics and clinical symptoms in their hosts. Further research is needed to understand the prevalence of various trematode-borne diseases, including ocular parasitosis, in South India.

Acknowledgments

We thank all participants who enrolled in this study; the Zoological Survey of India for help in identification of snails; Vinayaga Moorthy for help in field work and snail collection; the nursing sisters of Aravind Eye Hospitals for help in recruiting patients; and the Department of Ocular Microbiology at the Aravind Medical Research Foundation for help in sample collection.

The study was funded by the Indian Council of Medical Research, New Delhi, India, and supported by Aravind Medical Research Foundation, Madurai, Tamil Nadu, India.

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Association between Landscape Factors and Spatial Patterns of *Plasmodium knowlesi* Infections in Sabah, Malaysia

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The zoonotic malaria species *Plasmodium knowlesi* has become the main cause of human malaria in Malaysian Borneo. Deforestation and associated environmental and population changes have been hypothesized as main drivers of this apparent emergence. We gathered village-level data for *P. knowlesi* incidence for the districts of Kudat and Kota Marudu in Sabah state, Malaysia, for 2008–2012. We adjusted malaria records from routine reporting systems to reflect the diagnostic uncertainty of microscopy for *P. knowlesi*. We also developed negative binomial spatial autoregressive models to assess potential associations between *P. knowlesi* incidence and environmental variables derived from satellite-based remote-sensing data. Marked spatial heterogeneity in *P. knowlesi* incidence was observed, and village-level numbers of *P. knowlesi* cases were positively associated with forest cover and historical forest loss in surrounding areas. These results suggest the likelihood that deforestation and associated environmental changes are key drivers in *P. knowlesi* transmission in these areas.

Since the initial description of a large cluster of human infections with the zoonotic malaria *Plasmodium knowlesi* in Malaysian Borneo in 2004, increasing numbers of *P. knowlesi* cases have been identified throughout Southeast Asia (*1,2*). Although most persons infected with *P. knowlesi* respond to treatment, infection can cause severe and fatal disease (*3*). Understanding the distribution of *P. knowlesi* malaria and risk factors associated with this disease is critical for designing appropriate public health interventions.

Carried by long- and pig-tailed macaques (*Macaca fascicularis* and *M. nemestrina*), the *P. knowlesi* parasite has a geographic range limited by the distribution of mosquito vectors and simian hosts (*2*). Within this range, risk for *P. knowlesi* infection in humans is highly variable. Although sporadic *P. knowlesi* cases have been reported in several Southeast Asia countries, *P. knowlesi* is the most common cause of human malaria Malaysian Borneo, the portion of the country that lies on the island of Borneo (*1,2*). In the state of Sabah, suspected *P. knowlesi* notifications increased from 2% (59/2,741) of total malaria notifications in 2004 to 62% (996/1,606) in 2013 (*4,5*).

Molecular studies indicate that zoonotic *P. knowlesi* is not a newly emergent malaria species and is likely to predate human settlement in Southeast Asia (*6*). *P. knowlesi* was first described in macaques in the 1930s, and the first naturally acquired human case was reported in 1965 in peninsular Malaysia (*7,8*). However, true incidence and effects of *P. knowlesi* are poorly understood because of its frequent misidentification by microscopy as other human malaria species and because of limited availability of *P. knowlesi*–specific molecular diagnostic capabilities. *P. knowlesi* appears microscopically similar to the human malaria species *P. malariae* but can also be misdiagnosed as *P. falciparum* or *P. vivax* (*3*). The extent to which improved detection has contributed to recent increases in numbers of human cases is difficult to determine; however, the rise in *P. knowlesi* relative to other malaria species strongly suggests that *P. knowlesi* transmission has increased in Malaysian Borneo (*4,5*).

Land use changes, such as deforestation and agricultural expansion, have been proposed as the main drivers of this apparent emergence (*3*). Deforestation and related forest activities have been associated with changes in malaria vector populations and related disease incidence globally (*9*). Changes in vegetation, microclimate, and soil composition can affect the species composition and abundance of mosquito populations (*10*). In Malaysia,

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DOI: http://dx.doi.org/10.3201/eid2202.150656

studies have implicated the primarily exophagic *Anopheles leucosphyrus* group of mosquitoes as the main vector of *P. knowlesi* and have found relatively high biting rates in farm edges bordering forests and forest areas (*11–14*). Human-disturbed environments have been associated with changes in behavior of nonhuman primates and their increased contact with humans (*15*). Fragmentation of existing habitats can also increase the frequency of disease transmission by creating transition areas with increased spatial overlap among human, mosquito, and wildlife populations or by altering vector ecology (*16,17*). The effects of these changes at forest edges have been described for malaria and other vector-borne zoonotic diseases (*18,19*) but not for *P. knowlesi*. A previous mathematic modeling study highlighted the potential for increased transmission resulting from increased spatial overlap among people, macaques, and mosquitoes at forest edges (*20*).

Despite these apparent links between land use and *P. knowlesi* transmission, detailed environmental risk factors for *P. knowlesi* infections in humans are unknown. Although variability of *P. knowlesi* risk has been reported at a regional scale, patterns of *P. knowlesi* transmission have not been described at a subdistrict spatial scale (*2,21*). Furthermore, studies that formally evaluate associations between *P. knowlesi* and characteristics of environment and landscape are lacking.

After obtaining approval from the Medical Research and Ethics Committee of the Ministry of Health in Malaysia, we examined the changing incidence of *P. knowlesi* in Kudat and Kota Marudu districts in northwestern Sabah, Malaysia, on the island of Borneo, an area with relatively high *P. knowlesi* transmission (*22*). Our aim was to describe the spatial and temporal patterns of *P. knowlesi* incidence within these districts and to explore potential associations between village-level incidence and deforestation and other environmental factors. Clarifying these relationships is vital to predicting and responding to future disease outbreaks and understanding the underlying mechanisms of *P. knowlesi* emergence.

Methods

Study Site and Population

This study was conducted in the districts of Kudat and Kota Marudu in northwestern Sabah, Malaysia (07.38°–06.19°N, 116.62°–117.46°E), an area of 3,204 km^2 with a population of ≈120,000 persons predominantly of Rungus and Dusun ethnicities (*23*) (Figure 1). The climate is tropical, with no dry season and increased rainfall during November–March; the area has both coastal and inland regions and elevations ranging from sea level to 1,000 m above sea level. Substantial environmental change is ongoing in the region because of conversion of land for oil palm plantations and other agricultural activities (*24*). Both districts have central referral hospitals that serve defined catchment areas where patients have access to diagnosis

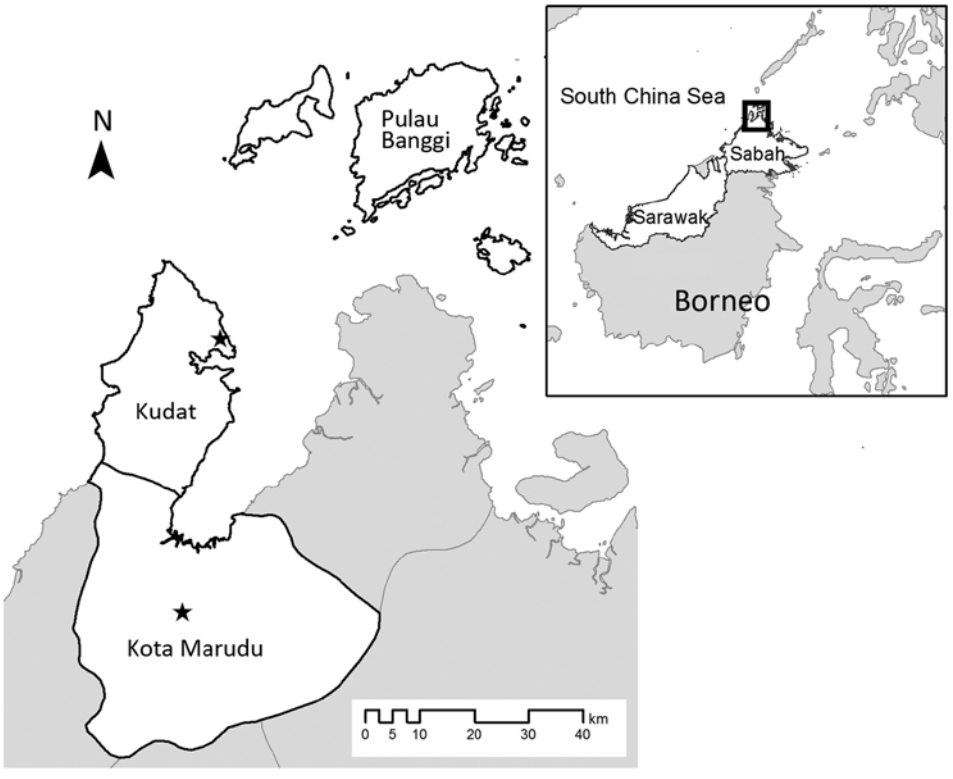

Figure 1. Location of Kudat and Kota Marudu districts in Sabah, Malaysia, where study of association of landscape and environmental factors and incidence of *Plasmodium knowlesi* transmission was conducted. Stars indicate location of the district hospitals. Inset shows location of these districts on the island of Borneo (box).

and treatment free of charge. All clinics refer patients to the central district hospital, where hospitalization is mandatory for malaria patients until a negative blood smear for malaria parasites has been obtained.

Geolocation of Patients and Villages

We conducted a retrospective review of malaria patients reported by Kudat and Kota Marudu district hospitals during 2008–2012. Data on diagnosis, admission date, demographics, and address of all malaria patients were obtained from hospital laboratory microscopy records. Villages and populations were identified from the 2010 Population and Housing Census in Malaysia (*23*), the most recent census conducted in this area, and the global positioning system coordinates of village centroids were recorded as part of a larger interdisciplinary study (MONKEYBAR ESEI project; http://malaria.lshtm.ac.uk/MONKEYBAR). Village populations were updated by using published population growth rates for Malaysia (*25*). All locations were confirmed by using imagery available through Google Earth (https://www.google.com/earth/) or other freely available satellite data. Patient addresses were matched to the census data or to the nearest reported village from the census data. Administrative boundaries were used to define the extent of urban areas, within which village data were combined.

Calculating the Proportion of Malaria Patients with *P. knowlesi*

P. knowlesi is microscopically similar to *P. malariae* but can also be misdiagnosed as *P. falciparum* or other human malaria species. In hospital microscopy records, the species of malaria is recorded, as determined by morphology, but no separate listing for *P. knowlesi* exists. Consequently, uncertainty in diagnosis resulted in some infections being recorded as *P. malariae* or *P. malariae/knowlesi*. To estimate the true proportion of *P. knowlesi*, we calculated the sensitivity and specificity of microscopy diagnosis of *P. malariae* for a subset of 539 malaria patients for whom both microscopy and molecularly confirmed results were available, including all patients from Kudat and Kota Marudu hospitals who were referred to a tertiary care hospital during this period and all patients recruited at these hospitals during 2013 and 2014 (*22,26*). The proportion of malaria cases reported as *P. knowlesi* per village per year was adjusted for this sensitivity and specificity by using a Bayesian estimation of true incidence from apparent incidence obtained by testing individual samples (*27*). This estimation is calculated as follows:

$$Pos_i \sim Binomial\,(p_i, n_i)$$

$$p_i = \pi * SE + (1 - \pi) * (1 - SP)$$

Models were fitted in R (http://www.R-project.org) through the prevalence and rjags packages interfacing with JAGS version 3.4.0 (http://mcmc-jags.sourceforge.net/) by using 2 chains containing 1,000 burn-in samples and 5,000 retained samples. Sensitivity and specificity parameters were determined by using β-PERT distributions of the minimum, maximum, and most likely values.

Environmental Data

Topography and land use data were extracted from various datasets derived from satellite-based remote-sensing data. These data were evaluated for buffer areas (i.e., areas within a certain radius of a village center) with a radius of 1, 2 and 5 km from the center of each village; these distances were chosen to explore a range of spatial scales at which environment may be relevant on the basis of the typical distribution of households, farming land, and local human and animal movements. Elevation data with a spatial resolution of 30 m were obtained from the ASTER Digital Global Elevation Model (*28*). The average annual normalized difference vegetation index (NDVI), which quantifies the greenness of vegetation, was calculated from Moderate Resolution Imaging Spectroradiometer 16-day composites at 250-m resolution (*29*). The NDVI is influenced by climatic factors (e.g., rainfall and temperature) and has been used extensively to predict malaria incidence and develop early warning systems in other contexts (*30,31*).

Tree cover data, derived from classified Landsat imagery at 30-m resolution, were obtained from Hansen et al. (*32*). Annual forest cover maps for the study districts were produced; forest was defined as ≥50% tree crown cover density. Although this land classification represents forested areas, it cannot distinguish types of forest or agroforestry such as rubber or oil palm. The proportion of forest coverage, proportion of forest loss during the year for which incidence was estimated, and proportion of cumulative forest loss for the previous 5 years (i.e., total forest loss for 2006–2010 was evaluated by using 2010 incidence) were calculated for each buffer radius for each village and time point. We used the Landscape Ecology Statistics plugin for Quantum GIS (*33*) to evaluate the effect of forest configuration as the number of forest patches per radius, a standard metric representing landscape fragmentation. Distributions of these variables were examined, and quartiles were used to categorize variables.

Because this analysis relied on passive reporting of malaria, we included travel time to the nearest clinic where patients would seek treatment for a febrile illness as a measure of access to care. Travel time to the clinic from each village was estimated by using travel times reported in community interviews and by patients recruited as part of a population-based case–control study (*26*).

Statistical Analysis

Annual *P. knowlesi* incidence for each village was mapped and smoothed incidence maps produced to visualize the data by using a kernel density estimation method, a standard method for interpolating point location data. Because the data were skewed relative to Poisson distribution, potential associations between environmental factors and reported *P. knowlesi* patients at the village level were assessed by using general linearized mixed models with a negative binomial distribution and an offset for population in R (*34*). To account for correlation between repeat measurements for the same village, we included the village variable as a random effect. Bivariable analysis was conducted for each covariate; variables for which $p<0.2$ were included in multivariable models. We used likelihood ratio tests to assess the significance of single variables and the Akaike Information Criterion (http://www.modelselection.org/aic/) for the final model selection. For correlated variables (e.g., mean elevation at different buffer radiuses), a single variable was selected for inclusion on the basis of marginal Akaike Information Criterion values. Potential bias from residual spatial autocorrelation in the model was explored for island and mainland areas through Moran's I. On the basis of this statistic, the negative binomial model was fit with a spatial correlation component estimated by using a distance-based Matern correlation function.

Results

Malaria in Kudat and Kota Marudu Districts from 2008–2012

A total of 405 villages were mapped in Kudat and Kota Marudu districts; median population per village was 168 persons (interquartile range 80–313), and median number of households was 44 (interquartile range 20–78). A total of 2,006 malaria patients were reported during 2008–2012: 833 cases in Kota Marudu district, 1,014 in Kudat district, and 159 from outside these districts. Standard reporting forms did not include age-specific data but classified patients as adults (65.7%, 1,318/2,006) or children (32.8%, 657/2,006), with a small number of records (1.5%, 31/2006) missing this information. Most malaria patients were male (66%, 1,330/2,006).

Most villages (60%, 245/405) reported at least 1 malaria patient during this period. The number of malaria patients reported varied annually, with marked seasonal variations in numbers of patients and amount of rainfall. Almost half (47%, 878/1,847) of reported malaria patients had suspected *P. knowlesi* or *P. malariae* infections diagnosed by microscopy. *P. falciparum* and *P. vivax* malaria were diagnosed in another 27% (512/1,847) and 25% (457/1,847) of patients, respectively.

Of 346 samples collected from patients for whom *P. knowlesi or P. malariae* was diagnosed by microscopy (including mixed infections) and sent for molecular confirmation, 90% (313/346) were confirmed as *P. knowlesi* by PCR. Sensitivity and specificity of microscopy diagnosis for *P. knowlesi* were 95% (95% CI 92%–97%) and 84% (95% CI 79%–89%), respectively (Table 1). Although studies have reported frequent misdiagnosis (*1–3,5*), few samples (3%, 16/539) were incorrectly identified as other species by microscopy. Because PCR results were available only for patients with confirmed malaria, we could not estimate the probability of detecting submicroscopic infections.

By using these values derived from collected samples, the true number of *P. knowlesi* patients was estimated as 739 (95% CI 664–794) for Kudat and Kota Marudu districts during 2008–2012. The range of estimated annual parasite incidence (API, expressed as cases/1,000 person/y) for *P. knowlesi* malaria calculated for each village was 0–102; overall mean API was 1.84. Of the 245 villages reporting malaria patients, 24% (59/245) had an estimated mean API for *P. knowlesi* of <l; the highest proportion, 44% (108/245), each had a mean API of 1–5; another 11% (26/245) had a mean API of 5–10; and 6% (15/245) had a mean API of 10–20. Two villages had a mean API of >20 (Figure 2, panel A).

Association with Environmental Variables

On the basis of estimates from remote sensing data (*32*), forest cover declined by 4.8% in Kudat and Kota Marudu during 2008–2012 (Figure 2, panel B). Although this loss was often highly localized, large tracts of forest were cleared in the interior of Pulau Banggi. Overall, substantial environmental change was observed in the districts; 39% (157/405) of villages lost >10% of forest cover within a 1-km radius during this 5-year period; 44% (179/405) lost >10% within a 2-km radius; and 51% (206/405) lost >10% within a 5-km radius.

Lower elevations, >65% of forest cover (within a 2-km radius), and higher historical forest loss were associated with greater incidence of *P. knowlesi* infections. Travel times to clinics, average annual NDVI, and number of forest patches were not significant at any radius in the bivariate analysis.

Table 1. Comparison of results of PCR and microscopy testing for *Plasmodium knowlesi*, Sabah, Malaysia, 2008–2012*

	No. samples		
Microscopy results	*P. knowlesi* PCR +	*P. knowlesi* PCR –	Total
P. malariae +	313	33	346
P. malariae –	16	177	193
Total	329	210	539

*+, positive; –, negative.

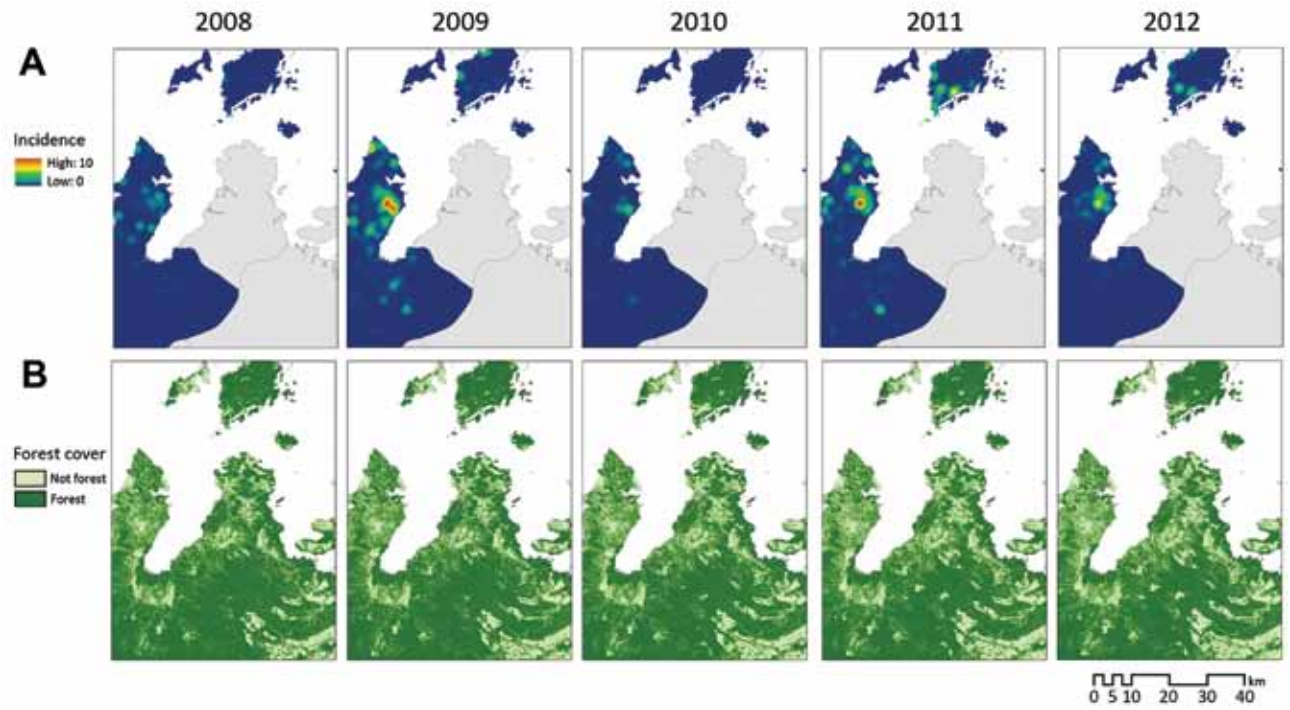

Figure 2. Comparison of estimated incidence (per 1,000 population) of *Plasmodium knowlesi* infection (A) and forest cover (B) and, Sabah, Malaysia, 2008–2012.

Spatial Patterns

Maps of interpolated *P. knowlesi* incidence show distinctive spatial patterns that vary from year to year, with some areas of high incidence persisting over time (Figure 2, panel A). To assess the degree of residual spatial correlation after fitting the negative binomial regression, the residuals of the best-fitting model were mapped and Moran's I was calculated (mainland Borneo, Moran's I 0.07, $p<0.0001$; islands off Borneo's coast, Moran's I 0.07, $p = 0.07$). Because Moran's I was significant, the final model was adjusted for spatial autocorrelation (Tables 2, 3).

Discussion

This study aimed to describe spatial and temporal patterns of *P. knowlesi* infection in northern Sabah, Malaysia, and to evaluate potential associations between village-level *P. knowlesi* incidence and key environmental factors. Although land use changes can affect emergence of infectious diseases, such associations have not been previously evaluated for *P. knowlesi.* We found links among deforestation, environmental characteristics, and reported incidence of *P. knowlesi* malaria in this region.

Village-level malaria data show marked spatial and temporal heterogeneity in *P. knowlesi* incidence in Kudat and Kota Marudu districts. After adjustment for the sensitivity and specificity of microscopy, *P. knowlesi* was the most common cause of human malaria, a finding consistent with other studies for this geographic area (*4,5,22*). Quantification of annual forest loss indicates that substantial environmental changes occurred during 2008–2012, with many villages losing substantial proportions of surrounding forest cover.

The proportion of forest surrounding a village was associated with incidence of *P. knowlesi* infections in the final model (Table 3); this association potentially reflects the role of forest environments as habitats of macaques and mosquito vectors. Studies report higher vectorial capacity and sporozoite rates for *P. knowlesi* in forest environments than in agricultural and settled lands (*12,35*). Long-tailed macaques have also been reported in various environments, including degraded secondary forest areas (*36*). The association of increased *P. knowlesi* incidence with both forest and forest loss likely confirms findings that transmission is occurring in forested areas undergoing substantial change, as with previously described frontier malaria (*37*).

Higher incidence of *P. knowlesi* was associated with higher proportions of forest loss surrounding villages during the 5-year period before year of reported incidence. This association could result from changes in macaque or mosquito habitats and from increased levels of human activity. Increased density of long-tailed macaques has been reported as a response to deforestation; loss of previous habitats can result in crowding within forest patches, with potential implications for disease transmission (*36*). Land

Table 2. Land use characteristics and bivariable statistics in *Plasmodium knowlesi* study, Sabah, Malaysia, 2008–2012*

Variable	Bivariable analysis	
	IRR (95% CI)	p value
% Forest remaining		
≤1 km radius		
<65	Reference	0.59
≥65	1.08 (0.82–1.43)	
≤2 km radius		
<65	Reference	0.06
≥65	1.30 (0.99–1.71)	
≤5 km radius		
<65	Reference	0.77
≥65	1.05 (0.78–1.40)	
% Forest lost in current year†		
≤1 km radius		
<1	Reference	0.11
1–2	0.94 (0.74–1.19)	
>2	1.22 (0.99–1.51)	
≤2 km radius		
<1	Reference	0.001
1–2		
>2	1.36 (1.09–1.71)	
≤5 km radius		
<1	Reference	0.01
1–2	0.97 (0.75–1.27)	
>2	1.57 (1.19–2.06)	
% Forest lost in past 5 years‡		
≤1 km radius		
<8	Reference	0.60
8–14	1.01 (0.76–1.33)	
>14	1.14 (0.85–1.53)	
≤2 km radius		
<8	Reference	<0.001
8–14	1.66 (1.25–2.20)	
>14	2.03 (1.46–2.80)	
≤5 km radius		
<8	Reference	0.32
8–14	0.93 (0.69–1.25)	
>14	1.13 (0.81–1.58)	
Mean elevation per 10 m above sea level		
≤1 km radius	0.99 (0.97–1.00)	0.12
≤2 km radius	0.98 (0.97–1.00)	0.06
≤5 km radius	0.97 (0.96–0.99)	0.01
Travel time to clinic, min	1.00 (0.99–1.00)	0.001

*Radius is calculated as distance from village center. IRR, incident rate ratio.
†Current year is the year for which incidence was calculated.
‡Five years before the specific year of reported incidence.

use changes have also been shown to affect abundance and community composition of potential vectors (*17,38*). Deforestation and associated agricultural development are also associated with changes in human risk because of altered distribution and behavior of humans; these changes result from more employment opportunities and shifts in human movement patterns because of forest clearing and agricultural activities (*19,37*). Although clearing of forests may initially deplete vector populations and thereby reduce malaria transmission, this reduction may be followed by colonization of cleared areas by more efficient vector species and subsequent increases in transmission (*9*). Historical forest loss was more significantly associated with *P. knowlesi* incidence than forest loss occurring during the same year of reported incidence (Table 2), suggesting that increased transmission is related to long-term changes in vector, host, or human populations involved. Additional longitudinal studies are required to investigate this hypothesis.

The effect of habitat may also be reflected in associations with elevation. Elevation was negatively correlated with *P. knowlesi* incidence, although the range of elevations within the study site was limited; most villages were at elevations <100 m. Both macaques and vectors are more frequently described in low elevation areas but have been reported at higher elevations (*36,39*).

Neither forest configuration nor vegetation indices appeared to be strongly associated with *P. knowlesi* incidence. Because NDVI is a measure of vegetation greenness, this measure may not differentiate between forest and other types of agriculture (e.g., oil palm) because NDVI tends to become saturated in tropical environments. Studies of other zoonotic diseases have found that the fragmentation and configuration of other types of land cover, in addition to forest, influence disease transmission (*18,19*). Other fragmentation metrics could be included to evaluate the effects of forest patch size and shape on *P. knowlesi* transmission.

The buffer sizes that we evaluated represent a range of potential scales at which variables (e.g., mean elevation) within different radiuses may affect *P. knowlesi* transmission. Human infections result from many factors interacting across different spatial scales, and the strength of association of these factors likely varies by distance. Our study covers an extensive area in which behaviors, distribution of villages, and ecology vary. Villages are typically within small spatial areas, but farming practices range from small-scale swidden farming to large-scale plantations and affect the scale of human interactions with the environment. Future analyses would benefit from including more detailed spatial data on household locations and human movement patterns.

The main limitation of this study is the reliance on records of malaria patients who sought care at hospitals; these patients may represent only a portion of malaria patients in the community. Although malaria is a notifiable disease and distance to a hospital was included as a measure of access to care, asymptomatic malaria cases and symptomatic cases that resolved without treatment are unaccounted for. Previous studies of other malaria species in similar transmission settings have described a large proportion of asymptomatic carriage within communities; however, asymptomatic carriage has not been evaluated for *P. knowlesi* (*40*). A cross-sectional survey of 2,019 persons in central Vietnam identified 3 persons whose samples were positive for *P. knowlesi*, yet all 3 were asymptomatic at the time of the survey and for the subsequent 6 months, showing that asymptomatic carriage of *P. knowlesi* can occur (*40*).

Table 3. Multivariable negative binomial regression of *P. knowlesi* incidence (including population offset) in *Plasmodium knowlesi* study, Sabah, Malaysia, 2008–2012*

Variable	IRR (95% CI)	p value
% Forest remaining, ≤2 km radius		
<65	Reference	0.0004
≥65	1.51 (1.42–1.99)	
% Forest lost in the past 5 y, ≤2 km radius		
<8	Reference	<0.0001
8–14	1.68 (1.27–2.22)	
>14	2.22 (1.53–2.93)	
Mean elevation per 10 m above sea level, ≤5 km radius	0.98 (0.96–0.99)	0.001

*Population was used as an offset to correct the number of infections reported for an estimate of the population size. Radius is calculated as distance from village center. IRR, incident rate ratio.

This study was also limited by the environmental data used. Data on forest cover and forest loss were aggregated by year, and finer-scale temporal associations between land cover (i.e., forest and other land types) and incidence could not be explored. This dataset was limited by defining forest as canopy cover, a definition that does not enable differentiation between types of forest or crops or between patches of different types of land. In addition, analysis was limited by the spatial resolution of satellite-based remote-sensing data, the use of a centroid point to represent village location, and the use of circular buffers rather than buffers of actual village shape. Exploratory spatial analysis suggested spatial heterogeneity in village-level data, and these spatial effects were included in the model. Additional work with more spatially specific outcomes and environmental data are needed to investigate these spatial patterns in more detail.

Despite inherent limitations in the outcome and covariate data used in this study, results strongly suggest a link between environmental change and reported incidence of emerging *P. knowlesi* in northern Sabah. Spatial analysis of environmental factors affecting disease emergence can be used to target surveillance and public health activities to areas expected to have increasing disease risk. Although additional population-based studies are needed to define environmental risk factors, this study indicates that deforestation is associated with human cases of *P. knowlesi* within northwestern Sabah, Malaysia.

Acknowledgments

We thank Albert M Lim, Lina Marlina Gulam, Beth Downe, and the MONKEYBAR team in Sabah for their help with this study. N. Alexander receives salary support from the Medical Research Council and Department for International Development (MR/K012126/1).

We acknowledge the Medical Research Council, Natural Environment Research Council, Economic and Social Research Council, and Biotechnology and Biosciences Research Council for funding received for this project through the Environmental and Social Ecology of Human Infectious Diseases Initiative, grant no. G1100796.

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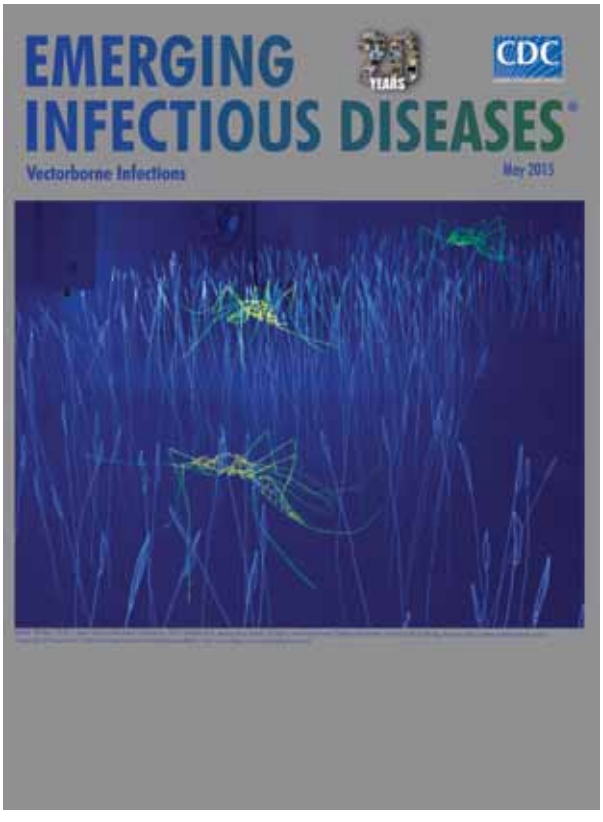

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Feasibility of Xpert Ebola Assay in Médecins Sans Frontières Ebola Program, Guinea

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Rapid diagnostic methods are essential in control of Ebola outbreaks and lead to timely isolation of cases and improved epidemiologic surveillance. Diagnosis during Ebola outbreaks in West Africa has relied on PCR performed in laboratories outside this region. Because time between sampling and PCR results can be considerable, we assessed the feasibility and added value of using the Xpert Ebola Assay in an Ebola control program in Guinea. A total of 218 samples were collected during diagnosis, treatment, and convalescence of patients. Median time for obtaining results was reduced from 334 min to 165 min. Twenty-six samples were positive for Ebola virus. Xpert cycle thresholds were consistently lower, and 8 (31%) samples were negative by routine PCR. Several logistic and safety issues were identified. We suggest that implementation of the Xpert Ebola Assay under programmatic conditions is feasible and represents a major advance in diagnosis of Ebola virus disease without apparent loss of assay sensitivity.

As of June 28, 2015, the recent Ebola virus disease (EVD) outbreak in West Africa had claimed >11,000 lives, and 27,443 confirmed, probable, and suspected cases have been reported in Guinea, Liberia, and Sierra Leone (*1*). One of the cornerstones of outbreak control has been rapid diagnosis of suspected cases. Timely confirmation of EVD status can lead to more rapid identification of EVD cases (decreasing potential transmission to contacts, and since the advent of treatment trials, expediting provision of potentially life-saving therapeutics); more immediate initiation of contact tracing; and more accurate epidemiologic surveillance. In addition, a rapidly obtained negative test result would decrease the time a suspected case-patient spends in an Ebola treatment center (ETC) or other Ebola-related health facility, and decrease the likelihood of infection with Ebola virus (EBOV) while waiting for the test result.

Since the start of the recent outbreak, international mobile laboratories were rapidly deployed, mainly near ETCs, to confirm the EVD status of suspected patients and of bodies recovered from the community, and to document the status of survivors. Current diagnostic testing is performed by using PCR of RNA extracted from venous blood samples or swab samples (e.g., oral swab samples for deceased patients). Although conventional PCRs have high specificity and sensitivity, the time between sampling and obtaining results can be considerable, in particular in settings in which a laboratory with PCR capacity is not readily available (*2*). Even in settings in which a laboratory is near an ETC, major delays can occur in obtaining results. A point-of-care instrument capable of diagnosing EVD with high sensitivity and specificity would preclude such delays. Even in environments in which control of an outbreak is (nearly) achieved, health structures are likely to be confronted with suspected cases of EVD for a considerable time. Thus, rapid, point-of-care diagnostics are likely to be invaluable in keeping health structures safe.

A novel Ebola diagnostic assay, the Xpert Ebola Assay (Cepheid Inc., Sunnyvale, CA, USA), was recently developed. This assay can be used with the Cepheid GeneXpert System, which is widely used for rapid detection of tuberculosis and rifampin resistance in decentralized settings (*3*). The Xpert Ebola Assay has been approved for emergency use by the US Food and Drug Administration for testing of venous blood samples on the basis of laboratory studies that used venous blood spiked with EBOV (*4*). The World Health Organization has issued a prequalification of the Xpert Ebola Assay (*5*).

However, the feasibility of implementing this technology inside a functioning ETC (compared with an external

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DOI: http://dx.doi.org/10.3201/eid2202.151238

laboratory), and the added value to Ebola programs are not known. Therefore, we conducted a study that assessed the added value of using the Xpert Ebola Assay in a Médecins Sans Frontières (MSF) ETC during the recent EVD outbreak in West Africa. Specifically, we compared total test time and time for obtaining results for the Xpert Ebola Assay with those for a routine in-house Ebola PCR used at the ETC to document any discordant results between the 2 tests and document information regarding biosafety and logistic and human resource requirements for implementation of the Xpert Ebola Assay.

Methods

Study Design

We conducted a cross-sectional study of laboratory analysis of paired venous blood samples. This study included a limited user satisfaction survey conducted by using semistructured interviews.

Ethics Statement

Verbal informed consent was obtained for all study participants, including patients who provided blood samples for analysis and laboratory staff who participated in user evaluation of the Xpert Ebola Assay. The study was approved by the Ethics Review Board of MSF (Geneva, Switzerland) and the Comité de Recherche Ebola and Comité National d'Ethique pour le Recherche en Santé (Conakry, Guinea).

Setting

The study was conducted in the MSF-managed Donka ETC in Conakry, Guinea. The Donka ETC was opened in March 2014 at the start of the outbreak in West Africa. It has 30 beds (85 at its peak capacity during October 2014–January 2015). By week 28 of 2015, Donka had admitted 818 patients with confirmed cases of EVD and discharged 789 patients (372 died and 417 recovered). MSF ETCs are based on the principle of providing patient care and support and isolating EVD-positive patients to break the chain of transmission. The centers aim to provide controlled access to patients or contaminated areas; controlled movement of staff, patients, and visitors inside these areas; disinfection facilities for persons leaving contaminated areas; and safe disposal of contaminated waste.

During the study, the Donka ETC hosted a clinical trial of treatment for EVD by transfusion of convalescence plasma. This trial (Ebola_Tx) was conducted by the Institute for Tropical Medicine (Antwerp, Belgium) (*6*). For routine diagnostics, the Donka ETC had samples tested at the Laboratoire National des Fièvres Hémorrhagiques, Gamal Abdel Nasser University of Conakry (Conakry, Guinea), which is supported by the Institut Pasteur (Dakar, Senegal). This laboratory was located in the same compound as the Donka ETC, but was not part of the center. An in-house, real-time PCR specific for the nucleocapsid protein gene of EBOV was used for routine diagnostics (*7*).

Sample Processing

Blood samples were obtained at the same time as the convalescent-phase plasma trial at the Donka ETC was conducted. Persons who fulfilled the definition for having suspected EVD were counselled by a health promotion team and then provided blood obtained by venipuncture. Venous blood for routine diagnosis was collected in a 4-mL serum tube (Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). An additional 2-mL blood sample was obtained from consenting persons into an EDTA tube (Vacutainer). Persons who did not provide consent, children <2 years of age, and persons who were not able to provide an additional blood sample (e.g., severely dehydrated persons) were excluded from the study (number was not recorded). For consenting persons admitted to the ETC after a positive routine diagnosis was made, additional 2-mL ml blood samples were collected into EDTA tubes 24 or 48 hours after a transfusion with convalescent-phase plasma and during convalescence (after 3 days without symptoms), each time in parallel with the 4-mL blood sample obtained for routine PCR testing.

Serum tubes were directly transferred to the routine laboratory for processing and testing. Samples in EDTA tubes were processed in a specifically developed MSF laboratory in the ETC that contained separated high-risk and low-risk areas. Samples in the high-risk area (deposited by ETC staff using complete personal protective equipment after sampling) could be handled by a technician in the low-risk area if the technician used sealed gloves and worked through a partition (glovebox-type setup). Technicians who worked only in the low-risk area did not require full personal protective equipment, used only latex gloves, and were dressed in scrubs and plastic boots in the laboratory (e.g., when using the Xpert Ebola Assay or handling inactivated samples). When these technicians worked through the partition and used the glovebox, they also wore surgical gowns and an additional layer of surgical gloves. The laboratory was staffed by 2 or 3 technicians at all times during the study.

In the high-risk area, 100-mL samples from each EDTA tube were directly transferred by using an automatic pipette with filter tips into a Cepheid sample reagent vial containing 2.5 mL of inactivating agent (4.5 mol/L guanidine thiocyanate). After 20 min of inactivation (twice the 10 min recommended by the manufacturer), the vial was transferred to the low-risk area in accordance with all biosafety procedures (decontamination of the vial in the high-risk area in a 0.5% hypochlorite solution during inactivation, and by incubation for 20 min in a 0.5% hypochlorite

solution in the low-risk area, as standard procedure for all material transferred from the high-risk to the low-risk zone; total time of 40 min). Subsequently, 1 mL of inactivated sample was transferred from the vial into the Xpert Ebola Assay cartridge. The cartridge was then inserted into the Cepheid GeneXpert instrument, and testing was conducted according to the manufacturer's recommendations.

GeneXpert Setup

The Cepheid GeneXpert setup has been in use in Guinea for tuberculosis diagnosis for several years. It consists of a GeneXpert instrument, personal computer, and disposable fluidic cartridges. Each instrument contains 4 individually accessible modules that are capable of independently performing testing. A specific cartridge (Xpert Ebola) specific for the EBOV Zaire strain was developed to target highly conserved sequences in the nucleocapsid protein (NP) and glycoprotein (GP) genes (*5*). The Xpert Ebola assay is fully automated and cartridge based (closed system) and includes automated controls for interference with the PCR and adequacy of sample input. The only manual step is inactivation of the blood sample and transfer to the Cepheid cartridge; sample processing (RNA extraction), reverse transcription, real-time PCR amplification, and detection of TaqMan probes are then performed automatically. Results are expressed as positive or negative, and a cycle threshold (C_t) for both gene targets was also calculated by the software automatically. The results for the NP gene were taken as the final GeneXpert result; no interpretation of amplification characteristics was conducted.

Data Collection and Analysis

All data was entered into a dedicated electronic data registry (Excel; Microsoft, Redmond, WA, USA), and data validation was performed by random checking of ≥10% of all records. Information was collected regarding patient characteristics and timing of each step in the Xpert Ebola Assay. For samples used as comparators, only time of sample submission to the laboratory and time of result could be recorded. Analysis was conducted by using EpiData Analysis version 2.2.2.183 software (EpiData Association, Odense, Denmark). Total times were calculated for each step of the testing procedure, descriptive data was presented as summary statistics, and differences in median timings were assessed by using the paired-sample Wilcoxon signed-rank test. Levels of significance were set at $p<0.05$.

Additional data for user experience was collected by using a structured questionnaire that included several statements about practical use of the Xpert Ebola Assay. Agreement was assessed on a scale of 1 through 5 (1 = complete disagreement to 5 = complete agreement). This questionnaire was completed twice by all 5 laboratory technicians who used the Xpert Ebola Assay: once at the start of work, and once after several weeks of work.

Results

Patient and Sample Characteristics

Data were analyzed for all samples collected during May 2-July 4, 2015; a total of 218 samples were collected from 148 persons (Figure 1). All samples collected were venous blood samples. Characteristics of samples and patients from which they were recovered are shown in Table 1. Median delay between estimated time of onset of symptoms and admission to the ETC was 3 days (interquartile range [IQR] 1–6 days).

Timeliness of Testing

Median time of each step in the testing process is show in Table 2. The median run time of the instrument was 94 min (IQR 94–95 min) for all successful runs (n = 218). Ten (5%) tests had to be repeated because of technical issues (8 power failures that were not buffered by an uninterrupted power supply, 1 temperature-related failure, and 1 invalid result); all repeated tests were successful. A full comparison of each step of the testing process could not be performed because data were only available from the routine laboratory on reception of the sample and provision of the results to the clinical staff of the ETC. However, when we compared the total result notification time (time between reception of sample in the MSF laboratory and availability of results), the median time for results to be available was reduced from 334 min (IQR 293–419 min) in the routine laboratory that used the in-house PCR to 163 min (IQR 151–196 min) in the laboratory that used the Xpert Ebola Assay ($p<0.0001$) (Figure 2). These times included the time required for repeat Xpert Ebola Assay for the 10 failed runs (n = 228 for the Xpert Ebola Assay); 11 samples were excluded from routine laboratory data

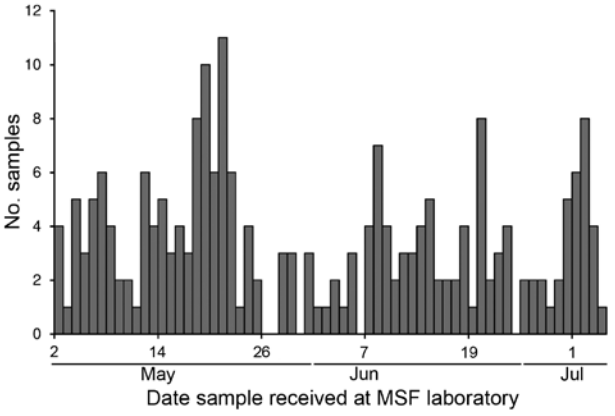

Figure 1. Frequency of sampling for Ebola virus at Médecins Sans Frontières (MSF) Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015.

Table 1. Characteristics of 148 patients and 218 blood samples collected for analysis by Xpert Ebola Assay at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015

Characteristic	No. (%)
Patient sex	
F	59 (40)
M	89 (60)
Patient age, y	
2–4	15 (10)
5–18	19 (13)
19–45	84 (57)
46–64	16 (11)
≥65	11 (7)
Not recorded	3 (2)
Sample type	
Diagnosis 1*	147 (67)
Diagnosis 2 (confirmation)	52 (24)
After transfusion	12 (6)
Convalescent phase	7 (3)

*One patient (2-year-old boy) did not have an initial diagnostic sample obtained for the study.

because the time for obtaining a result was not recorded (n = 207 for in-house PCR).

Test Discordance

Of 218 samples tested, the Xpert Ebola Assay identified 26 (12%) positive samples: 8 (5%) of 147 at initial diagnosis, 12 (100%) of 12 after transfusion, and 6 (86%) of 7 at convalescence. The routine laboratory identified 18 (69%) of the 26 positive samples identified by the Xpert Ebola Assay. No discordance was observed for diagnostic samples (Table 3), and no samples identified as negative in the Xpert Ebola Assay were identified as positive by the routine laboratory. The 8 samples identified as positive in the Xpert Ebola Assay and as negative by the routine laboratory (5 samples obtained during convalescence and 3 samples obtained after transfusion) had low viral loads (range C_t 33.0–40.8 for the NP gene) and were obtained from 4 patients. Detailed results for each of these patients are provided in the online Technical Appendix (http://wwwnc.cdc.gov/EID/article/22/2/15-1238-Techapp1.pdf).

The C_t for the NP gene in the Xpert Ebola Assay was compared with that for the GP gene and that for the NP gene in routine PCR (Figure 3). Although a clear correlation was observed (Pearson r = 0.95 with the GP gene and r = 0.80 with the NP gene in routine PCR), with only 1 exception, the C_t was lower (a higher viral load detected) in the Xpert Ebola Assay for the NP gene (Figure 3). Of 18 samples that had positive results for both tests, 12 (67%) had a difference of ≥3 C_t values, or approximately a 10-fold difference. When results were transformed to a linear scale, we found that viral load assessed by using the Xpert Ebola Assay (NP) was a median 22-fold higher (IQR 5–64 fold) than viral load assessed by the routine PCR (NP).

Table 2. Timing of steps in Xpert Ebola Assay for blood samples collected at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015*

Step	Median (IQR), min
Sampling obtained to sample received at laboratory†	11 (5–20)
Sample received to inactivation	14 (8–28)
Inactivation to start of assay	49 (43–56)
Start of assay to end of assay	94 (94–95)
End of assay to result available	2 (1–8)

*IQR, interquartile range.
†Only 67 samples were included; time of collection was not recorded for other samples.

User Satisfaction and Lessons Learned

Five laboratory technicians used the Xpert Ebola Assay during the study. One of these technicians had experience with the assay for diagnosis of tuberculosis. Opinions were queried on several aspects of the practical implementation of the assay (Figure 4). An overall high appreciation of ease of use was observed, and concerns regarding logistical aspects (storage space, cleaning) and safety aspects (manipulation of inactivated samples in the low-risk zone) were observed (Table 4).

Discussion

This study represents an analysis of implementation of the Xpert Ebola Assay in field conditions and assesses the practical aspects of its implementation and the added value in a functional EVD program in West Africa. Our results

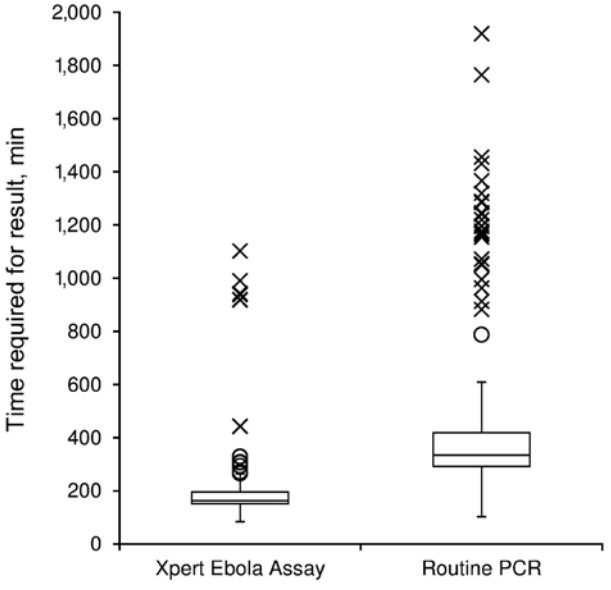

Figure 2. Tukey boxplot of time required from receiving sample in laboratory to obtaining results by Xpert Ebola assay and routine PCR at Médecins Sans Frontières Ebola Donka Treatment Center, Conakry, Guinea, May–June 2015. Boxes indicate first and third quartiles; vertical dashed lines indicate medians; whiskers indicate 1.5 times interquartile ranges (IQRs); asterisks indicate outliers >3 times the IQR; and circles indicate outliers 1.5–3 times the IQR.

Table 3. Blood samples identified as positive for Ebola virus by Xpert Ebola Assay at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015

Sample type	No. positive by Xpert Ebola Assay	No. (%) positive by routine PCR
Total	26	18 (69)
Diagnosis 1	8	8 (100)
Diagnosis 2	0	0
After transfusion	12	9 (75)
Convalescent phase	6	1 (17)

demonstrate the feasibility of introducing the Xpert Ebola Assay as a routine diagnostic tool for venous blood samples in an ETC and indicate a major decrease in time needed for obtaining results by using this point-of-care device. This assay halves the time required for the in-house PCR test used by the routine laboratory in the study setting without an apparent loss in sensitivity for detection of EVD cases. Sample sizes of positive cases were limited, but this assay seemed capable of better identifying positive cases with a low viral load than the routine diagnostic PCR used in the national laboratory in Donka. Thus, this assay might have the potential for earlier detection of EVD cases, although in this analysis, differences were only observed for convalescent-phase cases.

The longer time required for detection of convalescent-phase cases might have repercussions for patient discharge and length of stay in the ETC. More wide-scaled studies of Xpert Ebola Assay sensitivity and specificity in patient samples compared with in vitro–spiked samples used for validation are recommended, in particular, for diagnostic samples. In addition, given the higher C_t for the GP gene than for the NP gene, an in-depth analysis of associations between Ct values in different systems for different targets and viral loads might be indicated.

Several concerns were raised on the practical implementation of this novel system, in particular concerning biosafety and logistics, which might be relevant to persons seeking to implement a similar system for EVD diagnostics. At the level of sample collection, the approach does not differ from sampling for routine PCR diagnosis, and all standard ETC

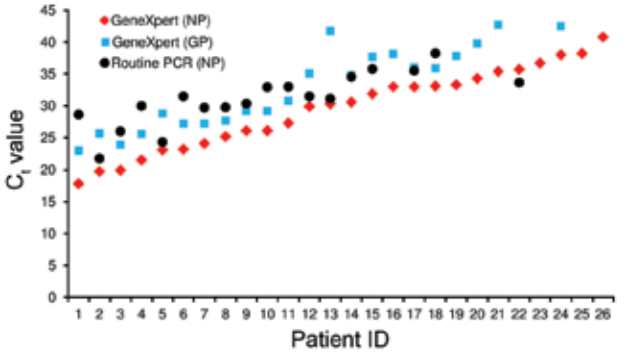

Figure 3. Ebola virus cycle threshold (C_t) values for GeneXpert Ebola Assay (nucleocapsid protein [NP] and glycoprotein [GP] genes) and routine PCR (NP gene) for patient samples identified as positive for Ebola virus by Xpert Ebola Assay at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015. ID, identification.

precautions (personal protective equipment, safe handling, safe packaging of samples) need to be in place as a prerequisite for implementing the Xpert Ebola Assay. In addition, laboratory technicians need comprehensive training on the safe manipulation of the instrument and samples if they do not have experience working with EBOV biologic material. In terms of logistics, implementers need to address the need for sufficient working space, provision of air-conditioning, a robust system for managing power failures, and a safe way of handling the critical step of transferring the biologic material to the inactivation vial.

Other studies have reported similar advances in providing point-of-care diagnostics in Ebola programs, focusing mainly on rapid diagnostic tests (*8,9*). Although such technology is expedient as a screening tool, approaches that rely on antigen detection might have difficulties matching the sensitivity of molecular techniques. In an outbreak setting, even 1 false-negative result, which would result in not identifying an EVD case, could initiate a new chain of transmission. Thus, the Xpert Ebola Assay might represent

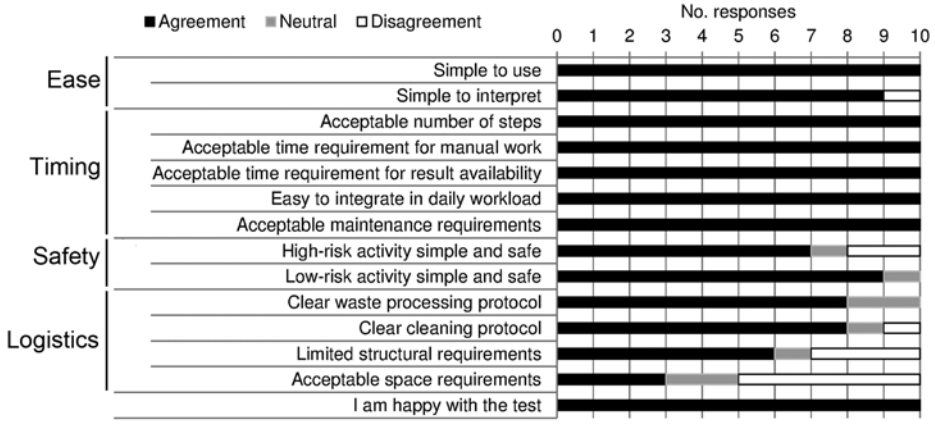

Figure 4. Laboratory staff feedback on key aspects of implementation of Xpert Ebola assay at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015.

Table 4. Main user concerns for Xpert Ebola Assay at Médecins Sans Frontières Donka Ebola Treatment Center, Conakry, Guinea, May–June 2015

Concern
Biosafety
• There were difficulties in preventing the rim of the inactivation vial from being touched by the tip of the pipette (which was contaminated with blood). Because material on the rim is not inactivated, this situation could be a considerable biohazard; the only strategy to avoid this situation was close observation of the vial rim by laboratory staff.
• Three incidents were reported in which vials containing inactivation fluid were dropped because of difficulties in handling vials with required biosecurity gloves; no incidents occurred with sample already added.
• The decontamination process for vials and transferring these vials to a low-risk zone did not compromise assay performance.
Instruments
• It was not possible to automatically export Xpert Ebola Assay data into an existing database; thus, manual coding was required.
• More detailed information on assay characteristics (e.g., PCR efficiency) was not available for users.
• Compatibility issues were identified when we attempted to set up a laboratory in which French was spoken; this problem remained unresolved throughout the study.
Logistics
• Power failures could usually be corrected by an uninterrupted power supply, which could support a full Xpert Ebola Assay run without depleting >25% of the power supply. However, the computer to which the Xpert Ebola Assay was linked could not be supported by the uninterrupted power supply and ran on its own battery. On several occasions, the uninterrupted power supply was reconfigured because of daily switching between day and night generators and failed to support the assay.
• Failure of air conditioning resulted in ambient temperatures of 28°C–31°C, but this failure did not have a major effect on instruments used (manufacturer's recommendations are to work at a temperature <30°C); only 1 run failure was observed.
• At the time of the study, insufficient information was available on storage conditions for reagents.
• Sufficient space was needed to store all equipment and consumable materials and handle chemicals (e.g., chlorine solution) for sample processing. However, the field laboratory (area ≈2.5 m × 5.5 m) was too small for storage of all materials.

a promising strategy toward rapid EVD diagnosis because its sensitivity is at least equivalent with that of conventional PCR. In settings in which an outbreak is being brought under control, this assay might be of considerable value because it has been shown to be implementable at the primary care level for treatment of tuberculosis (*10,11*).

The Xpert Ebola Assay could represent an advantageous strategy for screening in health structures that have no cases of EVD if (and only if) the following conditions can be met: 1) safe handling of samples of patients with suspected cases of EVD (including full personal protective equipment); 2) capacity to identify suspected case-patients and refer confirmed case-patients for treatment; and 3) capacity to practically manage the assay at the level of laboratory biosafety, logistics, and training of laboratory staff. However, such conditions may not easily be met under operational conditions. Validation of the Xpert Ebola Assay for finger-prick blood and swab samples, both at the level of test performance and of biosafety and feasibility, might facilitate such decentralized use of the test.

The study had some limitations. A national laboratory providing routine diagnosis was used as a comparative setting. However, this laboratory used an in-house PCR for diagnostic purposes. Thus, sample turnaround time might have not been representative for other laboratories deployed for EVD diagnostics. A more detailed breakdown of the timing of each step in the routine procedure would have enabled a more refined comparison and identification of potential room for improvement, but this information was not available. However, by reporting the breakdown of time intervals for the Xpert Ebola Assay, we hoped to provide a reference for other potential implementers.

In addition, because the study was conducted during May-June 2015, caseloads were relatively low at the Donka ETC and were not comparable to overwhelming caseloads observed in the EVD outbreak in West Africa during August–September 2014. Nevertheless, the staff feedback on time management was unequivocally positive, and it seems plausible that the time gains observed in this study would also be apparent under higher caseloads. However, a GeneXpert instrument with a higher number of modules could be required in settings with high caseloads. If the available 16-module instrument was used and ≥4 runs/day were conducted, ≥64 samples/day could be processed.

In conclusion, we show that implementation of the Xpert Ebola Assay under programmatic conditions in an MSF ETC in Guinea is feasible and represents a major decrease in time required to obtain results and a possible increase in sensitivity compared with routine diagnostic procedures that use PCR in this setting. Wider implementation of the Xpert Ebola Assay is recommended for facilities capable of supporting safe sampling and patient management if training of laboratory staff and standard operating procedures can be provided. Additional research into sensitivity and specificity of this test for patient samples is encouraged.

Acknowledgments

We thank all health staff in Guinea and other Ebola-affected countries for working tirelessly to halt the Ebola outbreak and all patients for participating in the study.

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Prognostic Indicators for Ebola Patient Survival

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To determine whether 2 readily available indicators predicted survival among patients with Ebola virus disease in Sierra Leone, we evaluated information for 216 of the 227 patients in Bo District during a 4-month period. The indicators were time from symptom onset to healthcare facility admission and quantitative real-time reverse transcription PCR cycle threshold (C_t), a surrogate for viral load, in first Ebola virus–positive blood sample tested. Of these patients, 151 were alive when detected and had reported healthcare facility admission dates and C_t values available. Time from symptom onset to healthcare facility admission was not associated with survival, but viral load in the first Ebola virus–positive blood sample was inversely associated with survival: 52 (87%) of 60 patients with a C_t of ≥24 survived and 20 (22%) of 91 with a C_t of <24 survived. C_t values may be useful for clinicians making treatment decisions or managing patient or family expectations.

The epidemic of Ebola virus (*Zaire ebolavirus*) disease (EVD) in West Africa began in eastern Guinea in December 2013 (*1*) and quickly spread into Liberia and Sierra Leone, eventually overwhelming the fragile healthcare infrastructures in these countries (*2*). During the peak of the epidemic, many healthcare facilities were quickly filled beyond capacity, which often forced clinicians to make difficult decisions about how to triage patients and how to manage patient and family expectations regarding probable outcomes. Reliable prognostic indicators available at the time of patient admission could help clinicians make these decisions.

We therefore assessed the reliability of 2 potential prognostic indicators: 1) the total elapsed time from reported symptom onset to healthcare facility admission and 2) cycle threshold (C_t), which can serve as an approximation of viral load, at the time of EVD diagnosis. Early treatment, which is made possible by early admission, is thought to improve chances of survival (*3*–*5*), but there is little supporting empirical evidence. Analyses of EVD patients in Ebola treatment units (ETUs) have shown that C_t values predict outcomes (*6*–*8*), but these studies do not account for those who died before ETU admission. By using onset-to-outcome data for all identified EVD patients during a 4-month period in Bo District, Sierra Leone, we explored the extent to which these indicators predicted outcome.

Methods

Population

Bo District is 1 of 14 districts in Sierra Leone and is located in the southern part of the country. Bo Town is the district capital, a major urban center, and the second largest city in Sierra Leone. Bo District consists of 15 chiefdoms, many of which are in rural areas, and includes ≈1,000 villages.

When patients suspected to have EVD were identified in Bo District, they were taken to the Ebola isolation unit in the government hospital in Bo Town. After a patient was admitted, blood was collected for Ebola virus testing and supportive care was provided (included oral rehydration therapy, paracetamol for fever, and sometimes presumptive care for other diseases such as malaria). During the first 2 months of the study period, the isolation unit did not

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DOI: http://dx.doi.org/10.3201/eid2202.151250

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consistently provide supportive care, but during the second 2 months, after the unit was reorganized by a new management team, the unit did provide such care. Throughout the 4-month study period, patient blood samples were transferred from this isolation unit to the field diagnostic laboratory, located a few kilometers away.

Patients with confirmed EVD were transferred to an ETU managed by Médecins Sans Frontières. In the ETU, located in the same compound as the field laboratory, the patients received care for EVD (fluid replacement, fever and pain medication, and antidiarrheal and antiemetic drugs), as well as presumptive care for other diseases, nutritional support, and psychosocial counseling (*9*). Occasionally, patients sought care first at the ETU and were admitted directly into that facility. Data for evaluating the treatment provided at either of these facilities were not available.

Data Sources

We collected data for all identified persons from Bo District who had confirmed EVD and a symptom onset date from September 12, 2014, through January 7, 2015. To have the most complete and accurate data, we relied on multiple sources: 1) demographic information and symptom onset dates from the case investigation forms; 2) admission dates and death reports from the government hospital isolation unit; 3) ETU admission dates and patient outcomes (survival or death); 4) EVD diagnostic test results; and 5) confirmation of deaths from the district burial team, which buried the bodies of deceased EVD-positive patients.

These sources routinely reported this information to the Bo District surveillance team, which maintained a database by using the Epi Info Viral Hemorrhagic Fever application (https://epiinfovhf.codeplex.com/). In the event of missing or conflicting information, we requested verification or additional information from the original sources. For each infected person, we compiled symptom onset date; healthcare facility admission dates; outcome type and date; patient age, sex, and place of residence; and laboratory test results. To ensure a complete linkage and to identify persons with duplicate records, we reviewed all information for errors.

This assessment was considered to be a nonresearch public health response activity and thus did not undergo institutional review board review. Because this secondary analysis used only information that had already been collected for public health surveillance and clinical management purposes, informed consent was not obtained.

Measurement of Time from Symptom Onset to Admission

Time from symptom onset to healthcare facility admission was calculated by subtracting the reported symptom onset date from the admission date and was recorded in days. We used as many as 3 recorded admission dates: dates of admission to the local clinic where EVD was first suspected, to the isolation unit, and to the ETU. To better examine the changing circumstances during the epidemic, we created 3 groups of patients according to the type of facility where they were admitted: 1) all EVD patients admitted to any healthcare facility (primary cohort), 2) only patients admitted to the ETU (ETU subgroup), and 3) patients admitted to the isolation unit during the last 2 months of the assessment (November 16–January 10) when patients were consistently receiving care in the unit (final 2 months subgroup).

Measurement of C_t

The field laboratory, operated by the US Centers for Disease Control and Prevention (CDC), tested persons suspected of having EVD by using nucleoprotein (NP) and viral protein (VP) 40 quantitative real-time reverse transcription PCRs and a β-2-microglobin control. These tests detect Ebola viral RNA in blood specimens (*10*,*11*). C_t is defined as the number of cycles of RNA replication that have occurred when the Ebola virus–specific RNA signal is detected. A total of 40 cycles of replication are run for a given specimen; if no RNA signal is detected and the β-2-microglobin control result is positive, the test result is negative. Therefore, the lower the C_t for a positive specimen, the higher the relative quantity of virus.

For this analysis, only the C_t values from the VP40 assay were used because this assay was slightly more sensitive than the NP assay (i.e., the VP40 detected positive cases that the NP did not). The association between C_t and 50% tissue culture infective doses per milliliter ($TCID_{50}$/mL) is provided in the CDC document Ebola Virus VP40 Real-Time RT-PCR Assay (*11*). Each 3-point decrease in C_t was associated with an ≈10-fold increase in Ebola viral load; a C_t of 39 corresponded to ≈40 $TCID_{50}$/mL and a C_t of 19 corresponded to ≈40 million $TCID_{50}$/mL (*11*). Standard curves were not determined for each run; therefore, the viral load for each patient was an approximation. Samples with a C_t of ≤40 were classified as EVD-positive. If a person was tested within 72 hours of symptom onset and the test result was negative, that person was generally retested to confirm the negative result (*12*). Confirmatory tests for deceased persons were performed by using body fluids collected from oral swab samples, whereas testing of live patients was performed on whole blood, serum, or plasma.

Statistical Analyses

Because it is unknown whether C_t values from swab samples and blood tests yield comparable results, we excluded from the primary cohort and the 2 subgroups all infected persons for whom EVD was detected after death (and thus tested by oral swab sampling). We also excluded patients for whom C_t values or admission dates were not available.

We calculated the case-fatality proportion for all patients in Bo District for whom outcome was known, who were admitted to a healthcare facility, and who were admitted to the ETU. We stratified the primary cohort and the 2 subgroups by patient outcome and described those who survived and those who died in terms of sex, average age, average C_t at first test, and average number of days from symptom onset to healthcare facility admission.

We examined the distribution of C_t values for the Ebola patients and created a scatterplot with a LOESS (locally weighted scatterplot smoothing) curve to serve as a graphical representation of patient survival by C_t. We did this with the LOESS function in R (https://www.r-project.org/) by using the default span of 0.75 and degree of 2. We determined the C_t that was most accurate when used as a dichotomous predictor for survival. We also categorized C_t into 3 levels according to visual inspection of the relationship between C_t and survival.

We ran unadjusted logistic regression analyses in R to determine if the following covariates are associated with patient survival: sex, age (continuous and categorical variable), C_t (continuous and categorical variable), and days from symptom onset to admission to a healthcare facility (any facility, ETU, isolation ward during last 2 months of the assessment). We ran 3 multivariable logistic regression models in R (1 for each group) that included sex, age (continuous variable), C_t (categorical variable), and days from reported symptom onset to admission to a healthcare facility. We used the Pearson correlation coefficient to examine collinearity between time from symptom onset to healthcare facility admission and C_t.

Results

During our study period, the surveillance system identified 227 Bo District residents with EVD. Outcome (death or recovery) could be confirmed for 216 patients, but outcome information was missing for 11. Of the 216 patients with outcome data, 164 were detected and admitted to a healthcare facility, but the other 52 died in the community before being detected. Of the 164 patients, 6 died before blood could be collected for confirmatory testing, C_t values were missing for 2, and admission dates were missing for 5. The primary cohort comprised the remaining 151 patients. Although 123 patients were admitted to the ETU, admission dates were known for only 99; the ETU subgroup comprised these 99 patients. Dates of admission to the isolation ward during the final 2 months of the study period (when treatment was consistently provided) were known for another 68 patients; the final 2 months subgroup comprised these 68 patients (Figure 1).

Outcome was known for 216 persons, of whom 142 (66%) died. Among the 164 persons admitted to a healthcare facility, 90 (55%) died. Among the 123 persons admitted to the ETU, 49 (40%) died (including 8 admitted directly to the ETU, 3 [38%] of whom died). All survivors were ultimately discharged from the ETU.

Approximately half of the patients in the primary cohort (52%), the ETU subgroup (49%), and the final 2 months subgroup (50%) died. Of the 151 patients in the primary cohort, 90 (60%) were female; of these, 47 (52%) died. The 2 subgroups had similar proportions. The mean age (in years) of survivors in each of the 3 groups was low- to mid-20s, and the mean age for those who died was low- to mid-30s. The mean C_t for the survivors in the 3 groups was in the upper 20s and the mean C_t for the deceased was in the low 20s. The mean number of days from symptom onset to healthcare facility admission was nearly same for those who survived and those who died (Table 1).

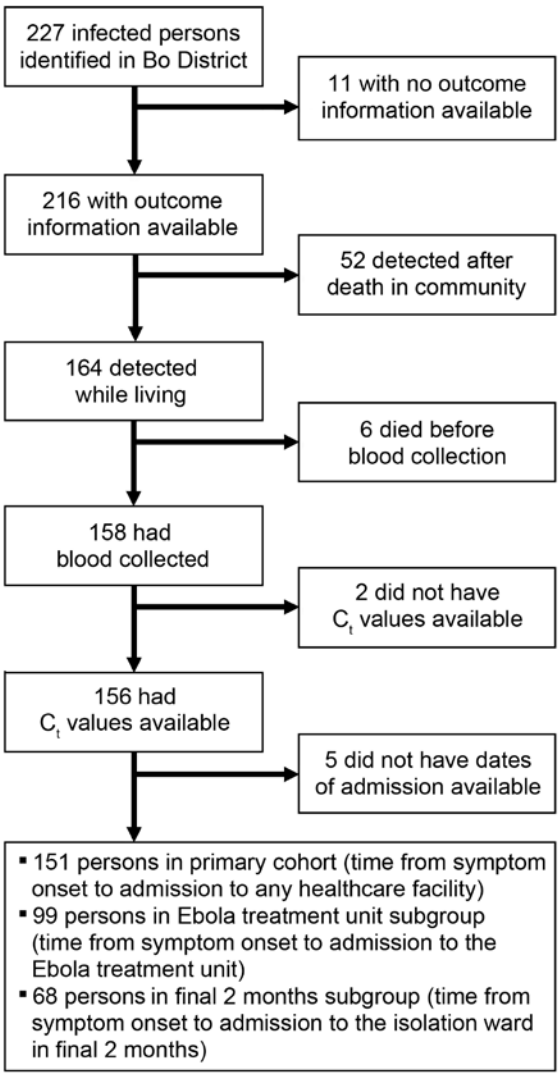

Figure 1. Classification of patients with Ebola virus disease into study groups, Bo District, Sierra Leone, September 2014–January 2015.

The average chance of survival among patients in the primary cohort showed a sharp increase for those with C_t values in the low- to mid-20s (Figure 2). Of note, 52 (87%) of 60 patients for whom C_t was ≥24 survived, whereas only 20 (22%) of 91 with a C_t of <24 survived. Of all 72 survivors, C_t was ≥24 for 52 (72%); of the 79 who died, C_t was <24 for 71 (90%).

Unadjusted logistic regression models indicate that C_t—as both a continuous and a categorical variable—is strongly associated with survival (Table 2). Among those in the primary cohort, older age in years (as a continuous variable) also was inversely associated with survival (odds ratio [OR] 0.97, 95% CI 0.95–0.99), meaning that younger patients were more likely to survive. Male patients had nearly the same odds of surviving as did female patients (OR 0.99, 95% CI 0.52–1.90). Also among those in the primary cohort, symptom onset to admission to any healthcare facility was not associated with survival (OR 0.97, 95% CI 0.87–1.08) (Table 2).

In the adjusted analysis of the primary cohort, the association of C_t with survival was not attenuated; the OR point estimates were more extreme in all parameterizations of C_t. The association found in the unadjusted models between age and survival, and the lack of association between patient sex and survival, remained virtually the same in the adjusted analysis. After adjustment of the analysis, time from symptom onset to admission was not significantly associated with survival for those in the primary cohort (OR 0.88, 95% CI 0.76–1.02). Analysis results for the 2 subgroups were similar (ETU subgroup OR 0.88, 95% CI 0.74–1.03; final 2 months subgroup OR 0.85, 95% CI 0.64–1.11) (Table 2) and produced very similar ORs for the other covariates (data not shown). This tenuous association between time from symptom onset to admission and survival may be driven by a small number of patients with long times from symptom onset to admission; when the primary cohort is restricted to patients for whom reported time of symptom onset to admission was ≤10 days (145 of 151 patients), the magnitude of the association was greatly diminished (adjusted OR 0.97, p = 0.82). The Pearson correlation test between the time from symptom onset to admission to a healthcare facility and C_t yielded a small but statistically significant relationship (r = 0.19, p = 0.01). C_t values were slightly higher for patients for whom time from symptom onset to admission was longer than for those for whom this time was shorter (Figure 3).

Discussion

The overall observed case-fatality proportion of 66% in this assessment is higher than some other case-fatality estimates for this epidemic but less than those reported for many previous outbreaks of EVD (*13*–*15*). As of November 18, 2015, the World Health Organization reported a 41% (3,589/8,704) case-fatality proportion in Sierra Leone (*16*), and an ETU in Freetown, the country's capital city, reported a mortality rate among its patients of 31% (*17*). Although the higher case-fatality proportion for Bo District could reflect specific circumstances (such as differences in severity of illness, access to care, or patient care-seeking behavior), it could also reflect more complete outcome ascertainment. For instance, the case-fatality proportion was lower (55%) when community deaths were excluded or when only those who survived long enough to be admitted to the ETU (40%) were considered; these proportions are more in line with some estimates based on ETU patients only (*6*,*17*). Another analysis, from a subset of patients in Sierra Leone for whom outcomes were known, estimated a mortality rate of 69%, similar to that found in this study (*18*). Including deaths from community and healthcare facility sources could increase the estimated lethality of EVD in Sierra Leone and perhaps more generally in West Africa (*19*).

Community members were commonly told that patients who receive care for EVD soon after symptom onset have a better chance of survival (*3*,*4*), in part because severe diarrhea is a prominent feature of the disease (*20*,*21*). Accordingly, the sooner EVD patients receive care that counteracts the deleterious effects of substantial fluid loss, the less likely is development of hypovolemia and multiple organ failure (*22*,*23*). This position is both intuitive and biologically plausible, so we were surprised that the association between survival and time from symptom onset to admission to a healthcare facility did not reach statistical significance. Although seemingly counterintuitive, our finding is similar to that of a recent analysis conducted in another area of Sierra Leone (*6*). One explanation for these findings is that the average time to admission (3.5 days) was too long to demonstrate an association with survival. Many patients were far along in the course of the disease by the time they received supportive care; 43% died before reaching the ETU. The goal of reducing the time to receipt

Table 1. Characteristics of patients with confirmed Ebola virus disease, Bo District, Sierra Leone, September 2014–January 2015

	Primary cohort, n = 151		Ebola treatment unit subgroup, n = 99		Final 2 months subgroup, n = 68	
Characteristic	Survived	Died	Survived	Died	Survived	Died
No. patients	72	79	50	49	34	34
No. female	43	47	31	32	16	23
Mean age, y	24.1	31.7	24.8	30.1	20.2	33.0
Mean cycle threshold	27.9	20.5	27.9	21.2	26.8	21.4
Mean time from symptom onset to admission, d	3.5	3.7	6.0	5.6	3.4	3.5

of care is laudable but could be challenging in a setting like West Africa, where many villages are located far from healthcare facilities. More likely, improving the availability of more sophisticated supportive care or developing advanced therapies, such antiviral drugs, will be needed to improve outcomes, particularly among the sickest patients. However, earlier detection and treatment remains a priority for every Ebola response because quicker isolation of EVD patients probably reduces transmission (*24–27*).

Similar to the results of some analyses of ETU patients (*6–8*), we found that C_t of the first Ebola virus–positive sample was strongly associated with survival. Infected persons with lower C_t values (thus higher viral loads) at time of detection were more likely to die than were those with higher C_t values. This finding was consistent across patients regardless of time from symptom onset to admission to any healthcare facility (Figure 3), suggesting variability in the severity and course of the illness. The differences among patients can be stark: a patient with mild illness (and lower viral load) might not notice the initial onset of symptoms, wait longer for treatment, and still be more likely to survive than someone with rapid and severe illness onset who is immediately sent to the ETU.

To date, quantitative real-time reverse transcription PCR has been used in the Ebola response to distinguish EVD cases from non-EVD cases and to determine when a convalescing patient can be released from an ETU. However, evidence that C_t might also be useful as a prognostic tool is increasing. This finding was first reported >10 years ago after an outbreak of Sudan virus infection (*28*). Since then, EVD analyses supporting this position have been conducted in Sierra Leone and Guinea by using data from a few healthcare facilities in each country (*6–8*). Our assessment supports and adds to this body of evidence as a population-based sample that includes patients who died before admittance to an ETU.

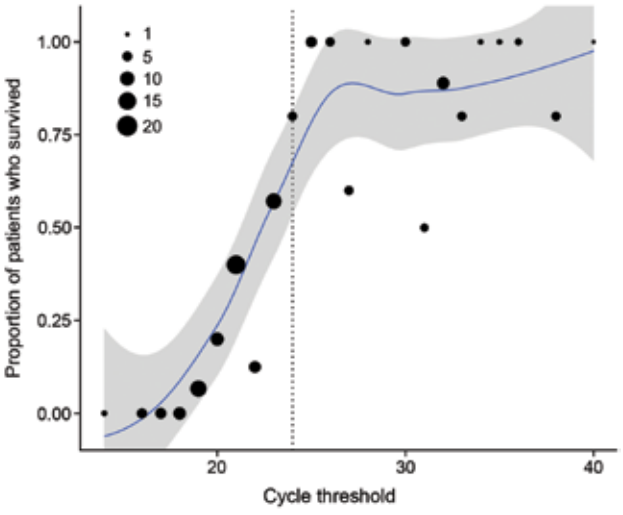

Figure 2. Percent survival among 151 patients in the Ebola virus disease (EVD) primary cohort by cycle threshold (C_t) rounded to nearest integer, Bo District, Sierra Leone, September 2014–January 2015. Locally weighted smoothing line and 95% uncertainty intervals added to illustrate trend; The area of each dot is scaled to represent the number of confirmed EVD cases, by C_t. The trend line suggests a sharp increase in survival for patients with C_t values in the mid-20s (dotted line).

Using C_t as a prognostic indicator could have several benefits for clinicians. First, it could guide patient triage and help clinical staff determine the best treatment course for the gravely ill, particularly when intravenous fluids or advanced supportive therapies are in short supply. Such therapies might help improve patient outcomes when used

Table 2. Logistic regression models assessing association of patient sex, age, C_t, and time from Ebola virus disease symptom onset to healthcare facility admission with patient survival, Bo District, Sierra Leone, September 2014–January 2015*

Cohort	No. patients	Unadjusted		Adjusted†	
		OR for survival (95% CI)	p value	OR for survival (95% CI)	p value
Primary cohort					
Male, vs. female	151	0.99 (0.52–1.90)	0.98	0.96 (0.38–2.44)	0.94
Age, y, increasing, continuous	151	0.97 (0.95–0.99)	0.009	0.97 (0.94–0.99)	0.01
Age ≥20 y, vs. <20 y	151	0.54 (0.27–1.06)	0.076		
C_t, decreasing, continuous	151	0.73 (0.65–0.80)	<0.001		
C_t <20, vs. >24	151	0.0044 (0.0002–0.0245)	<0.001	0.003 (0.001–0.018)	<0.001
C_t 20–24, vs. >24	151	0.12 (0.04–0.28)	<0.001	0.086 (0.028–0.22)	<0.001
C_t <24, vs ≥24	151	0.04 (0.02–0.10)	<0.001		
Days from symptom onset to admission to any healthcare facility, increasing, continuous	151	0.97 (0.87–1.08)	0.59	0.88 (0.76–1.02)	0.089
ETU subgroup					
Days from symptom onset to admission to ETU, increasing, continuous	99	0.95 (0.83–1.07)	0.37	0.88 (0.74–1.03)	0.11
Final 2 months subgroup					
Days from symptom onset to admission to isolation ward, increasing, continuous	68	0.98 (0.79–1.20)	0.84	0.85 (0.64–1.11)	0.23

*C_t, cycle threshold; ETU, Ebola treatment unit; OR, odds ratio.
†Adjusted for sex, age (continuous), and C_t (<20, 20–24, >24).

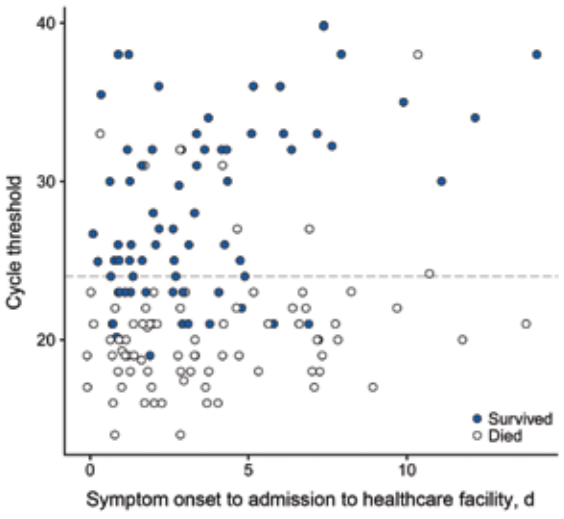

Figure 3. Scatterplot of outcome by cycle threshold (C_t) at time of first Ebola virus–positive test result and time to admission at any healthcare facility (primary cohort, n = 151), Bo District, Sierra Leone, September 2014–January 2015. Each circle represents an infected person. The dashed line indicates the classification threshold of the C_t value of 24. Observations are slightly horizontally jittered to reduce overplotting.

on those who have higher viral loads at the time of initial care and those who are more ill than their peers. Second, if a patient has mild symptoms but a high viral load, clinicians can be prepared for the patient's condition to deteriorate quickly. Third, healthcare staff also can use this information to manage patient and family expectations regarding probable outcomes.

Limitations of this study include probable misreporting of patient age and symptom onset date, as well as the relatively small number of cases. Although we included all known deaths among persons with EVD to calculate the overall case-fatality proportion, the 58 persons who died before or shortly after detection were excluded from the analysis because comparable blood samples or reliable symptom onset dates were not available. This exclusion is potentially a source of bias if the time from symptom onset to death for these 58 persons is longer than the time from onset to admission for the remaining patients (Technical Appendix). In addition, although this study included all detected cases, it is unknown how many cases might not have been detected by the surveillance system. Outcomes of these undetected infected persons might have differed from those of detected persons, leading to an incorrect estimate of survival. The quality of care probably varied between facilities and between time periods, particularly in the isolation unit, which could have affected our findings. Furthermore, the C_t values used for this assessment are specific to the CDC laboratory equipment and VP40 assay used. Use of other laboratory equipment, procedures, or assays could yield different results, thus affecting the optimal C_t. Standardization of equipment, procedures, and assays would facilitate the use of C_t as a prognostic indicator. Last, determining an optimal C_t for prognosis probably depends on specific patient characteristics and the effectiveness of care provided; the strength of the association between C_t and survival could be confounded by these or other unmeasured factors.

In summary, the case-fatality proportions found by this study were higher than estimates that do not include all deaths of known infected persons or that are limited to ETU patients only. A C_t of ≥24 in the first Ebola virus–positive sample was a strong predictor of survival among persons who were alive when detected by the surveillance system. In this population, the time from reported symptom onset to healthcare facility admission was not associated with survival. Additional studies are needed to validate these findings and to continue to explore how C_t values can be combined with other biomarkers (*29*,*30*) to provide insights into the effectiveness of treatment and prognosis.

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Invasive Group A *Streptococcus* Infection among Children, Rural Kenya

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To determine the extent of group A *Streptococcus* (GAS) infections in sub-Saharan Africa and the serotypes that cause disease, we analyzed surveillance data for 64,741 hospital admissions in Kilifi, Kenya, during 1998–2011. We evaluated incidence, clinical presentations, and *emm* types that cause invasive GAS infection. We detected 370 cases; of the 369 for which we had data, most were skin and soft tissue infections (70%), severe pneumonia (23%), and primary bacteremia (14%). Overall case-fatality risk was 12%. Incidence of invasive GAS infection was 0.6 cases/1,000 live births among neonates, 101/100,000 person-years among children <1 year of age, and 35/100,000 among children <5 years of age. Genome sequencing identified 88 *emm* types. GAS causes serious disease in children in rural Kenya, especially neonates, and the causative organisms have considerable genotypic diversity. Benefit from the most advanced GAS type–specific vaccines may be limited, and efforts must be directed to protect against disease in regions of high incidence.

Worldwide, childhood deaths have decreased, largely attributable to fewer deaths from pneumonia, measles, and diarrhea (*1*); some of these reductions have been achieved through vaccination against common bacterial pathogens such as *Streptococcus pneumoniae* and *Haemophilus influenzae* type b (*2*). However, progress in reducing deaths among children has been slower in sub-Saharan Africa, where approximately half of all such deaths occur, a third during the first month of life (*1*). To achieve further disease reductions, it is essential to address other, potentially preventable, causes of invasive bacterial disease, such as group A *Streptococcus* (GAS). It is estimated that >660,000 cases of invasive GAS infection occur each year; >95% cases occur in resource-poor regions, and >160,000 patients die (*3*). Despite these estimates, data on invasive GAS infections in resource-poor settings are limited.

The Young Infant Study of invasive bacterial disease conducted in the late 1990s in The Gambia, Ethiopia, Papua New Guinea, and the Philippines reported GAS in 29 (17%) of 167 bacterial isolates from blood cultures and in 3 (7.5%) of 40 cerebrospinal fluid (CSF) cultures (*4*). Although this finding meant that GAS was the third most commonly isolated bacterium after *S. pneumoniae* and *Staphylococcus aureus*, research into associated invasive GAS infections has been limited. To our knowledge, in sub-Saharan Africa, only 1 estimate of invasive GAS incidence has been published: 29 cases/100,000 person-years (definite cases of bacteremia only) among children <5 years of age in Kenya and 96 cases/100,000 person-years among children <1 year of age (*5*). These incidences are higher than those reported from other resource-poor settings. Data from Fiji, in the Pacific, report an incidence of 26 cases/100,000 person-years among children <5 years and 45 cases/100,000 person-years among children <1 year of age (*6*). In New Caledonia, the incidence for children <5 years of age was 7 cases/100,000 person-years (*7*).

Vaccines for GAS are being developed; the most advanced is a 30-valent serotype-specific vaccine. Data about the *emm* types causing invasive GAS disease in sub-Saharan Africa are critical for assessing potential vaccine serotype coverage. Through comprehensive prospective clinical and microbiological surveillance (1998–2011), we determined incidence, clinical characteristics, and outcomes among children with invasive GAS

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DOI: http://dx.doi.org/10.3201/eid2202.151358

infections in a hospital in rural Kenya. We used whole-genome sequencing to determine e*mm* types and phylogenetic variations of invasive GAS isolates.

Materials and Methods

Study Design and Participants

Since 1998, the Kenya Medical Research Institute/Wellcome Trust Research Programme has undertaken prospective systematic clinical surveillance, including standardized clinical documentation and systematic microbiological investigation, for invasive bacterial disease among all children admitted for medical care to Kilifi County Hospital (in Kilifi, a rural area of coastal Kenya), as described elsewhere (*5*,*8*). Our observational study identified cases of invasive GAS disease during this surveillance of all children admitted to Kilifi County Hospital from August 1, 1998, through December 31, 2011. The study size was determined by admissions during the study period. The study was approved by the National Ethics Committee, Nairobi, Kenya (ERC 2144), and the Oxford Tropical Research Ethics Committee (OXTREC 151–12).

The denominator population was determined by using the Kilifi Health and Demographic Surveillance System, which covers 891 km^2 surrounding the hospital and in 2011 included ≈260,000 residents (*9*); household enumerations are performed quarterly. We calculated the population age structure at the midpoint of the study (mid-2004) and the total number of live births.

Clinical Surveillance and Case Definitions

At the time of patient admission to the hospital, a standardized set of clinical symptoms and signs were recorded and prospectively entered into a database. At the time of patient discharge, outcome was recorded. Anthropometry for the presence of kwashiorkor (edematous malnutrition) was systematically undertaken at admission and used to define severe acute malnutrition (*10*). For all nonelective admissions, samples were collected for complete blood count, malaria slide, and blood culture. If clinically indicated, culture was performed for CSF, urine, and pus swab samples. Inpatient treatment was provided according to World Health Organization guidelines (*10*).

Starting in January 2007, in line with national guidelines, HIV testing by rapid test was offered for all children admitted. For children who had invasive GAS disease before 2007 and were not tested during our previous study of bacteremia (*5*), a trained counselor visited households and offered voluntary counseling and testing (*9*). For children who had died or were untraceable, a stored blood sample was tested by PCR for HIV. Sickle cell disease testing by electrophoresis was undertaken as clinically indicated; for children admitted with bacteremia during 1998–2008, PCR was used to retrospectively test for sickle cell disease, as described previously (*11*).

For this analysis, data were extracted from clinical and laboratory databases. All paper clinical records were reviewed for signs and symptoms relevant to invasive GAS disease, including the presence of pharyngitis, burns, scabies, and a vesicular rash suggestive of varicella or herpes zoster infection.

Cases of invasive GAS were defined as definite if GAS was isolated from a normally sterile site (blood, CSF, or other sterile fluid/tissue) or if necrotizing fasciitis with evidence of GAS infection was present (e.g., typical gram-positive cocci found after Gram staining or serologic testing results positive for streptococci). Cases of invasive GAS were defined as probable if any of the following were found: classic necrotizing fasciitis without microbiological confirmation; cellulitis in a patient who was moderately or severely unwell (i.e., unwell and history of parenteral receipt of antimicrobial drugs, admission to hospital, or both); microbiological confirmation (i.e., growth of GAS on culture of swab sample or serologic test results positive for streptococci); or other clinically relevant infection in a patient who is moderately or severely unwell (i.e., unwell and history of parenteral receipt of antimicrobial drugs, admission to hospital, or both), in conjunction with positive GAS culture from deep wound swab sample or biopsy sample from surgical infection site (*6*).

Clinical syndromes of invasive GAS disease vary. These syndromes were categorized as meningitis, severe pneumonia, skin or soft tissue infection, joint and bone infection, necrotizing fasciitis, urinary tract infection, acute glomerulonephritis, abdominal disease, endocarditis, bacteremia with no focus, and streptococcal toxic shock syndrome (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/15-1358-Techapp1.pdf).

Microbiological and Molecular Methods

Blood cultures were undertaken by using the BACTEC Peds Plus system (Becton Dickinson, Franklin Lakes, NJ, USA) according to the manufacturers' instructions. Positive broth cultures and CSF, urine, and surface swab samples were subcultured on 5% horse blood agar and chocolate agar. GAS isolates were identified by β-hemolysis, followed by Gram staining and catalase testing, and then grouped by latex bead agglutination. Penicillin susceptibility was tested by disk diffusion (http://www.bsac.org.uk/). Laboratory procedures were subject to internal quality control and external quality control by the UK National External Quality Assessment Service.

GAS isolates were subcultured on 5% horse blood agar from archived bacterial isolates (stored at –80°C) and transported to the Wellcome Trust Sanger Institute, Cambridge, UK. DNA was extracted by a QIAxtractor

(QIAGEN, Valencia, CA, USA), and DNA quality and quantity were documented by using NanoDrop (Thermo Scientific, Waltham, MA, USA) and Qubit (Life Technologies, Carlsbad, CA, USA) techniques. Whole-genome sequences were determined from Illumina 96-plex libraries by using the HiSeq2000 sequencing platform (Illumina, San Diego, CA, USA) to generate tagged 75-bp paired-end reads. To obtain the overall population structure of the sequenced genomes, we mapped individual Illumina read pairs to the MGAS5005 (*emm*1) reference genome (*12*) by using SMALT version 0.5.8 (http://www.sanger.ac.uk/resources/software/smalt/). The average coverage of the resulting whole-genome alignment was 190×. The minimum base-call quality for identifying a single nucleotide polymorphism (SNP) was set at 50, and the minimum mapping quality for SNP calling was set at 30. SNPs called in known MGAS5005 prophage regions and repeat regions were excluded from analyses. The final genome alignment was 1,629,062 bp and comprised 125,233 SNPs. To examine the genomic relationships between the sequenced genomes, we generated a maximum-likelihood tree from the SNP alignment by using FastTree (*13*). Draft genome assemblies were compiled by using an iterative sequence assembly process as defined previously (*14*). An initial quality control screen of the short-read sequences to identify mixed isolates and low-quality sequences was determined by examining genome assembly length and SNP heterogeneity. A total of 43 (11.6%) sequences had an assembly length of >2 mega basepairs and were excluded from phylogenetic analyses because of possible contamination. The *emm* type and multilocus sequence type (MLST) were obtained from in-house BLAST analysis of draft genome assemblies and compared with those in centralized databases (http://www.cdc.gov/streplab/m-proteingene-typing.html, http://pubmlst.org/spyogenes/). New *emm* and MLST alleles were assigned by database curators. Allocation of *emm* cluster was derived as previously described (*7*). Heterogeneity observed within the typing schemes was investigated by using maximum-likelihood associations in whole-genome sequence data.

Epidemiologic Analysis

Epidemiologic analyses were undertaken by using STATA version 13 statistical software (StataCorp LP, College Station, TX, USA). Clinical characteristics of children with invasive GAS disease were tabulated, and the frequency of clinical syndromes of invasive GAS disease and associated case-fatality risks (by age group) were calculated. Incidence rates were calculated by using the invasive GAS cases resident within the Kilifi Health and Demographic Surveillance System, the age structure of the population at the study mid-point (2004), and the total number of live births. Trends in admissions were examined by using rolling averages, and a comparison between seasons (wet and dry) was made by using the Poisson distribution.

Results

During the study, 64,761 children were admitted to the hospital with acute illness. From 370 children with invasive GAS infection, 391 GAS isolates were identified. Of these 391 isolates, 154 (39.4%) were from blood, 9 (2.3%) from CSF, 214 (54.7%) from a swab sample (wound, skin breach, or pus), 8 (2.0%) from joint aspirates, and 6 (1.5%) from urine. From 20 children, >1 GAS isolate was identified: 7 children had invasive GAS isolated from both blood and CSF; 2 children had repeat positive blood cultures; 2 children had invasive GAS isolated from blood and a swab sample; 1 child had invasive GAS isolated from CSF and a swab sample; 7 children had invasive GAS isolated from 2 swab samples; and 1 child had invasive GAS isolated from 3 swab samples. No isolates were resistant to penicillin.

Characteristics of Children and Risk Factors for Definite Invasive GAS Disease

Full clinical information was available for 369 of the 370 children: 152 children had definite and 217 had probable invasive GAS disease as defined. A total of 94 (25.5%) cases of invasive GAS were in neonates (Table 1). Among the 152 children with definite invasive GAS disease, 5 (3.3%) had burns, 4 (2.6%) had concurrent scabies, 1 (0.7%) had a vesicular rash (consistent with herpes zoster or varicella), and 2 (1.3%) had a history of trauma. Among the 217 with probable invasive GAS disease, 26 (12.0%) had burns, 3 (1.4%) had scabies, 1 (0.5%) had a vesicular rash, and 4 (1.8%) had a history of trauma (risk factors were not mutually exclusive). No reports of pharyngitis were documented for patients who had definite or probable invasive GAS disease. Among the 152 children with definite invasive GAS disease, prevalence of common risk factors for invasive bacterial disease was high: 81 (53.3%) had any risk factor; 30 (19.7%) had severe acute malnutrition, including 9 (5.9%) with kwashiorkor; 28 (18.4%) had malaria (slide positive for *Plasmodium falciparum*), and 24 (15.8%) had HIV infection.

Clinical Syndromes of GAS Disease and Case-Fatality Risk

Among the 369 children with invasive GAS disease, the most frequent infection was skin or soft tissue infection, occurring in 258 (69.9%); followed by severe pneumonia in 86 (23.3%), of which 59 (69%) were complicated by sepsis; then bacteremia without focus in 53 (14.4%) (Table 2). Also among these 369 children, 17 (4.6%) had bone and joint infections, 11 (3.0%) had meningitis, 6 (1.6%) had

Table 1. Characteristics of children with GAS disease admitted to Kilifi County Hospital, Kenya, 1998–2011*

Characteristic	All GAS disease, n = 369, no. (%)	Definite invasive GAS disease, n = 152, no. (%)	Probable invasive GAS disease, n = 217, no. (%)
Age			
0–6 d	33 (8.9)	13 (8.6)	20 (9.2)
7–28 d	61 (16.5)	38 (25.0)	23 (10.6)
29–59 d	17 (4.6)	12 (7.9)	5 (2.3)
60 d–1 y	63 (17.1)	40 (26.3)	23 (10.6)
>1 and <5 y	125 (33.9)	41 (27.0)	84 (38.7)
5–12 y	70 (19.0)	8 (5.3)	62 (28.6)
Sex			
M	219 (59.3)	84 (55.3)	135 (62.2)
F	150 (40.7)	68 (44.7)	82 (37.8)
Severe acute malnutrition			
No	294 (79.7)	106 (69.7)	188 (86.6)
Yes (wasting)	47 (12.7)	30 (19.7)	17 (7.8)
Yes (kwashiorkor)	11 (3.0)	9 (5.9)	2 (0.9)
Not known	17 (4.6)	7 (4.6)	10 (4.6)
Malaria (positive slide result)			
No	313 (84.8)	123 (80.9)	190 (87.6)
Yes	56 (15.2)	29 (19.1)	27 (12.4)
HIV infection			
No	209 (56.6)	116 (76.3)	93 (42.9)
Yes	28 (7.6)	24 (15.8)	4 (1.8)
Not known	132 (35.8)	12 (7.9)	120 (55.3)
Sickle cell disease			
No	136 (36.9)	95 (62.5)	41 (18.9)
Sickle cell trait	14 (3.8)	9 (5.9)	5 (2.3)
Sickle cell disease	3 (0.8)	1 (0.7)	2 (0.9)
Not known	216 (58.5)	47 (30.9)	169 (77.9)

*Malaria incidence (slide-positive admissions data from Kilifi Health and Demographic Surveillance System) decreased from 28.5 to 3.45 cases per 1,000 person-years during 1999–2007. HIV prevalence was 4.9% (routine antenatal screening, 2004–2007) with no evidence of a temporal trend. Sickle cell disease prevalence among infants in the Kilifi Health and Demographic Surveillance System (2006–2009) was 15% for genotypes HbAS and 1% with HbSS (*11*). Severe acute malnutrition is referenced against World Health Organization population standards (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/15-1358-Techapp1.pdf). GAS, group *A Streptococcus*.

a urinary tract infection, 2 (0.5%) had acute glomerulonephritis, 1 (0.3%) had endocarditis, 1 (0.3%) had nonspecific abdominal signs, and 1 (0.3%) had necrotizing fasciitis. A total of 19 (5.1%) cases met the criteria for streptococcal toxic shock syndrome (*15*). Of the 369 children, 45 (12.2%) died. The case-fatality risk was highest among those with severe pneumonia (20/86, 23.3%), followed by primary bacteremia (11/53, 20.8%) and meningitis (2/11, 18.2%). Pneumonia and primary bacteremia occurred most frequently among children <1 year of age.

Incidence of Invasive GAS Disease

The minimum incidence (cases/100,000 person-years) for definite and all (definite and probable) invasive GAS disease, respectively, among children <5 years of age was 17 (95% CI 14–21) and 35 (95% CI 30–40); among children <1 year of age, incidence was 59 (95% CI 45–74) and 101 (95% CI 83–121). Among neonates, incidence (cases/1,000 live births) for definite and all invasive GAS, respectively, was 0.3 (95% CI 0.2–0.4) and 0.6 (95% CI 0.4–0.7). The incidence of death was 0.1 (95% CI 0.1–0.2) deaths

Table 2. Common clinical syndromes of GAS disease among children admitted to Kilifi County Hospital, Kenya, 1998–2011*

Clinical syndrome	Age						
	0–6 d	7–28 d	29–59 d	60 d–1 y	>1–<5 y	5–12 y	Overall
All cases							
No. (%)	33 (100)	61 (100)	17 (100)	63 (100)	125 (100)	70 (100)	369 (100)
Deaths, CFR	10 (30.3)	23 (37.7)	1 (6.3)	7 (11.1)	14 (11.2)	1 (1.4)	45 (12.2)
Skin and soft tissue infection							
No. (%)	22 (66.7)	33 (54.1)	5 (29.4)	37 (58.7)	99 (79.2)	62 (88.6)	258 (69.9)
Deaths, CFR	6 (27.3)	4 (12.1)	0	1 (2.7)	6 (6.1)	1 (1.6)	17 (4.5)
Severe pneumonia†							
No. (%)	7 (21.2)	17 (27.9)	8 (47.1)	28 (44.4)	21 (16.8)	3 (4.3)	86 (23.3)
Deaths, CFR	2 (28.6)	5 (29.4)	0	5 (17.9)	8 (38.1)	0	20 (23.3)
Primary bacteremia							
No. (%)	8 (24.2)	17 (27.9)	3 (17.6)	9 (14.3)	13 (10.4)	3 (4.3)	53 (14.4)
Deaths, CFR	2 (25.0)	5 (29.4)	1 (33.3)	2 (22.2)	1 (7.7)	0	11 (20.8)

*CFR, case-fatality risk; GAS, group A *Streptococcus*.
†59 of the 86 severe pneumonia cases were complicated by sepsis.

Table 3. Estimated minimum incidence of definite and probable invasive GAS disease and deaths associated with invasive GAS disease in the catchment area of Kilifi County Hospital, Kenya, 1998–2011*

Incidence†	Age group				
	Neonate, 0–27 d, n = 9,828‡	Infant, 28–59 d, n = 10,463‡	Infant, 2–11 mo, n = 92,070‡	Child 1–4 y, n = 453,857‡	Child 5–12 y, n = 730,512‡
Probable and definite invasive GAS disease incidence (95% CI)	631 (484–808)	105 (52–188)	43 (31–59)	19 (15–23)	6 (4–9)
Definite invasive GAS disease incidence (95% CI)	326 (223–459)	86 (39–163)	27 (18–40)	7 (5–10)	1 (0–1)
Death associated with all invasive GAS disease (95% CI)	163 (93–264)	10 (0–53)	5 (2–13)	2 (1–3)	0 (0–1)

*GAS, group *A Streptococcus*.
†Per 100,000 person-years.
‡Population denominator in person-years.

per 1,000 live births (Table 3). No trend was detected in the number of cases admitted over the study period (online Technical Appendix Figure 1). Invasive GAS cases occurred less frequently during the dry months across all years (December–March, 26 cases/month) than during months of the short and long rains (April–October, 33 cases/month) (p = 0.029).

Molecular Epidemiology of GAS

Of the 391 original GAS isolates, we retrieved 371 and generated high-quality genome sequences for 328 (online Technical Appendix Table 2). From another 29 GAS isolates (combined total of 357) with lower quality genome sequences, we were able allocate an *emm* type. The remaining 14 samples were subsequently excluded from molecular analyses because they were not GAS or were mixed cultures, affecting accurate SNP calling (but not epidemiologic analyses because these isolates had been subcultured, stored, and then subcultured again, potentially introducing contamination). Through BLAST analysis of the 357 genome sequences against the *emm* typing database, we assigned 88 different *emm* types (97 including subtypes). Of the *emm* subtypes, 21 were new variants. No *emm* types represented >5% of the isolates studied, showing that no single *emm* type was predominant in the GAS population irrespective of clinical association (Figure 1; online Technical Appendix Figure 2).

Of the 357 GAS isolates, we assigned an *emm* cluster designation to 329 on the basis of the recently described *emm* cluster classification scheme (*16*). Of the 48 *emm* clusters described, 24 were represented within the Kilifi invasive GAS population of isolates (online Technical Appendix Table 3). Of the 140 MLSTs identified, only 24 sequence types were represented within the MLST database (78/328 strains with high-quality whole-genome sequence data). We identified 89 new allelic variants among the 7 housekeeping genes and assigned 116 new MLSTs. Crude phylogenetic analyses of the Kilifi invasive GAS population as a whole revealed a star-like topology (Figure 2) indicative of diverse core genotypes. Collectively, these data illustrate substantial heterogeneity within invasive GAS genotypes in the Kilifi population.

In terms of vaccine coverage, 99 (28%) of 357 GAS isolates are included within the current 30-valent vaccine (*19*), and another 104 (29%) exhibit a degree of *emm* cross-reactivity in vitro (Figure 1) (*20*). Of the remainder, 27 (8%) were not included in the vaccine and are not cross-reactive, and 127 (36%) have not yet been investigated for cross-reactivity.

Discussion

Incidence of invasive GAS disease in this rural sub-Saharan African setting was strikingly high, particularly among children in the first year of life among whom GAS was a major cause of sepsis and severe pneumonia. The minimum incidence of invasive GAS infection was highest among neonates (0.6 cases/1,000 live births; more than one third of all case-patients died). Minimum incidence in the first year of life overall was also high (101 cases/100,000 person-years), twice that for Fiji, the only other resource-poor setting from which an incidence estimate is available (*6*). The incidence estimates presented here are probably underestimates because inclusion in the study relied on hospital admission; hence, they are referred to as minimum incidence estimates. Residents living nearer to Kilifi County Hospital are more likely to access care than those living farther from it (*21*), and care-seeking behavior varies (*22*). The incidence of invasive GAS is probably accompanied by high prevalence of the spectrum of GAS infections, including acute poststreptococcal glomerulonephritis and acute rheumatic fever, which can lead to rheumatic heart disease (*23*); however, data for sub-Saharan Africa are limited (*24*,*25*).

In rural Kenya, unlike in other settings, pharyngitis, varicella, and scabies did not seem to be major drivers of invasive GAS disease (*23*,*26*), and impetigo was not differentiated from skin infections. These conditions are probably underascertained because they would not in themselves result in hospital admission, and unlike most of the clinical and microbiological data (systematically sought and collected), these diagnoses relied on observations being recorded. Also, despite the high frequency of skin and soft tissue infections, we detected only 1 case of necrotizing fasciitis, which may again be underascertainment from clinical information.

Invasive GAS was, however, associated with concurrent conditions driving other bacterial diseases in sub-Saharan Africa: HIV, severe acute malnutrition, and malaria (*5*,*27*,*28*) but not sickle cell disease (as reported elsewhere) (*11*,*29*–*31*).

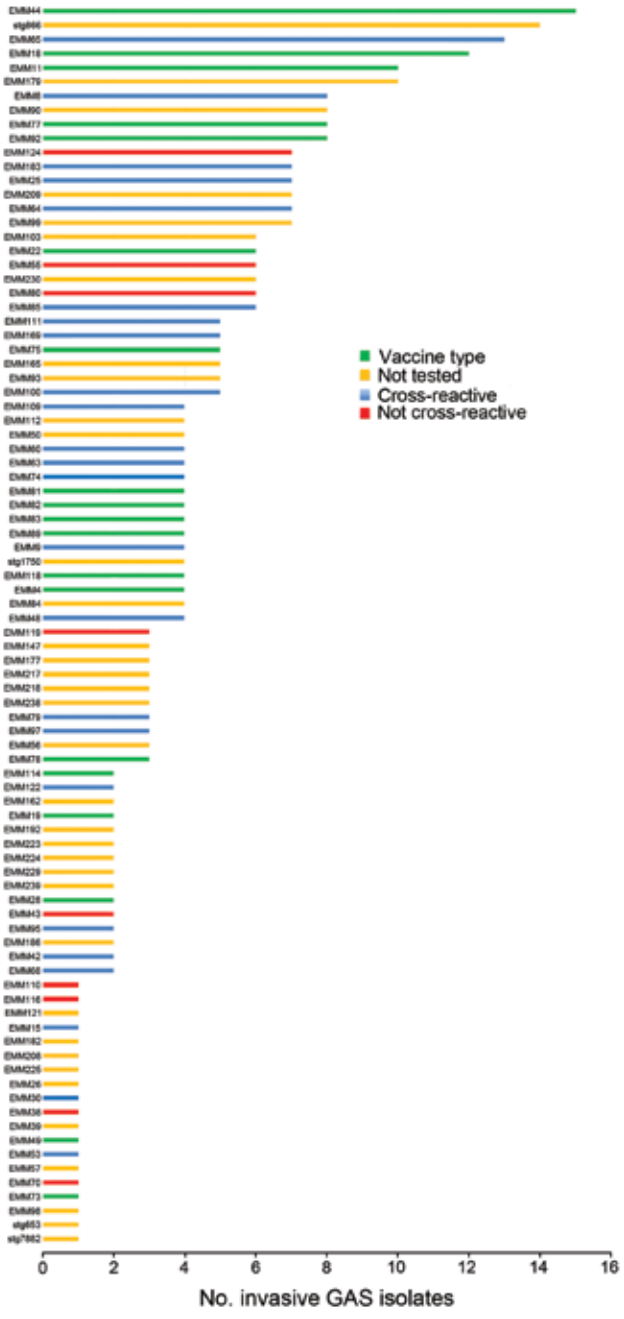

Figure 1. *emm* types of group A *Streptococcus* (GAS) isolates from children with GAS disease admitted to Kilifi County Hospital, Kenya, 1998–2011. *emm* types shown in green are included in the 30-valent vaccine; *emm* types in blue are not included in the 30-valent vaccine, but this vaccine may provide immunity to this *emm* type through cross-reactivity; *emm* types in red are not included in the 30-valent vaccine, and there is no evidence of cross-reactivity; *emm* types in yellow are not included in the 30-valent vaccine, and their cross-reactivity has not yet been tested.

In this study, the invasive GAS *emm* types and *emm* clusters were extremely heterogeneous and differed from those that cause disease in resource-rich settings. The presence of several *S. dysgalactiae* subsp. *equisimilis*–like *emm* types within a *S. pyogenes* genomic backbone supports previous observations of interspecies genetic transfer of *emm* alleles (*32*). The overall diversity of *emm* types we describe supports findings of increased heterogeneity in other resource-poor settings (*33*). One published study reports noninvasive GAS *emm* types from sub-Saharan Africa. In that study, school children in Ethiopia were investigated for GAS carriage; 43 different *emm* types were identified in 82 colonizing GAS isolates (*34*). Less than one third of *emm* types identified in our study were also identified in the Ethiopia study, suggesting that the pool of GAS *emm* types in circulation, even within neighboring countries, is larger than that described here.

Reducing the incidence of invasive GAS infection in this setting could be achieved by reducing risk factors such as severe acute malnutrition and HIV (e.g., through prevention of mother-to-child transmission), as well as by supporting antisepsis measures at delivery, including antiseptic neonatal cord care (*35*–*37*). Early and improved treatment of skin infections, including impetigo, and burns could also reduce invasive GAS disease. However, prevention through effective vaccination will probably lower disease incidence the most, as has occurred for other pathogens, such as *S. pneumoniae* (*38*) and *H. influenzae* type b (*39*). The difficulty with *emm* type–specific GAS vaccine approaches (*19*) is the heterogeneity of GAS *emm* types and limited data on many of the *emm* types identified in this study. From current information, only 57% of invasive GAS disease cases would be covered (either directly or through cross-reactivity) by the most advanced 30-valent vaccine being developed (*19*). Furthermore, serotype replacement could occur, as described for *S. pneumoniae* (*40*), and would require detailed surveillance.

The high incidence of invasive GAS disease in rural sub-Saharan Africa underlines the contribution of invasive bacterial disease in this region to childhood deaths, particularly among neonates and young infants; associated case-fatality risk is high. Invasive GAS may also be causing puerperal sepsis in this setting; more studies are needed. Reductions in childhood illness and death could, however, be achieved through effective GAS vaccination. Further development of GAS vaccines followed by clinical trials must be prioritized, targeted at settings with the highest disease incidence.

Acknowledgments

We thank the core informatics, library, and sequencing teams at The Wellcome Sanger Institute for whole-genome sequencing; and we thank all those at the Kenya Medical Research Institute/

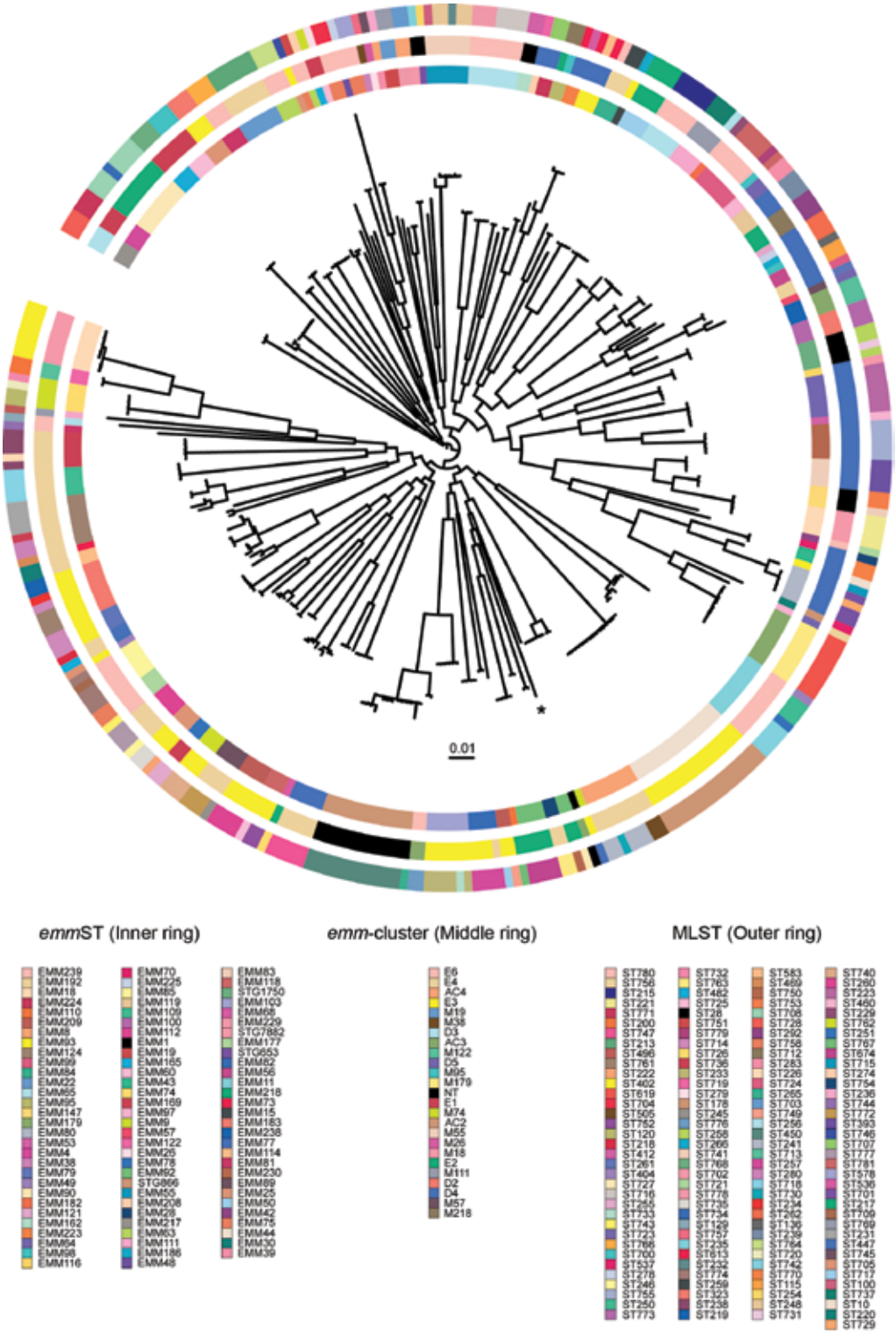

Figure 2. Population structure of 328 *Streptococcus pyogenes* strains from children with group A *Streptococcus* (GAS) disease admitted to Kilifi County Hospital, Kenya, 1998–2011. Unrooted maximum-likelihood phylogeny based on the whole-genome associations of mapped *S. pyogenes* genomes to the MGAS5005 reference genome indicates extensive genomic diversity within the population. The rings surrounding the central phylogeny correspond to standard GAS molecular typing methods; colors indicate different STs. Inner ring, *emm* ST (*16*); middle ring, *emm* cluster (*17*); outer ring, multilocus sequence type (*18*). NT, nontypeable *emm* clusters; ST, sequence type. *Position of the MGAS5005 reference genome. Scale bar indicates genetic change of 0.01.

Wellcome Trust Research Programme who were involved with surveillance at Kilifi County Hospital. We also thank and acknowledge those who set up and managed the MLST global database and the Centers for Disease Control and Prevention global *emm* database.

This study is published with permission from the director of the Kenya Medical Research Institute. This work was supported by the European Society for Paediatric Infectious Disease, The Wellcome Trust, UK (grant nos. 093804, 091758/Z10/Z, 098532, 083579, 077092 to A.C.S., M.R.D., T.N.W., J.A.G.S., G.D., J.B.), and KEMRI-Wellcome Trust and the National Health and Medical Research Council of Australia (grant no. 35250 to M.R.D.).

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Randomized Controlled Trial of Hospital-Based Hygiene and Water Treatment Intervention (CHoBI7) to Reduce Cholera

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The risk for cholera infection is >100 times higher for household contacts of cholera patients during the week after the index patient seeks hospital care than it is for the general population. To initiate a standard of care for this high-risk population, we developed Cholera-Hospital-Based-Intervention-for-7-Days (CHoBI7), which promotes hand washing with soap and treatment of water. To test CHoBI7, we conducted a randomized controlled trial among 219 intervention household contacts of 82 cholera patients and 220 control contacts of 83 cholera patients in Dhaka, Bangladesh, during 2013–2014. Intervention contacts had significantly fewer symptomatic *Vibrio cholerae* infections than did control contacts and 47% fewer overall *V. cholerae* infections. Intervention households had no stored drinking water with *V. cholerae* and 14 times higher odds of hand washing with soap at key events during structured observation on surveillance days 5, 6, or 7. CHoBI7 presents a promising approach for controlling cholera among highly susceptible household contacts of cholera patients.

Severe cholera without adequate rehydration kills up to half of affected persons (*1*). The World Health Organization estimates that 3–5 million cholera cases occur worldwide each year (*2*). Studies have identified multiple risk factors for *Vibrio cholerae* infection, such as drinking street-vended water, placing one's hands into stored household water, lack of drinking water treatment, eating food prepared by a recently ill food handler, and not washing hands with soap before eating food (*3*–*8*). These findings suggest that cholera is transmitted through contaminated water and poor hygiene practices. Therefore, interventions targeting improved water treatment and storage practices and hand washing with soap have the potential to substantially reduce cholera transmission (*8*).

Previous studies in Bangladesh have demonstrated that household contacts of cholera patients are at >100 times higher risk for cholera infections during the 1-week period after the index patient seeks hospital care (*5*,*7*,*9*–*11*). Although the average rate of cholera in National Institute of Health–sponsored surveillance areas of Bangladesh is 1.6 cases/1,000 persons, a study in urban Dhaka, Bangladesh, found that 210 household contacts/1,000 index patients were infected with *V. cholerae* during a 21-day surveillance period (>90% of these infections occurred during the first week after the index patient sought care) (*7*,*12*). This high rate of cholera among household contacts probably results from a shared contaminated environmental source, such as water or food in the household, or secondary transmission from infected household members because of poor hygiene (*6*,*9*,*13*).

In Bangladesh, the current standard of care for cholera patients at hospital discharge is to provide oral rehydration solution (ORS) packets. No standard of care exists for household contacts of these patients despite their very high risk for cholera (*5*,*7*). The time that patients and their accompanying family members spend at a health facility during a severe diarrheal episode provides an opportunity for health providers to communicate information about water sanitation and hygiene (WASH) behavior change when perceived severity of diarrheal disease and perceived benefits of water treatment and hand washing with soap are likely to be highest (*14*). However, only a few studies have evaluated the effects of health facility–based WASH interventions, and none have evaluated the effects of these interventions in reducing enteric infections among household contacts of hospitalized diarrhea patients (*15*–*22*).

To initiate a standard of care for household contacts of cholera patients during the 1-week high-risk period after the index patient seeks care, we evaluated the efficacy

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DOI: http://dx.doi.org/10.3201/eid2202.151175

of a hospital-based intervention promoting hand washing with soap and treatment of water called Cholera-Hospital-Based-Intervention-for-7-Days (CHoBI7) in Dhaka, Bangladesh, during June 2013–November 2014. We hypothesized that, in comparison with the standard message given to cholera patients at hospital discharge on ORS, CHoBI7 would significantly reduce cholera infections and increase hand washing with soap and treatment of water among highly susceptible household contacts of cholera patients.

Methods

All study participants (household contacts and cholera index patients) provided informed consent; consent comprised adult participants (≥18 years of age) signing an informed consent and/or parental consent form and children 12–17 years of age signing an assent form. If a study participant could not read, the consent form was read to him or her, and the participant then was asked to document his or her consent with an X in the presence of a witness. All study procedures were approved by the research Ethical Review Committee of icddr,b, Dhaka, and the Institutional Review Board of The Johns Hopkins Bloomberg School of Public Health (Baltimore, MD, USA).

We evaluated the efficacy of CHoBI7 by conducting a cluster randomized controlled trial in Dhaka during June 2013–November 2014. Suspected cholera patients seeking care at the icddr,b Dhaka Hospital were defined as persons with acute watery diarrhea (≥3 loose stools during a 24-h period) and moderate to severe dehydration using the World Health Organization definition. These patients were screened for *V. cholerae* in their feces by using the Crystal VC Rapid Dipstick test (Span Diagnostics, Surat, India) (*23*,*24*). All dipstick-positive findings were confirmed by bacterial culture. All patients suspected to have cholera who resided within a police thana (ward) of Dhaka and were admitted to icddr,b Dhaka Hospital were screened for eligibility for the CHoBI7 trial. A cholera case was defined as a fecal bacterial culture result positive for *V. cholerae* in a suspected cholera patient. Cholera patients were excluded from the study if a household contact already was enrolled (currently or previously) or if they had received cholera vaccine, to avoid confounding from an ongoing cholera vaccine trial.

Household contacts were defined as persons sharing the same cooking pot as the index patient for the previous 3 days. To be eligible for the study, household contacts had to plan to reside in the household of the index patient for the following week and could not have received cholera vaccine. Eligible household contacts in the hospital attending their ill family member at the time of cholera patient enrollment were invited to participate, and the household was visited to recruit household contacts within 36 hours after patient enrollment. Typically, cholera patients stayed at Dhaka Hospital for 24–48 hours before returning home. A cluster was defined as the index cholera patient and his or her household contacts.

The design of CHoBI7 was informed by factors from the Integrated Behavioral Model for Water, Sanitation and Hygiene interventions and constructs from the Health Belief Model (*25*,*26*). CHoBI7 was tailored to residents living in slum areas of Dhaka during 3 months of piloting and previous formative research (*27*). CHoBI7 includes 1) a pictorial ("Chobi" in Bangla) module on how cholera can spread through the environment (e.g., contamination of household drinking water sources and stored water), how persons can spread cholera to each other by contaminating food and water in their home, and instructions on proper hand washing with soap and treatment of water (Figure 1); and 2) a cholera prevention package containing a 3-month supply of chlorine tablets (Aquatabs sodium dichloroisocyanurate; Medentech, Wexford, Ireland, UK) for water treatment, soapy water bottles (a low-cost alternative to bar soap made using detergent), a hand washing station, a sealed water vessel with cover to ensure safe water storage, and cue-to-action cards with instructions about promoted behaviors (Figure 2). A trained health promoter at Dhaka Hospital delivered this pictorial module and cholera prevention package to cholera patients and their accompanying family members during a consultation session in the hospital. These messages were reinforced through daily household visits by the health promoter for the 1-week intervention period. The cost per household for CHoBI7 was US $45.50 (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/15-1175-Techapp1.pdf); the cost included intervention hardware, transport cost, and the promoter's salary.

Study recruitment at Dhaka Hospital occurred Saturday–Thursday each week during the study period. Each

Figure 1. Cholera-Hospital-Based-Intervention-for-7-Days (CHoBI7) Intervention hardware, Dhaka, Bangladesh, June 2013–November 2014. The kit contained a water vessel with cover, chlorine tablets, hand washing station, and bottle of soapy water.

Figure 2. Promotional flipbook and cue cards about hand washing with soap and treatment of water, Dhaka, Bangladesh, June 2013–November 2014. Cue cards are placed next to intervention hardware as a cue to action on hygiene and water treatment–related behaviors.

week, half of the surveillance days were randomly selected to be intervention days, and half were randomly assigned to be control days by using a random number generator. The principal investigator (C.M.G.) assigned randomization; this scheme limited the likelihood of seasonal variations in study arm assignment and selection bias. The control arm received the standard message given at health facilities in Bangladesh about the use of ORS to treat diarrhea, and the intervention arm received this standard message and CHoBI7. To minimize bias, we used 2 separate teams for intervention and evaluation activities.

Households were visited on days 1 (baseline), 3, 5, 7, and 9 (visits 1–5) after the cholera patient sought care at Dhaka Hospital for clinical surveillance and to assess intervention uptake indicators. For clinical surveillance, household contacts were asked whether they had diarrhea (≥3 loose stools during a 24-hour period) or vomiting in the previous 48 hours, and a rectal swab sample was collected from willing household contacts at each household visit to test for *V. cholerae* in feces by bacterial culture. Because of limitations in our study personnel capacity, rectal swab samples were available only from household contacts enrolled during June 2013–June 2014.

To assess indicators of intervention fidelity, we collected a water sample from the household's water source and drinking water stored in the home at each household visit to test for *V. cholerae* by bacterial culture and for the presence of free chlorine, as a proxy measure of water treatment, by using a digital colorimeter (Hach, Loveland, CO, USA). The US Centers for Disease Control and Prevention–recommended cutoff for free chlorine of ≥0.2 mg/L in household stored drinking water was used (*28*).

Spot checks were conducted at each household visit in all study households to observe whether soap was present near (within 10 steps of) the latrine and cooking areas as a proxy measure of hand washing with soap (*29*). To observe hand washing with soap practices, a 5-hour structured observation substudy was conducted once in all households recruited during October 2013–November 2014 (59 intervention and 56 control households) on surveillance day 5, 6, or 7. Hand washing with soap was recorded at the following key events promoted in CHoBI7: 1) after using the toilet, 2) after cleaning a child's anus, 3) before eating, and 4) before preparing food.

Rectal swab samples were collected on Cary-Blair media, and water samples were collected in 500-mL bottles and transported to the Enteric Microbiology Laboratory at icddr,b. Fecal specimens and water samples were analyzed for *V. cholerae* and serotyped according to previously

published methods (*30*,*31*). The laboratory was blinded to the study arm of specimens received.

Our primary outcomes were 1) the incidence of *V. cholerae*–infected household contacts, defined as a culture result positive for *V. cholerae*, and 2) the incidence of symptomatic *V. cholerae* infection, defined as diarrhea or vomiting in the past 48 hours in a *V. cholerae*–infected household contact. Our secondary outcomes were the percentages of 1) hand washing with soap at key events during 5-hour structured observation, 2) households with soap at the latrine and cooking areas, 3) households with stored drinking water with detectable *V. cholerae*, and 4) households with free chlorine concentrations ≥0.2 mg/L in stored drinking water. We excluded the baseline household visit from analyses of the intervention efficacy because the intervention had not yet been provided to household members. The cost per cholera case and case averted was calculated by using the assumptions in online Technical Appendix Table 2.

We used Optimal Design software (University of Michigan, Ann Arbor, MI, USA) for the sample size calculation to determine the number of cholera cases (clusters of household contacts) needed to reject the null hypothesis that the incidence of cholera did not significantly differ by study arm at a 95% CI and 80% power (*32*). We assumed that cholera infection would occur in 20% of household contacts in the control arm and that the intervention would reduce this rate to 10% with an average cluster size of 3 household contacts (*7*). On the basis of these assumptions, we estimated needing 156 index cholera patients and 468 household contacts (78 cholera patients and 234 household contacts in each study arm).

To compare baseline household- and individual-level characteristics by study arm, we conducted a χ^2 test for categorical variables, a 2-sample *t* test for continuous variables, and a Fisher exact test when ≤5 values were in a category. Logistic regression models were performed to estimate the odds of developing cholera and to compare intervention uptake indicators during visits 2–5 by study arm using generalized estimating equations to account for clustering within households and approximate the 95% CI. These analyses were performed by using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). For study variables where 1 study arm had no events, we used Fisher exact tests at the household level to determine a significant difference between study arms. To calculate exact 95% CIs in this instance, we used an algorithm to invert test statistics in R (R Core Team, Vienna, Austria) (*33*).

Results

Of the 655 suspected cholera cases screened by Crystal VC Rapid Dipstick for the presence of *V. cholerae* in feces, 255 (39%) were dipstick positive; 400 (61%) results were negative or indeterminate (Figure 3). Of persons with dipstick-positive samples, 71 declined to participate, and 16 were negative for *V. cholerae* by bacterial culture.

We invited all eligible household members in study households during the baseline surveillance visit to participate in the trial. Of household members in cholera patient households, 27% (229/853) were unavailable for the baseline interview and therefore were not enrolled in the trial. The proportion of household members available for the baseline interview did not differ significantly by study arm (26% intervention arm vs. 29% control arm, p = 0.45). Of the 453 household contacts screened for eligibility, 5 declined to participate, and 6 were not home during the clinical surveillance period.

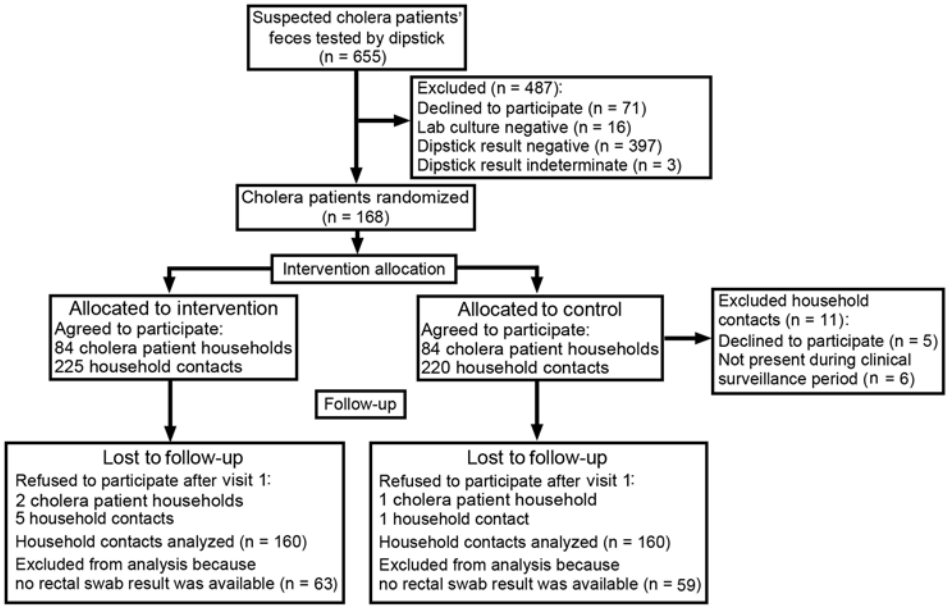

Figure 3. Flowchart of study participation in randomized controlled trial of cholera hospital-based intervention for 7 days, Dhaka, Bangladesh, June 2013–November 2014.

Two intervention and 1 control household refused to participate after the baseline visit. Therefore, 84 cholera patients and 225 household contacts were allocated to the intervention arm and 84 cholera patients and 220 household contacts to the control arm. Baseline index patient, household contact, and household characteristics did not differ significantly by study arm (Table 1). During the study period, 27% of control households had at least 1 water source (e.g., water pump) sample that tested positive for *V. cholerae* compared with 33% of intervention households (p = 0.4).

Culture results for *V. cholerae* were available for 320 (73%) household contacts. Enrolled household contacts with or without rectal swab culture results available did not differ significantly in clinical or demographic characteristics (online Technical Appendix Table 3).

A total of 148 (93%) control and 140 (88%) intervention household contacts were negative for *V. cholerae* at the baseline visit (p = 0.30) (Table 2). Intervention contacts had a 47% lower incidence of *V. cholerae* infection (symptomatic and asymptomatic) than control contacts (odds ratio [OR] 0.50, 95% CI 0.21–1.18) during the intervention period. Furthermore, intervention contacts had no symptomatic *V. cholerae* infections, compared with 5% of control contacts (OR 0.00, 95% CI 0–0.623). On the basis of these findings, we determined the cost per cholera case (symptomatic *V. cholerae* infection) averted would be US $227.50 ($227.50–$598.68) (online Technical Appendix Table 2). We calculated the range for the cost estimate using the 95% CI for the OR of a symptomatic *V. cholerae* infection.

The odds of hand washing with soap at key events during the structured observation period were 14 times higher in the intervention arm than in the control arm (OR 14.68, 95% CI 8.32–25.90) (Table 3), and the odds of hand washing with soap after toileting were 12 times higher in the intervention arm than in the control arm (OR 12.14, 95% CI 5.68–25.93). A significantly higher proportion of household visits in the intervention arm than in the control arm had soap present at the cooking area (99.7% vs. 15%, p≤0.0001) and latrine area (98% vs. 13%, p≤0.0001) during the intervention period (Table 3). *V. cholerae* was

Table 1. Demographic and environmental characteristics of households of patients with cholera, by study arm, Dhaka, Bangladesh, June 2013–November 2014*

Characteristic	Control arm	Intervention arm	p value†
No. households	83	82	
No. enrolled household contacts, median ± SD (min–max)	2 ± 0.9 (2–6), n = 220	2 ± 0.8 (2–5), n = 219	0.9
Index patient			
Female sex, no. (%)	56 (67)	52 (63)	0.5
Age, y, median ± SD (min–max)	25 ± 17.6 (0.67–95)	25 ± 15 (1–65)	0.3
<5, no. (%)	5 (6)	8 (10)	0.6
5–14, no. (%)	16 (19)	17 (21)	
>14, no. (%)	62 (75)	57 (70)	
Household contact‡			
Female sex, no. (%)	135 (61)	126 (58)	0.3
Age, y, median ± SD (min–max)‡	13 ± 15 (0.75–67), n = 220	13 ± 16 (0.58–75), n = 219	0.3
<5 years, no. (%)	36 (16)	45 (21)	–
5–14, no. (%)	84 (38)	68 (31)	0.3
>14, no. (%)	103 (46)	106 (48)	0.4
Television ownership, no. (%)	42 (51)	45 (55)	0.5
Electricity, no. (%)	82 (99)	82 (100)	0.3
Refrigerator ownership, no. (%)	12 (14)	9 (11)	0.5
A household member can read and write, no. (%)	67 (81)	72 (88)	0.2
Educational level of person responsible for primary drinking water collection, no. (%)			
No formal education	40 (48)	40 (49)	0.3
Primary school	31 (37)	24 (29)	
Secondary school	11 (13)	17 (21)	
Higher secondary school	0	0	
Bachelor's degree	1 (1)		
Master's degree	0	1 (1)	
Water source type, no. (%)			
Groundwater	45 (54)	46 (46)	0.3
Piped water supply	380 (46)	34 (41)	
Baseline presence, no. (%)			
Any type of soap in latrine area of household	13 (16)	9 (11)	0.3
Any type of soap in cooking area of household	10 (12)	8 (10)	0.6
Vibrio cholerae in stored drinking water	5 (6)	9 (11)	0.2
V. cholerae in source water	10 (12)	12 (15)	0.6
Presence of *V. cholerae* in source water during study period	22 (27)	27 (33)	0.4

*Unless otherwise specified, the denominator for the control are is 83 and for the intervention arm 82.
†χ^2 test for categorical variables and 2-sample *t* test for continuous variables.
‡p values were calculated by using generalized estimating equations to account for the clustering of the data at the household level.

Table 2. Evaluation of intervention efficacy to reduce *Vibrio cholerae* infection among household contacts of cholera patients during the intervention period (visits 2–5), Dhaka, Bangladesh, June 2013–November 2014

Household contact characteristic	No. (%) contacts		Odds ratio (95% CI)	p value*
	Control arm	Intervention arm		
Culture results available	160 (100)	160 (100)	–	–
Negative for *V. cholerae* infection at baseline	148 (93)	140 (88)	1.15 (0.88–1.51)	0.30
Initial *V. cholerae* infections during the intervention period	20 (14)	10 (7)	0.50 (0.21–1.18)	0.11
Initial symptomatic *V. cholerae* infections during intervention period†	8 (5)	0	0.00 (0–0.623)‡	0.006§

*Calculated with logistic regression model by using generalized estimating equations to account for clustering within study households.
†Symptomatic infection defined as a *V. cholerae*–infected household contact with diarrhea or vomiting in the past 48 hours.
‡To calculate exact 95% CIs, an algorithm was used to invert household-level test statistics.
§Fisher exact test calculated at the household level.

present in no stored drinking water samples in households in the intervention arm and in 6% of samples in the control arm during the intervention period (OR 0, 95% CI 0–1.08). The proportion of households with free chlorine concentrations ≥0.2 mg/L was significantly higher in the intervention arm than in the control arm (94% vs. <1%, p<0.0001).

Discussion

CHoBI7 significantly reduced symptomatic *V. cholerae* infections and reduced overall *V. cholerae* infections by nearly half during the intervention period. Consistent with these findings, the odds of hand washing with soap at key events during the structured observation were 14 times higher in the intervention arm than in the control arm, and nearly all intervention households had free chlorine concentrations in stored drinking water in the Centers for Disease Control and Prevention–recommended range. In addition, no stored drinking water samples in intervention households had detectable *V. cholerae*. These findings demonstrate that CHoBI7 was highly effective in reducing symptomatic cholera and increasing hand washing with soap and treatment of water during the 1-week high-risk period for household contacts of cholera patients.

We attribute the success of the CHoBI7 intervention to several key factors. First, this intervention was delivered during a time of severe illness in these households, when perceived severity of diarrheal disease and perceived benefits of hand washing with soap and treatment of water were likely to be high. Previous studies have found that during outbreaks of severe disease, such as cholera, households have higher perceived severity of diarrheal disease and greater perceived benefits of water treatment (*14*,*34*,*35*). Consistent with this observation, in Dhaka in 2013, use of a community-level point-of-use chlorine dispenser peaked after cholera-associated deaths in a slum area (L. Unicomb, pers. comm.). Second, we provided hardware that was pretested in a pilot study and facilitated the promoted behaviors (hand washing with soap and treatment of water) (*27*). Third, we trained health promoters to reinforce the promoted behaviors by using the CHoBI7 pictorial module, which probably led to a favorable environment for habit formation (*25*).

CHoBI7 significantly reduced symptomatic *V. cholerae* infection but not overall infection. We suspect the reason is our small sample size and the intervention reducing the infecting inoculum size within households to a level below which symptomatic infection could occur. Consistent with this hypothesis, a previous challenge trial found that symptomatic infection could occur at an inoculum size of 10^4 CFUs of *V. cholerae* and that illness severity was based on the size of the infecting inoculum (*36*).

Major advantages of the CHoBI7 intervention are its focus on high-risk persons during the 1-week period when

Table 3. Odds ratios for hand washing with soap and water treatment and indicators of water quality in an intervention study of *Vibrio cholerae*, Dhaka, Bangladesh, June 2013–November 2014

Outcome	No. complying/no. persons (%)		Odds ratio* (95% CI)	p value*
	Control arm	Intervention arm		
Hand washing with soap events at key times during 5-h structured observation	50/629 (8)	418/759 (55)	14.68 (8.32–25.90)	<0.0001
Hand washing with soap events after toileting during 5-h structured observation	23/123 (19)	144/197 (73)	12.14 (5.68–25.93)	<0.0001
Household visits with soap in latrine area, visits 2–5†	50/332 (15)	326/327 (99.7)	1,842.36 (241.53–145,054.53)	<0.0001
Household visits with soap in kitchen area, visits 2–5†	43/332 (13)	317/327 (97)	213.64 (62.59–729.24)	<0.0001
Households visits with detectable free chlorine ≥0.2 mg/L in household stored drinking water, visits 2–5‡	1/332 (<1)	308/327 (94)	4,878.62 (799.30–4.503 × 10^{15})	<0.0001
Household visit with stored water with detectable *V. cholerae*, visits 2–5	5/83 (6)	0/82 (0)	0.00 (0–1.08)§	0.06¶
Household visit with source water with detectable *V. cholerae*, visits 2–5	15/83 (18)	22/82 (27)	1.66 (0.79–3.49)	0.18

*Logistic regression using generalized estimating equations.
†Soap present within 10 steps of the latrine or cooking area at household visits during the intervention period.
‡Cutoff recommended by the Centers for Disease Control and Prevention (Atlanta, GA, USA).
§To calculate exact 95% CIs, an algorithm was used to invert test statistics.
¶Fisher exact test.

they are most susceptible to cholera infections and its dissemination in a clinical setting in which cholera cases can be rapidly identified by dipstick test. Furthermore, the intervention is relatively inexpensive (US $227.50/cholera case averted) and would be likely to be more cost effective than a similar WASH intervention implemented as a community-based intervention, given the much lower prevalence of cholera in the general population (1.6 cholera cases/1,000 general population vs. 50 cholera cases/1,000 household contacts of cholera patients) (*12*,*37*). A recent study that used a cholera vaccine cost-effectiveness calculator found that a cholera vaccination program targeting geographic hotspots for cholera (cholera incidence >10 cases/1,000 year) in Bangladesh would cost US $226 per cholera case averted, similar to the cost of CHoBI7 (*38*).

To our knowledge, only 1 intervention study has been published that evaluated the effectiveness of safe water storage and water treatment on cholera transmission among household contacts of cholera patients. This study, conducted in Calcutta, India, resulted in a 59% reduction in overall cholera infections in the chlorine water treatment arm and a 76% reduction in the narrow-necked water pitcher arm during the 5-day intervention period (*39*). An earlier intervention study in Dhaka found that promotion of hand washing with soap among household contacts of shigellosis patients resulted in an 85% reduction in symptomatic *Shigella* infections during the 10-day intervention period (*40*). These findings are consistent with those from our trial and suggest that WASH interventions directed toward the high-risk period for household contacts of hospitalized diarrhea patients might be a promising approach for reducing transmission of enteric pathogens in this susceptible population.

Our study has a few limitations. First, because CHoBI7 combined hand washing with soap and treatment of water, we cannot establish the effect of these interventions individually. Second, our sample size was small, and we were unable to obtain culture results from as many household contacts as anticipated because of limited study personnel capacity. This limitation reduced our power to detect a significant difference in primary outcome between the 2 study arms (80% vs. 69%). Third, 27% of household members of cholera patients were not present during the baseline surveillance visit and therefore were not enrolled as household contacts. These persons are likely to have been the household members who spent the most time outside the home during the study period. However, the proportion of household members who were unavailable for the baseline interview did not differ significantly by study arm. Fourth, the study could not be unblinded; however, to minimize potential bias, the evaluation and intervention teams were separate, and the laboratory was blinded to intervention assignment. Fifth, this study was an efficacy trial. Our objective was to evaluate whether hand washing with soap at key events and treatment of household stored drinking water consistently would significantly reduce *V. cholerae* infections. Future studies should conduct an effectiveness trial to identify whether a hospital-based intervention only (without home visits) can lead to sustained uptake of the promoted hand washing with soap and treatment of water during the 1-week high-risk period for these households.

In our study, CHoBI7 significantly reduced symptomatic *V. cholerae* infections among household contacts of cholera patients in urban Dhaka, Bangladesh. These findings suggest that this hospital-based intervention is a promising, cost-effective approach that could be initiated as a standard of care for household members of cholera patients. Future studies should investigate the efficacy of CHoBI7 in other settings affected by cholera globally, evaluate the effects of CHoBI7 on other enteric pathogens, and identify effective low-cost approaches to take CHoBI7 to a larger scale.

Acknowledgments

We thank the study participants and the following research assistants who conducted the field work for this study: Ismat Minhaz Uddin, Rafiqul Islam, Al-Mamun, Maynul Hasan, Kalpona Akhter, Khandokar Fazilatunnessa, Sadia Afrin Ananya, Akhi Sultana, Sohag Sarker, Jahed Masud, Abul Sikder, Shirin Akter, and Laki Das.

This research was supported by the Center for Global Health at Johns Hopkins University and the National Institute of Allergy and Infectious Diseases, National Institutes of Health. icddr,b thanks the governments of Australia, Bangladesh, Canada, Sweden, and United Kingdom for providing core/unrestricted support.

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Sustained Transmission of Pertussis in Vaccinated, 1–5-Year-Old Children in a Preschool, Florida, USA

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Learning Objectives

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

- Evaluate the epidemiology and prevention of pertussis
- Assess the efficacy of the pertussis vaccine in preventing clinical pertussis in the current study
- Analyze the attack rate of pertussis in a preschool in the current study
- Distinguish the most common symptoms of pertussis among children in the current study

CME Editor

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

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DOI: http://dx.doi.org/10.3201/eid2202.150325

In September 2013, local county health officials in Tallahassee, Florida, USA, were notified of a laboratory-confirmed pertussis case in a 1-year-old preschool attendee. During a 5-month period, 26 (22%) students 1–5 years of age, 2 staff from the same preschool, and 11 family members met the national case definition for pertussis. Four persons during this outbreak were hospitalized for clinical management of

pertussis symptoms. Only 5 students, including 2 students with pertussis, had not received the complete series of vaccinations for pertussis. Attack rates in 1 classroom for all students who received the complete series of vaccinations for pertussis approached 50%. This outbreak raises concerns about vaccine effectiveness in this preschool age group and reinforces the idea that recent pertussis vaccination should not dissuade physicians from diagnosing, testing, or treating persons with compatible illness for pertussis.

In the United States, incidence of pertussis is greatest among infants, children 7–10 years of age, and adolescents (*1*). During 2000–2012, reported pertussis cases increased >6-fold from 7,867 cases to 48,277 cases (*2*). One potential contributing factor for increased incidence of pertussis is waning immunity after acellular pertussis vaccination (*3*,*4*).

In September 2013, the Florida Department of Health in Leon County (DOH–Leon, Tallahassee, FL, USA) was notified of a PCR result positive for *Bordetella pertussis* for a 1-year-old vaccine-exempt preschool student. Treatment, chemoprophylaxis, and pertussis education were provided to household close contacts, classmates, parents of classmates, and staff associated with the classroom for this student. The investigation identified a 3-year-old sibling who had illness clinically compatible with pertussis before onset of pertussis in the 1-year-old student. This sibling did not attend the preschool.

On December 11, DOH–Leon received a report that a 1-month-old infant had a PCR result positive for pertussis. A public health investigation determined that the vaccinated 3-year-old sibling and mother of this infant had illnesses clinically compatible with pertussis and disease onset before that of the infant. The sibling attended the same preschool as the initial 1-year-old student, and the mother was a substitute teacher at the preschool. Less than 1 week later, another 3-year-old child who attended the preschool showed a PCR result positive for pertussis.

In response to these reports, an outbreak investigation was initiated. On December 19, the local DOH staff conducted a site visit to the preschool to prevent further transmission and determine the incidence of pertussis among students, household close contacts, and staff.

Methods

The preschool had 117 students 10 months–6 years of age and 26 staff. On December 19, the preschool director and staff were queried about any additional students or staff members with a cough illness. At this time, the preschool instituted school wide cough illness surveillance and reported any new cough illnesses to DOH. For newly identified case-patients and their contacts, treatment and prophylaxis were administered according to guidelines from the Centers for Disease Control and Prevention (CDC; Atlanta, GA, USA). All symptomatic persons, or their parents if they were <18 years of age, were interviewed by using a standardized case report form to record onset dates, demographics, symptoms, healthcare visits, laboratory testing, treatment, and vaccination status. Pertussis vaccination status for case-patients and all students was cross-referenced in the Florida immunization registry and with preschool vaccination records.

The Council of State and Territorial Epidemiologists (CSTE) 2013 case definition for pertussis was used to classify cases, with the exception that serologic analysis performed at CDC was considered a confirmatory laboratory test (*5*). In addition, persons with only school-based epidemiologic links to a laboratory-confirmed case were classified as probable cases, rather than confirmed cases. All other epidemiologic links, such as household links, were classified as confirmed per CSTE case definition. One residual nasopharyngeal specimen that showed a PCR result positive for pertussis at a commercial laboratory and 3 serum samples collected retrospectively from consenting case-patients (adult) were forwarded to CDC for confirmation of *B. pertussis* infection.

On January 7–8, 2014, DOH staff administered an onsite cough illness questionnaire to student and staff households (completion rate 98%). The questionnaire sought to capture any cough illness, classic symptoms of pertussis, and duration of illness since December 1, 2013. All but 3 student households and 1 staff member household completed the cough illness questionnaire. Case data were analyzed for several factors, including age, classroom, number of vaccinations, duration from most recent vaccination to symptom onset, and case classification status.

Vaccine effectiveness was calculated as (1 – relative risk) × 100 for the cohort of children attending the preschool (*6*). Relative risk was defined as the attack rate (AR) in fully vaccinated children divided by the AR in children whose vaccination status was not up to date. All children attending the preschool were age-eligible to have received ≥3 doses of pertussis vaccine (DTaP). Children <18 months of age were considered fully vaccinated if they had received 3 doses of DTaP. All other children were considered fully vaccinated if they had received ≥4 doses of DTaP.

Results

Eleven cases were detected during September 2013–January 2014 and classified as confirmed: 5 laboratory confirmed (3 by PCR specific for IS*481*; 1 by PCR specific for IS*481*, HIS*1001*, PIS*1001I*, *ptxS1*, and RNaseP; and 1 by serologic testing at CDC) and 6 epidemiologically linked household contacts (Figure). Twenty-eight cases were classified as probable (total of 39 confirmed and probable cases). Twenty-six students 1–5 years of age (AR 22%) and 2 staff (AR 7%) were identified as having pertussis

(Table). The remaining 11 case-patients were linked to the preschool: 9 were household contacts and 2 were camp counselors who had contact with a sibling of a laboratory-confirmed case-patient who attended the preschool.

Four case-patients 1 month–2 years of age, including 2 students, were hospitalized. Only 1 of the hospitalized case-patients had received ≥3 doses of pertussis vaccine (2 children were too young for 3 doses and 1 child who had received 1 dose was on a delayed schedule). Lengths of hospitalization ranged from 1 to 5 days, and duration of cough ranged from 14 to 50 days. Three of the four hospitalized case-patients had posttussive vomiting, 2 had paroxysmal cough, 2 had inspiratory whoop, and 1 had stridor.

All 39 case-patients had a cough illness for >2 weeks, which is consistent with the CSTE case definition (*5*). The average duration of illness for all case-patients was estimated to be 23 days, which is an underestimate because 25 case-patients were still symptomatic at time of last interview. Fourteen (54%) of 26 students and 11 (85%) of 13 case-patients who were not students had additional symptoms consistent with pertussis (Table). A paroxysmal cough (56%) was the most common additional symptom identified.

Only 5 of 117 children in the preschool had not received the complete series of vaccinations. Of these 5 children, 2 were case-patients and both had received ≥1 dose of pertussis vaccine: the hospitalized 1-year-old child who had received a single dose and a 3-year-old child who had received only 2 doses. The other 3 children were unvaccinated but did not have pertussis.

Of the 33 children who had pertussis, 28 had received ≥3 pertussis vaccinations, and 23 had received ≥4 vaccinations. Vaccine effectiveness among children attending the preschool was estimated to be 45.0% (95% CI -70.4% to 82.2%). The average number of days from last vaccination to onset of symptoms for the students was only 667 days (≈22 months), and 7 (27%) children had been vaccinated within the previous year.

ARs among students by classroom ranged from 0% to 47%; 6 classrooms had an AR >20% for students. The 2 classrooms with 3-year-old students had the highest ARs. Moreover, an AR of 48% was identified in 1 of these classrooms in which all 17 students had received the complete series of vaccinations. This classroom had an infectious staff member with laboratory-confirmed pertussis during the outbreak. Children 2–3 years of age were at 2.2 times (95% CI 1.0–4.9 times) greater risk for pertussis infection than children 4–6 years of age. When this analysis was restricted to only children 3 years of age, the risk increased to 2.5 times (95% CI 1.1–5.5 times).

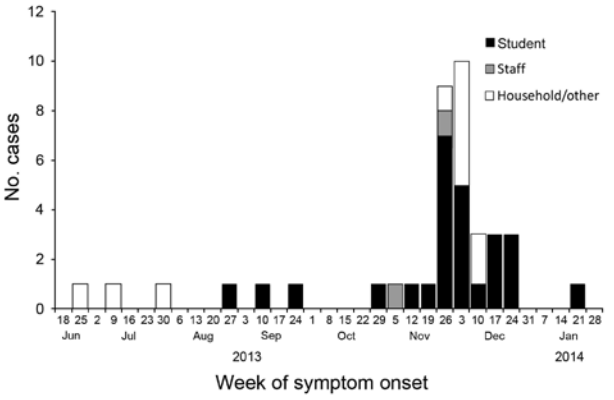

Figure. Epidemic curve of confirmed and probable pertussis cases during an outbreak in a preschool, by week of symptom onset, Florida, USA, 2013–2014. A total of 26 students (black bars), 2 staff (gray bars), and 11 household/other epidemiologically linked persons (white bars) were involved in this outbreak.

Discussion

This investigation highlights an outbreak of pertussis in a preschool with few vaccine exemptions. To our knowledge, sustained transmission of pertussis in a vaccinated cohort of 1–5-year-old children has not been reported in the United States. Short-duration vaccine effectiveness estimates for children receiving ≥3 doses of acellular pertussis vaccines have been described at ≥80% (*4*,*7*,*8*). Although the small number of nonvaccinated children in the preschool resulted in a vaccine effectiveness that had extremely wide CIs that overlap 0, the low estimate, coupled with documented sustained transmission over a period of months, raises concerns about inadequate protection against pertussis in an age group believed to be well protected by acellular pertussis vaccination.

Poor performance of a vaccine in a defined cohort might suggest a provider-level failure to store, use, and administer the vaccine properly. Although we did not assess vaccine storage and handling practices, children from this investigation were seen by multiple providers in the community. Moreover, no general increase in reported pertussis incidence was observed in the county at the same time as this outbreak.

Although the number of cases confirmed by laboratory testing was low (13%), many of the case-patients had substantial illness consistent with pertussis. In addition, *B. pertussis* infections were confirmed in persons from 5 households over a 5-month period. Confirmatory laboratory testing at CDC provided further evidence of a *B. pertussis* outbreak. The 3 nonvaccinated students in whom illness did not develop were not assessed for prior evidence of infection.

The use of a focused cough illness questionnaire for case ascertainment might have captured cough illnesses that met the case definition, but might not have been pertussis, during December 1, 2013–January 7, 2014. However, because the questionnaire focused on this narrow period, to minimize recall bias, additional cases of pertussis before December 1, 2013, might not have been identified.

Table. Characteristics of students, staff, and household members associated with an outbreak of pertussis in a preschool, Florida, USA*

Characteristic	Case classification status			Characteristic comparisons	
	Confirmed	Probable	Noncase	% Cases, n = 39	Attack rate, %
Link to preschool					
Student	4	22	91	67	22
Staff	2	0	26	5	7
Household/other†	5	6	ND	28	ND
Hospitalized	2	2	ND	10	ND
Symptoms					
Apnea	2	2	ND	10	ND
Cough >2 wk	11	28	ND	100	ND
Inspiratory whoop	5	2	ND	18	ND
Paroxysmal cough	9	13	ND	56	ND
Posttussive vomiting	4	4	ND	21	ND
Age‡					
<18 mo	1	1	7	8	22
18 mo–4 y	2	13	41	58	27
≥4 y	1	8	43	35	17
Up-to-date pertussis vaccinations‡	3	21	88	92	21
No. pertussis vaccinations‡					
<3	1	1	3	8	40
3	0	1	4	4	20
≥4	3	20	81	88	22
Time from vaccination to symptom onset, y‡§					
<1	1	6	39	27	15
1–<2	1	7	22	31	27
2–3	1	5	13	23	32
>3	1	4	14	19	26
Classroom‡					
1A	1	1	11	8	15
2A	0	1	11	4	8
2B	0	3	10	12	23
3A	0	5	12	19	29
3B	2	6	9	31	47
4A	1	1	7	8	22
4B	0	1	9	4	10
4C	0	2	7	8	22
4D	0	2	6	8	25
Kindergarten	0	0	9	0	0

*ND, not determined.
†Other, symptomatic camp counselors unaffiliated with the preschool who had contact with children or siblings that attended the preschool.
‡Values and calculations are only for students in the preschool.
§For noncases, time from vaccination to symptom onset used January 1, 2014, as onset date.

Over the course of the outbreak, mass prophylaxis was provided only to the classroom with 1-year-old children after laboratory confirmation of the first reported case because of concerns about the risk for severe pertussis in this younger age group. Given the limited laboratory testing early during the outbreak and to be consistent with adherence to CDC guidelines, classroom-wide chemoprophylaxis was not provided for laboratory-confirmed cases in older children. Chemoprophylaxis was provided to household and high-risk contacts. As a result, effects of school-wide or classroom-wide chemoprophylaxis were not assessed. No staff were identified as being pregnant during the outbreak. However, early during the outbreak, the 1-year-old child of a rotating staff member (use of postexposure prophylaxis by this staff member was not known) was hospitalized with laboratory-confirmed pertussis in December, despite use of prophylaxis in September by the class in which the mother worked.

As part of this investigation, it was apparent that many physicians were hesitant to provide a diagnosis of pertussis and did not test for this disease, given the recent vaccination history of the patients and despite reporting of an ongoing laboratory-confirmed pertussis outbreak. The spectrum of illness for pertussis in vaccinated children can vary widely and is often mild, with few classic symptoms of pertussis (*9*). Hesitation by providers in reporting presumptive pertussis delays public health response to prevent continued transmission of pertussis in the community. Thus, recent pertussis vaccination should not preclude diagnosis, testing, and treatment of presumptive pertussis cases (*10*).

Although all children in the classroom had received the complete series of vaccinations for pertussis, the classroom with the highest AR was one in which a teacher with a laboratory-confirmed case of pertussis who had not received a Tdap booster vaccination, worked throughout her illness. Three of the students showed symptom onset before the

teacher, and 5 students, including 1 laboratory-confirmed case-patient, had symptom onset 6–13 days after the teacher showed symptom onset. Improved efforts toward early diagnosis and appropriate treatment to mitigate transmission and booster vaccinations for adults in situations in which prolonged close contact between children, especially children <1 year of age, and adults occur could be considered (*11*).

Reports of genetic changes in circulating *B. pertussis* have raised concern that this organism could be adapting to vaccine-induced immunity (*12*,*13*). Given these reports and the increased levels of circulation of pertussis among older age groups with documented waning of immunity, further monitoring of acellular pertussis vaccine performance in preschool-age children is necessary to determine if this outbreak was an isolated finding or possibly identification of an emerging epidemiologic trend.

Acknowledgments

We thank the preschool director, staff, and student families for their participation, assistance, and general curiosity, and laboratory staff at CDC, especially Maria Lucia Tondella, Jennifer Liang, Pam Cassiday, and Lucia Pawloski, for their assistance in confirming patient samples and expert guidance on pertussis.

This study was supported by an Epidemiology Laboratory and Capacity Cooperative Agreement with the Centers for Disease Control and Prevention.

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Molecular Characterization of Invasive *Streptococcus dysgalactiae* subsp. *equisimilis*, Japan

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Learning Objectives

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

- Distinguish clinical characteristics of *Streptococcus dysgalactiae* subsp. *equisimilis* infections, based on a study of isolates from Japanese patients with invasive streptococcal infections
- Describe *emm* and multilocus sequence typing types of *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from Japanese patients with invasive streptococcal infections
- Identify the characteristics of antibiotic resistance genes of *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from Japanese patients with invasive streptococcal infections.

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DOI: http://dx.doi.org/10.3201/eid2202.141732

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We collected β-hemolytic streptococci (1,611 isolates) from patients with invasive streptococcal infections in Japan during April 2010–March 2013. *Streptococcus dysgalactiae* subsp. *equisimilis* (SDSE) was most common (n = 693); 99% of patients with SDSE infections were elderly (mean age 75 years, SD ±15 years). We aimed to clarify molecular and epidemiologic characteristics of SDSE isolates and features of patient infections. Bacteremia with no identified focus of origin and cellulitis were the most prevalent manifestations;

otherwise, clinical manifestations resembled those of *S. pyogenes* infections. Clinical manifestations also differed by patient's age. SDSE isolates were classified into 34 *emm* types; *stG6792* was most prevalent (27.1%), followed by *stG485* and *stG245*. Mortality rates did not differ according to *emm* types. Multilocus sequence typing identified 46 sequence types and 12 novel types. Types possessing macrolide- and quinolone-resistance genes were 18.4% and 2.6%, respectively; none showed b-lactam resistance. Among aging populations, invasive SDSE infections are an increasing risk.

Streptococcus dysgalactiae subspecies *equisimilis* (SDSE) belongs to the pyogenic group of streptococci first designated by Vandamme et al. in 1996 as a new subspecies within the species *S. dysgalactiae* (*1*). Previously isolated from humans, as commensal microorganisms, these streptococci have been designated β-hemolytic groups C and G because they are agglutinated by serum against Lancefield group C or G antigens. On blood agar plates, SDSE typically appears as large glossy colonies surrounded by a broad zone of strong β-hemolysis (*2*). For SDSE to be distinguished according to current taxonomy (*3*), specific biochemical properties need to be ascertained.

Although SDSE has long been considered much less virulent than *S. pyogenes*, many clinical and epidemiologic studies have determined that SDSE can cause a variety of severe invasive infections resembling those caused by *S. pyogenes* (*4*–*12*). These include not only cellulitis and deep abscesses but also streptococcal toxic shock syndrome (STSS) (*13*), necrotizing fasciitis, meningitis, endocarditis, and others. In addition, severity of invasive SDSE (iSDSE) infection approximates that seen with invasive *S. pyogenes* infection (*6*,*9*).

SDSE and *S. pyogenes* are considered to be closely related phylogenetically and may have originated from a common precursor (*14*). Moreover, recent genomic research has demonstrated that many pathogenically notable virulence factors in SDSE, including M protein, streptokinase, and streptolysin, were all encoded by genes highly homologous with those identified in *S. pyogenes* (*15*–*17*). However, SDSE lack several virulence factors, such as a cysteine protease (designated erythrogenic toxin B); a hyaluronic acid capsule (*hasA* and *hasB*); and an inhibitor of complement activation (*sic*) (*17*), in addition to many superantigens (*18*,*19*).

Despite this absence of some virulence factors, clinical (*4*,*13*,*20*) and epidemiologic reports (*5*,*6*,*8*–*10*,*21*) indicate that SDSE is pathogenic for humans, particularly, elderly persons with coexisting conditions. Surveillance that we conducted in 2006 implicated SDSE as a major causative pathogen in invasive β-streptococcal infections affecting the elderly in Japan (*22*). In industrialized countries, SDSE infections are frequent among elderly persons, especially among those with underlying medical conditions (*23*,*24*).

In Japan, we have organized large-scale epidemiologic surveillance for β-streptococci that are causing invasive infections and have identified SDSE as the most prevalent β-streptococcal pathogen since 2003 (*22*,*25*). However, information is limited regarding molecular characteristics of isolates and early indicators of prognosis for patients with these infections.

On the basis of *emm* genes that show polymorphisms similar to *S. pyogenes* (*26*), gene sequence analysis has been applied to *emm* typing for epidemiologic study of SDSE. According to the Centers for Disease Control and Prevention (CDC; http://www2a.cdc.gov/ncidod/biotech/strepblast.asp), >90 *emm* types have been recognized among SDSE. We previously reported that in Japan, *stG485* and *stG6792* were more prevalent in isolates from iSDSE infections, whereas *stG10* and *stG6* were more prevalent in noninvasive strains (*22*). Predominance of *emm* types also has been found to vary by geographic region.

In this study, we aimed to clarify molecular and epidemiologic characteristics of isolates from patients with iSDSE infections and the clinical features of these infections. The analysis included assessing clinical manifestations according to specific patient age group, conducting *emm* typing and multilocus sequence typing (MLST), and determining antimicrobial agent susceptibility and mechanisms of resistance to antimicrobial agents.

Materials and Methods

Study Design and Case Definition

We conducted nationwide surveillance of iSDSE infections during April 2010–March 2013, supported by a grant from the Japanese Ministry of Health, Labour and Welfare. After we obtained written permission from the laboratory director or hospital director, 341 general hospitals with a clinical microbiology laboratory participated in this surveillance project. Participating hospitals were located throughout Japan. Surveillance for iSDSE was carried out in parallel with 3 other investigations concerning invasive pneumococcal diseases (*27*), invasive *S. pyogenes* diseases, and invasive *S. agalactiae* diseases (*28*).

Infections with iSDSE were defined as cases in which SDSE was isolated from normally sterile clinical samples such as blood, cerebrospinal fluid, joint fluid, or pus obtained from within a closed space. Strains were sent by the various participating institutions when SDSE was reidentified by β-hemolysis on sheep blood agar (Becton Dickinson, Tokyo, Japan) and met the following criteria: agglutination results indicated Lancefield group A, C, or G; resistance to bacitracin; lack of L-pyrrolidonyl arylamidase, according to the Manual of Clinical Microbiology

(*2*); and, for some isolates, 16S rRNA sequencing results consistent with SDSE. Isolates were stored at -80°C in 10% skim milk until use (Becton Dickinson, Sparks, MD, USA).

Requested Information

We asked attending physicians to complete and anonymously submit questionnaires along with iSDSE isolates. Requested data included patient age at onset, patient sex, origin of sample, clinical manifestation or diagnosis, underlying diseases, prior administration of antimicrobial agents, antimicrobial agent used for the infection, clinical laboratory data obtained at hospitalization, and outcome at discharge. Clinical manifestations and diagnoses were verified by a pulmonologist, according to the diagnostic criteria for sepsis based on the guidelines of the American College of Chest Physicians and the Society of Critical Care Medicine (*29*,*30*), as well as input from attending physicians, in the context of the definition of STSS established by CDC (*31*).

emm Typing and MLST

Typing of the *emm* gene was performed as described (*22*,*25*), by amplification by PCR, after which resulting PCR fragments were sequenced. Each *emm* type was identified by using the CDC *emm* sequence database (http://www2a.cdc.gov/ncidod/biotech/strepblast.asp).

MLST was performed according to the method of Ahmad et al. (*32*). First, 7 housekeeping genes, *gki* (glucose kinase*)*, *gtr* (glutamine transport protein), *murI* (glutamate racemase), *mutS* (DNA mismatch repair protein), *recP* (transketolase), *xpt* (xanthine phosphoribosyl transferase), and *atoB* (acetoacetyl-coathioloase) were amplified, and all amplified DNA fragments were sequenced. Sequencing results for the 7 housekeeping genes in every strain each were assigned a sequence type (ST) by using the MLST website (http://sdse.mlst.net/). Relationships of each ST were analyzed by eBURST version 3.1 (http://eburst.mlst.net/v3/).

Antimicrobial Agent Susceptibility

Susceptibilities to 8 oral and 7 parenteral antimicrobial agents for SDSE strains were determined by agar-dilution methods by using Mueller-Hinton agar supplemented with 5% defibrinated sheep blood. Antimicrobial agents were obtained from their respective manufacturers. We used the following breakpoints recommended by the Clinical Laboratory Standards Institute (*33*): penicillin G (susceptible, ≤0.12 μg/mL); cefotaxime (susceptible, ≤0.25 μg/mL); meropenem (susceptible, ≤0.5 μg/mL); vancomycin (susceptible, ≤1 μg/mL); clarithromycin (susceptible, ≤0.25 μg/mL; intermediate, 0.5 μg/mL; resistant, ≥1 μg/mL); clindamycin (susceptible, ≤0.25 μg/mL; intermediate, 0.5 μg/mL; resistant, ≥1 μg/mL); and levofloxacin (susceptible, ≤2 μg/mL; intermediate, 4 μg/mL; resistant, ≥8 μg/mL).

Identification of Antimicrobial Resistance Determinants

Three macrolide-resistant genes, *erm*(A), *erm*(B), and *mef*(A), were identified in iSDSE strains by PCR methods as described (*25*,*34*). To determine fluoroquinolone resistance, quinolone-resistant determining regions of *gyrA*, *gyrB*, *parC*, and *parE,* we sequenced genes and deduced amino acid substitutions (*34*,*35*).

Statistical Analysis

We assessed statistical significance of differences for age group and specific infectious disease, macrolide or quinolone resistance, and *emm* type. We performed χ^2 tests or the Fisher exact test using Ekuseru-Toukei 2012 software for statistics (Social Survey Research Information, Tokyo, Japan).

Results

Age Distributions of Patients

Age distributions of patients with invasive β-streptococcal infection caused by iSDSE, *S. pyogenes*, and *S. agalactiae* are shown in Figure 1. Infections caused by iSDSE were most prevalent (n = 693). During 3 successive periods, iSDSE infections accounted for the following numbers of cases: 231 during April 2010–March 2011, 216 during April 2011–March 2012, and 246 during April 2012–March 2013. Of all patients infected with iSDSE, 687 were adults ≥18 years of age (99.1%); only 6 were children. The mean age of adult patients with iSDSE infection was 75 years (SD ± 15 years), significantly older than those infected with *S. pyogenes* (61 years, SD ± 17 years) and *S. agalactiae* (70 years, SD ± 15 years) ($p<0.001$ for each).

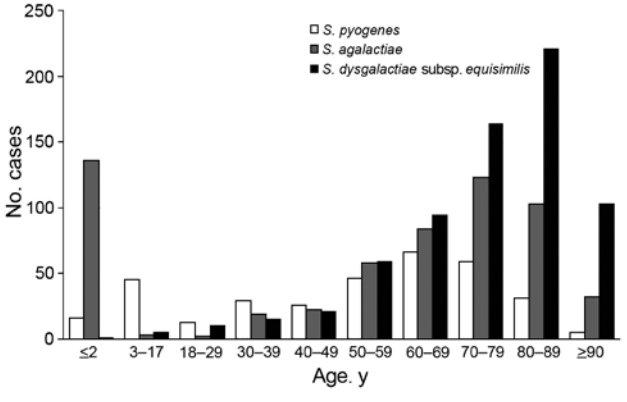

Figure 1. Age distribution of patients with invasive β-streptococcal infections, Japan, April 2010–March 2013. *Streptococcus pyogenes,* n = 336; *Streptococcus agalactiae,* n = 582; *Streptococcus dysgalactiae* subsp. *equisimilis,* n = 693. Means and SDs of ages in patients ≥18 years of age for each pathogen were the following: *S. pyogenes* (mean 61 years; SD ± 17), *S. agalactiae* (mean 70 years; SD ±15), and *S. dysgalactiae* subsp. *equisimilis* (mean, 75 years; SD ±15).

Relationships between Age Group and Clinical Manifestations

Relationships between age group and clinical manifestations in patients with iSDSE are shown in Table 1. Comorbid conditions, including a variety of underlying diseases, were found in 76.8% of patients: diabetes (22.7%), malignancies (16.7%), cardiac diseases (21.4%), and liver or renal dysfunction (16.3%). The male-to-female ratio was 1.2:1.

SDSE caused a variety of invasive infections. Most common was bacteremia without an identified primary focus (39.0%), followed by cellulitis (33.8%) and septic arthritis (6.8%); pneumonia with a positive blood culture accounted for 5.9%. STSS (0.4%) and necrotizing fasciitis (2.3%) occurred infrequently, as did endocarditis (1.6%), cholangitis/peritonitis (2.0%), and osteomyelitis/spondylitis (2.0%). Pneumonia occurred in patients ≥80 years of age (p = 0.006); in contrast, septic arthritis and noncutaneous abscesses tended to occur in patients ≤59 years of age (p = 0.076 and p<0.001, respectively).

emm Type, Clonal Complex, and ST

Correlations between *emm* type and clonal complex (CC) in iSDSE strains are shown in Table 2. The *emm* types were classified into 34 groups. The most prevalent was *stG6792*, which accounted for 27.1% of isolates, followed by *stG485* (13.3%), *stG245* (10.7%), *stG652* (6.8%), *stG10* (6.2%), and *stG6* (5.5%). These 6 *emm* types accounted for 69.6% of types in all strains. Among strains typed as *stG485* or *stG245*, 6 had Lancefield group A antigen rather than C or G.

MLST performed for all iSDSE strains yielded 46 STs and 12 novel STs. Allele numbers are being requested for the novel STs. Novel STs accounted for 3.0% of strains.

Results of eBURST analysis are shown in Figure 2. These STs were classified into 8 CCs and 10 singletons. Among them, CC17 was most prevalent (41.4%, n = 287), followed by CC25 (21.5%, n = 149) and CC29 (11.1%, n = 77).

To clarify relationships between *emm* type and CC, we identified a dominant CC for each *emm* type. Except for *stG652* strains, CCs in almost all strains in *stG6792* and *stG653,* and all of those in *stG2078* and *stG4974*, were identified as CC17. Similarly, strains in *stG245, stG6, stG166b*, and *stG5420* were assigned to CC25, *stC74a* to CC29, *stG10* to CC15, and *stC6979* to CC129. Several *emm* types, *stG485*, *stC652*, *stC36*, *stG480*, and *stG4222,* belonged to ≥2 different CCs. No significant correlation was found between *emm* type and fatality rate for infected patients (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/2/14-1732-Techapp1.pdf; p = 0.830).

Identification of Novel *emm* Types

We identified 2 novel *emm* types, *emmG2.0* and *emmG3.0,* among iSDSE strains (online Technical Appendix Figure 2). *G2.0* was a new *emm* type in which 21-bp deletions occurred in *stG245.0*, and *emmG3.0* had a chimeric structure derived from *stG3251.0* and *stG485.0.* In these novel *emm* type strains, the STs were ST33 and ST128, which belonged to CC25 and CC128, respectively. These findings suggest that strains in *emmG2.0* and *emmG3.0* were derived from those with *stG245*-CC25 and *stG485*-CC128.

Antimicrobial Agent Resistance and *emm* Type

Relationships between macrolide resistance or quinolone resistance and *emm* type are shown in Table 3. A total of 18.5% (n = 128) of the strains showed macrolide resistance mediated by 3 genes. Resistance conferred by the *erm*(A) gene, representing inducible resistance to macrolides, lincosamide, and streptogramin B, was found in 7.9% of isolates; that arising from the *erm*(B) gene conferring constitutive macrolide resistance was found in 8.9%; and that mediated by the *mef*(A) gene conferring intermediate

Table 1. Clinical manifestations and age of patients with invasive *Streptococcus dysgalactiae* subsp. *equisimilis* infection, Japan, April 2010–March 2013*

Clinical manifestation	No. (%) cases by age, y						Total no. (%) cases	p value†
	<18	18–59	≥60	≥70	≥80	≥90		
Cellulitis	1 (0.4)	36 (15.4)	26 (11.1)	59 (25.2)	78 (33.3)	34 (14.5)	234 (33.8)	0.702
Pneumonia		2 (4.9)	4 (9.8)	5 (12.2)	18 (43.9)	12 (29.3)	41 (5.9)	0.006
Arthritis	1 (2.1)	11 (23.4)	9 (19.1)	13 (27.7)	8 (17.0)	5 (10.6)	47 (6.8)	0.076
Abscess, noncutaneous	1 (3.2)	13 (41.9)	5 (16.1)	5 (16.1)	5 (16.1)	2 (6.5)	31 (4.5)	<0.001
Endocarditis		3 (27.3)		5 (45.5)	2 (18.2)	1 (9.1)	11 (1.6)	–
Meningitis	1	1		3		1	6 (0.9)	–
STSS		1	1	1			3 (0.4)	–
Necrotizing fasciitis	1 (6.3)	3 (18.8)	3 (18.8)	3 (18.8)	4 (25.0)	2 (12.5)	16 (2.3)	0.803
Cholangitis/peritonitis		2 (14.3)	1 (7.1)	5 (35.7)	5 (35.7)	1 (7.1)	14 (2.0)	0.740
Osteomyelitis/spondylitis		2 (14.3)	5 (35.7)	4 (28.6)	3 (21.4)		14 (2.0)	–
Bacteremia without primary focus	1 (0.4)	30 (11.1)	39 (14.4)	58 (21.5)	97 (35.9)	45 (16.7)	270 (39.0)	0.058
Others‡		1	1	3	1		6 (0.9)	–
Total	6 (0.9)	105 (15.2)	94 (13.6)	164 (23.7)	221 (31.9)	103 (14.9)	693 (100)	

*STSS, streptococcal toxic shock syndrome; –, not determined because of small number of strains; blank cells indicate 0.
†p values were calculated for differences between the 5 age groups, except the <18 y group.
‡Lymphangitis (n = 5) and keratitis (n = 1).

Table 2. Correlation with *emm* type and clonal complex among *Streptococcus dysgalactiae* subsp. *equisimilis* isolates from invasive infections, Japan, April 2010–March 2013*

	Clonal complex, no. (%)										Total no.
emm type	CC17	CC25	CC29	CC128	CC15	CC129	ST138/153	ST78/130	Singleton	Novel ST†	(%)
stG6792	183		1		1					3	188 (27.1)
stG485	3		50	37						2	92 (13.3)
stG245	1	68		5							74 (10.7)
stG652	6	19	7		1		3		2	9	47 (6.8)
stG10	2				41						43 (6.2)
stG6	2	26							10		38 (5.5)
stG653	25			1						1	27 (3.9)
stG2078	26										26 (3.8)
stC36	9					1			14	1	25 (3.6)
stC74a			18								18 (2.6)
stG166b	1	12								4	17 (2.5)
stG480	7				6				3		16 (2.3)
stG5420	1	14									15 (2.2)
stC6979						13					13 (1.9)
stG4974	10										10 (1.4)
stG4222	4	6									10 (1.4)
Others‡	7	4	1	2		1		2	16	1	34 (4.9)
Total	287 (41.4)	149 (21.5)	77 (11.1)	45 (6.5)	49 (7.1)	15 (2.2)	3 (0.4)	2 (0.3)	45 (6.5)	21 (3.0)	693 (100)

*ST, sequence type. Blank cells indicate 0.
†Allele numbers for novel STs are being requested.
‡Others include *stG11, stG211, stG2691, stG495, stG5345, stG97, stG643, stG62647, stC1400, stC46, stC5345, stC9431, stC10, stC839, emmG2, emmG3, stGM220,* and *stL1929*.

resistance to macrolides was found in 1.6%. Resistant strains were distributed among >10 *emm* types. In particular, 51.4% of the *stG245* strains and 44.2% of *stG10* strains showed macrolide resistance mediated by the *erm*(A) or *erm*(B) gene. These types were related significantly to macrolide resistance ($p<0.001$).

Fluoroquinolone-nonsusceptible strains accounted for 2.6% of isolates (n = 18). These possessed amino acid substitutions in quinolone resistance-determining regions of GyrA and ParC, encoded by *gyrA* and *parC* genes, respectively. Strains (n = 11) for which levofloxacin MICs were at least 16 μg/mL had both substitutions of Ser81Phe or Ser81Tyr in GyrA and Ser79Phe in ParC, whereas remaining strains for which levofloxacin MICs were 4–8 μg/mL had Ser79Phe (n = 5), Ser79Tyr (n = 1), or Asp83Gly (n = 1) in ParC. These fluoroquinolone-resistant strains were distributed in 9 *emm* types, including *stG6792* (n = 5) and *stG485* (n = 4).

Susceptibilities to 8 oral and 7 parenteral antimicrobial agents, among 693 iSDSE strains, are shown in online Technical Appendix Table 1. Except for cefazolin and cefotiam, which were preferred by physicians in Japan, the

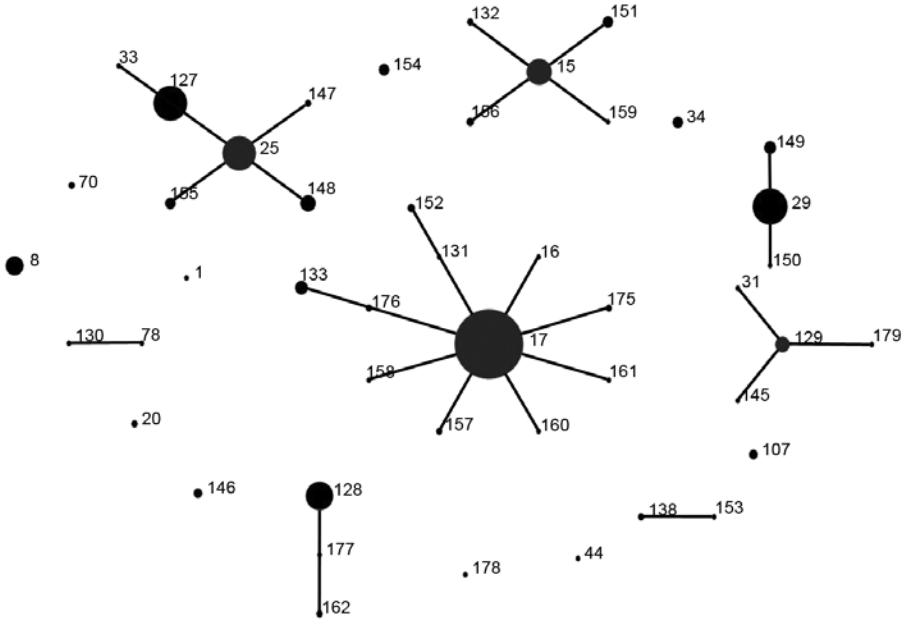

Figure 2. eBURST analysis (http://eburst.mlst.net/v3/) of *Streptococcus dysgalactiae* subsp. *equisimilis* from invasive infections, Japan, April 2010–March 2013. Eight clonal complexes (CC) were identified: CC15, CC17, CC25, CC29, CC128, CC129, ST78/ST130, and ST138/ST153.

Table 3. Correlation of *emm* type and macrolide or quinolone resistance genes among *Streptococcus dysgalactiae* subsp. *equisimilis* isolates, Japan, April 2010–March 2013*

emm type	Total no. strains	No. (%) macrolide resistance			Total no. (%) resistance	p value	No. (%) quinolone resistance†		Total no. (%) resistance	p value
		erm(A)	*erm*(B)	*mef*(A)			*gyrA* + *parC*	*parC*		
stG6792	188	10 (5.3)	7 (3.7)	7 (3.7)	24 (12.8)	0.018	2 (1.1)	3 (1.6)	5 (2.7)	0.950
stG485	92	12 (13.0)	1 (1.1)	2 (2.2)	15 (16.3)	0.565	4 (4.3)		4 (4.3)	0.257
stG245	74	1 (1.4)	37 (50.0)		38 (51.4)	<0.001		1 (1.4)	1 (1.4)	0.476
stG652	47	6 (12.8)	1 (2.1)		7 (14.9)	0.513	3 (6.4)		3 (6.4)	0.091
seG10	43	10 (23.3)	8 (18.6)	1(2.3)	19 (44.2)	<0.001	1 (2.3)		1 (2.3)	0.908
stG6	38	2 (5.3)	1 (2.6)	1 (2.6)	4 (10.5)	0.194			0	–
stG653	27				0	–		1 (3.7)	1 (3.7)	0.712
stG2078	26	6 (23.1)	1 (3.8)		7 (26.9)	0.656		1 (3.8)	1 (3.8)	0.725
stC36	25	1 (4.0)	1 (4.0)		2 (8.0)	0.169			0	–
stC74a	18		2 (11.1)		2 (11.1)	0.415			0	–
stG166b	17				0	–			0	–
stG480	16	2 (12.5)			2 (12.5)	0.534			0	–
stG5420	15				0	–		1 (6.7)	1 (6.7)	0.316
stC6979	13	1 (7.7)			1 (7.7)	0.312			0	–
stG4222	10				0	–			0	–
stG4974	10		2 (20.0)		2 (20.0)	0.900	1 (10.0)		1 (10.0)	0.138
Others	34	4 (11.8)	1 (2.9)		5 (14.7)	0.562			0	–
Total	693	55 (7.9)	62 (8.9)	11 (1.6)	128 (18.5)		11 (1.6)	7 (1.0)	18 (2.6)	

*Dashes indicate p value not determined because of the small number of strains. Blank cells indicate 0.
†Mutations in *gyrA* gene: Ser81Phe (n = 8) and Ser81Tyr (n = 3). Mutation in *parC* genes: Ser79Phe (n = 16), Ser79Tyr (n = 1), Asp83Gly (n = 1).

antibacterial activity of β-lactam agents was superior; MIC for 90% of strains tested ranged from 0.004 to 0.125 μg/mL. MICs of β-lactam agents or vancomycin were not excessive for any strain.

Discussion

We analyzed molecular characteristics of SDSE strains from invasive infections, including *emm* typing, MLST, and antimicrobial resistance determinants, together with clinical features. Molecular epidemiologic surveillance showed that the most prevalent *emm* type was *stG6792*, which has been true for iSDSE infection since 2003 (*22*,*25*). Surprisingly, this type has not been prevalent in other countries (*6*–*11*). The reason for variation in dominant *emm* type between countries remains to be determined.

MLST analysis indicated that CC17, particularly consisting of ST17, was the most prevalent CC, which was identified in a variety of *emm* types. *S. pyogenes* strains belonging to a single *emm* type usually have shown the same CC with only single- and double-locus variants (*28*). In contrast, SDSE strains included a variety of CCs (STs), a fundamental difference from *S. pyogenes* strains. Data reported by McMillan et al. indicated that SDSE strains belonging to *stG2078* were classified as ST17 (CC17), whereas those belonging to *stG6792* were assigned to ST4 (CC4) (*36*). These findings may indicate that CC17 in SDSE conveyed high virulence and that *emm* gene findings have recently become more apparent.

Genomic analysis suggests that SDSE obtained several virulence genes from *S. pyogenes* by horizontal transfer. Our results also show the possibility of novel *emm* types arising from recombination events among *emm* genes in SDSE, indicating that SDSE still is undergoing change. Cross-species transmission between SDSE and other streptococci suggests diversification of SDSE and evolution of highly pathogenic SDSE.

SDSE strains in this study were uniformly susceptible to β-lactam agents, and MICs of these agents were excellent, except for those of some cephalosporin agents. In contrast, macrolide resistance was found in 18.5% of strains, an increase from our previous findings (*25*). We also previously reported that macrolide resistance increased among *S. pyogenes* strains, exceeding 50% in invasive infections (*28*) and 60% in noninvasive infections (*34*). Similarly, macrolide-resistant strains may increase among SDSE strains. Although quinolone resistance was uncommon, we predict that its prevalence will increase with increasing quinolone administration.

SDSE isolates were collected at the same time as strains of *S. pyogenes*, *S. agalactiae*, and *S. pneumoniae* (*27*) for 3 years throughout Japan. The mean age of patients with iSDSE infections was greater than of those with *S. pyogenes* and *S. agalactiae* infection. As expected, the fatality rate was significantly higher for elderly patients, especially those with pneumonia, severe sepsis, septic shock, or disseminated intravascular coagulation (data not shown). Our results identify iSDSE as a common cause of community-acquired infections in an aging society. Immunologic senescence associated with aging as well as underlying diseases are suspected to contribute to risk. Differences in virulence factors between *S. pyogenes* and SDSE, including superantigens and cysteine proteases, may be key causative factors. Further clarification of the contribution of virulence factors present at

onset is needed because severe SDSE infections will probably become more frequent.

In Japan, community-acquired iSDSE infections first drew attention in 2003 (*12*,*13*), when persons ≥65 years of age accounted for nearly 20% of the total population. According to 2013 Japanese population statistics, the segment of the population ≥65 years of age had exceeded 25%, with Japan becoming the highest-ranking country in terms of average life expectancy (Statistics Bureau Japan, Ministry of Internal Affairs and Communications; http://www.stat.go.jp/english/index.htm). Given the relationship between age and iSDSE, we believe that our population dynamics particularly predispose the country to increases in iSDSE infection that may not yet be present in other countries.

In conclusion, SDSE may become a global concern as a causative pathogen with the potential for high mortality rates among elderly persons with community-acquired infections, especially in industrialized countries. Global surveillance of invasive SDSE infection is needed.

Acknowledgments

We thank everyone who participated actively in this large surveillance activity covering 3 periods. We also thank Madoka Naito and Shinji Masuyoshi for laboratory assistance.

This study was funded in part by a grant under the category, "Research on Emerging and Re-emerging Infectious Diseases" (H22-013).

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Population Effects of Influenza A(H1N1) Pandemic among Health Plan Members, San Diego, California, USA, October–December 2009

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Lacking population-specific data, activity of seasonal and pandemic influenza is usually tracked by counting the number of diagnoses and visits to medical facilities above a baseline. This type of data does not address the delivery of services in a specific population. To provide population-specific data, this retrospective study of patients with influenza-like illness, influenza, and pneumonia among members of a Kaiser Permanente health plan in San Diego, California, USA, during October–December 2009 was initiated. Population data included the number of outpatients accessing healthcare; the number of patients diagnosed with pneumonia; antimicrobial therapy administered; number of patients hospitalized with influenza, influenza-like illness, or pneumonia; level of care provided; and number of patients requiring specialized treatments (e.g., oxygen, ventilation, vasopressors). The rate of admissions specific to weeks and predictions of 2 epidemiologic models shows the strengths and weaknesses of those tools. Data collected in this study may improve planning for influenza pandemics.

Planning for pandemic influenza would be enhanced by accurate prediction of the percent of the population that would be infected and those who would access healthcare; the level of outpatient and inpatient services, from primary to tertiary care; and the number of patients who had complications such as pneumonia and needed specialized care such as ventilation and observation in an intensive care unit. The pandemic of influenza A(H1N1) that occurred during 2009 (pH1N1) provided an opportunity to answer some of these questions and provide information that could assist in planning for future pandemics. Therefore, I conducted a retrospective study of members of the Kaiser Permanente (KP) health plan in San Diego, California, USA, who reported influenza-like illness (ILI) during the pH1N1 pandemic.

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DOI: http://dx.doi.org/10.3201/eid2202.150618

Methods

Patients and Study Design

This study does not identify the number of pH1N1 infections among the population but does identify the number of outpatients and inpatients in this population who accessed medical care. Data on outpatients, for whom influenza diagnostic studies were not done, includes the number who had ILI or influenza A and those who were treated with oseltamivir, received a diagnosis of pneumonia, were confirmed to have pneumonia based on chest radiograph, and were treated for pneumonia. Antimicrobial regimens administered are also documented. For inpatients, the data include the number admitted to a hospital with a diagnosis of ILI, influenza A, or pneumonia; those who were treated for ILI, pneumonia, or both; the antimicrobial regimens administered; the level of care received (regular medical or a higher level such as telemetry, assignment to an intensive care unit [ICU], bilevel positive airway pressure [BiPAP]/continuous positive airway pressure [CPAP], ventilation, vasopressors, and hemodialysis); the length of stay (LOS) in the hospital; and the results of testing for influenza A. These data are provided to assist medical and public health professionals in estimating the demand for outpatient and inpatient care and pharmaceutical supplies.

The members of the KP health plan are predominantly employed or formerly employed persons, which may mean that this population is not generally representative of the general population of the United States. However, it is similar to the general population in San Diego County (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/15-0618-Techapp1.pdf).

Patient-specific data for KP members were extracted electronically from 2 sources: care provided to KP members by providers in the KP system and care provided by providers outside that system. For the months of October–December, 2009, the KP San Diego outpatient database was searched for all visits to a healthcare provider by

persons with the diagnoses of ILI, influenza, or pneumonia; the inpatient database was searched for all discharges coded as ILI or influenza. Each of the electronic charts for outpatients that included diagnoses of ILI, influenza, or pneumonia was reviewed for documentation of a provider's reading of chest radiograph, a radiologist's report of chest radiograph, and treatment with antiviral or antibacterial therapy. Adhering to KP policy, nasopharyngeal swabs from outpatients were not sent for testing for influenza RNA by using PCR.

Electronic charts for inpatients that included ILI, influenza, or pneumonia were also reviewed for diagnosis of any of the 3 conditions and a reading of a chest radiograph by a provider and a radiologist. Also documented were treatment with antiviral or antibacterial therapy; level and length of care in medical, telemetry, or ICUs; receipt of respiratory therapy (oxygen, BiPAP/CPAP, ventilation); vasopressor therapy; hemodialysis' LOS; and results of or lack of testing for influenza by culture, enzyme-linked immunosorbent assay (EIA), or PCR on secretions from a nasopharyngeal swab.

In addition, records of KP San Diego members who were seen by providers outside the KP system for whom influenza, ILI, and pneumonia were diagnosed were extracted electronically. For each of these patients, the LOS was available.

KP demographic data was electronically extracted from various databases. Annual median household income and education levels were determined on the basis of US Census Bureau–derived geocoding for the KP member's ZIP code of residence (http://geocoding.geo.census.gov/geocoder). KP members were stratified into 3 groups (low, medium, and high) on the basis of the percentage of household members with a high school diploma or higher degree.

Chronic conditions were extracted from a KP database that documents selected chronic conditions of particular interest to the health plan. Demographic data from San Diego County was supplied by an epidemiologist employed by the county (R.B.). Data on chronic kidney disease (CKD) was extracted from the United States Renal Data System based on data from the National Health and Nutrition Examination Survey (NHANES, http://www.usrds.org/atlas12.aspx).

Case Definition

A case was designated as ILI or influenza on the basis of the provider's diagnosis and the discharge diagnosis of the patient. Confirmation of the diagnosis of influenza was based on the results of a culture, an EIA, or PCR for influenza A RNA performed on nasopharyngeal swab samples. Diagnosis of pneumonia for all patients evaluated in a KP facility was based on a chest radiograph report by a radiologist, in contrast to diagnoses for patients evaluated in a non-KP facilities, which were based on the discharge diagnosis.

Attack Rate

This study only provides data for the persons who accessed health care and does not include data for those who did not; thus, the attack rate in this population could not be calculated. Other studies have provided information on the attack rate. In 2010, Kelly et al. estimated the cumulative incidence of infection during the first wave of the 2009 pandemic as 16%–28% in preschool-age children, 34%–43% in school-age children, 12%–15% in young adults, and 2%–3% in older adults (*1*); the mean attack rate was 19.1%. Gilbert et al. estimated the attack rate of the 2009 influenza A(H1N1) pandemic to be ≈20.6% (*2*). The Centers for Disease Control and Prevention (CDC) published summary estimates of the morbidity and mortality of the 2009 pandemic during April 2009–April 2010 (http://www.cdc.gov/h1n1flu/estimates_2009_h1n1.htm); mid-level range estimates were ≈61 million cases for all ages. The population of the United States in 2009 can be estimated to be 306,013,175 based on the populations in the 2000 and 2010 census reports and the average incremental increase in the population, which was 2,732,363 per year. Using the estimate of ≈61 million cases for all ages and the population estimate of 306,013,175, the mean attack rate for all ages would be ≈19.9% (61 million divided by 306,013,175; http://www.census.gov/prod/cen2010/briefs/c2010br-01.pdf)

Results

Demographic Data

Complete demographic data consisting of age and sex distribution, race/ethnicity, language, estimated income, estimated education level, obesity, and smoking for San Diego KP members and for San Diego County residents are listed in online Technical Appendix Table 1. Chronic conditions tracked among patients of KP and non-KP members in San Diego County are given in online Technical Appendix Table 2, and the criteria that KP used to acquire the data are in Diagnostic Criteria in the online Technical Appendix. Combinations of selected chronic conditions in KP health plan members, as of December 2009, are provided in online Technical Appendix Table 3.

Estimated Numbers of Infected Persons

On the basis of an attack rate of 20%, the estimated numbers of San Diego KP members with influenza A(H1N1) for October, November, and December 2009 were 99,144, 98,982, and 98,989, respectively (mean 99,038/3 months; Table 1). Of the estimated total San Diego KP health plan members infected, the numbers who accessed outpatient

Table 1. Estimated influenza-like illness among KP members treated as outpatients in KP and non-KP facilities, San Diego, California, USA, October–December 2009*

Characteristics	Total population				Rate per 100,000 members		
	October	November	December	Mean	October	November	December
KP health plan population, San Diego	495,718	494,911	494,947	495,192	100,000	100,000	100,000
Estimated infections (20% attack rate)	99,144	98,982	98,989	99,038	20,000	20,000	20,000
Number who accessed IPH	2,439	3,202	1,038	NA	492	647	209
Number of outpatients diagnosed with pneumonia	69	124	26	NA	13.9	25.1	5.3
Number of outpatients diagnosed with pneumonia by chest radiograph	31	57	17	NA	6.3	11.5	3.4
Number of outpatients diagnosed with pneumonia, OOHPC	23	17	18	NA	4.6	3.4	3.6
Number of outpatients diagnosed with pneumonia by chest radiograph, OOPHC	ND	ND	ND	NA	ND	ND	ND
Number of outpatients diagnosed with pneumonia	92	141	44	NA	18.5	28.5	8.9

*IPH, in-plan health care; KP, Kaiser Permanente; OOPHC, out-of-plan health care; NA, not applicable; ND, no data available.

care during October, November, and December 2009 were 2,432, (≈0.49%), 3,202 (≈0.64%), and 1,038 (0.21%), respectively (Table 1). During those 3 months, pneumonia was diagnosed in 105 outpatients based on a radiologist's report: 60 in the 0–18-year age group and 45 in the 19–>90-year age group. All were treated as outpatients: Online Technical Appendix Table 4 lists the number of persons with ILI, pneumonia by clinical diagnosis, and pneumonia by radiological diagnosis for age and gender. Table 1 lists by month the health plan population in San Diego, the estimated number of infections based on an attack rate of ≈20%, the number of members who accessed healthcare, the number diagnosed with pneumonia, and, for those evaluated in a KP facility, the number in whom pneumonia was diagnosed on the basis of a radiologist's report.

The most frequently prescribed antimicrobial regimens for outpatients evaluated in a KP facility in whom pneumonia was diagnosed were azithromycin (n = 60) and amoxicillin (n = 47) for patients ages 0–18; for patients ages >18, moxifloxacin, doxycycline, and azithromycin were most frequently prescribed. Oseltamivir was administered to 104 (age 0–18) of ≈136 and 46 (age 19–>90) of ≈83 outpatients with pneumonia (values are estimates because age groups did not align exactly; online Technical Appendix Table 5).

Inpatients Infected

During October–December 2009, a total of 90 patients with ILI were admitted to a KP hospital: 34 in October, 52 in November, and 4 in December. Of these, 24 (26.7%) were 0–19 years of age and 66 (73.3%) were 20–>90 years of age (online Technical Appendix Table 6). Nasopharyngeal swab samples for 55 of the 90 tested positive by PCR for influenza A; 2 tested positive by EIA and 1 by culture. No patient had a negative PCR and a positive EIA or culture (online Technical Appendix Table 7). Seven patients were admitted to the ICU; 6 were placed on ventilators, and 5 were treated with vasopressors (Table 2). Inpatients with pneumonia indicated by chest radiograph had a longer LOS than did patients with ILI alone (Table 3).

Of the 90 inpatients, 72 received antibacterial regimens (17 ceftriaxone/doxycycline and 13 ceftriaxone/azithromycin); 87 received oseltamivir (online Technical Appendix Table 8). All 5 patients 0–18 years of age whose chest radiographs were read as pneumonia had positive PCR results for influenza A(H1N1)pdm09. Of the 16 whose chest radiographs were read as no pneumonia, 7 had positive PCR results and 7 were negative; PCR testing was not done for the other 2. Of those ≥19 years of age with pneumonia, 23 of 27 had positive PCR results. Of the 36 inpatients who did not have pneumonia, 18 were positive for influenza A by PCR and 16 negative; testing was not done for 2 (online Technical Appendix Table 9).

Discussion

Among the KP San Diego membership, a stable population, this study identified outpatients and inpatients during October–December 2009 who were diagnosed with influenza or ILI. All outpatients with a clinical influenza/ILI diagnosis, and those with that diagnosis and pneumonia, and the antimicrobial regimens prescribed, were recorded. Inpatients with clinical influenza/ILI diagnosis, with that diagnosis and pneumonia and the level/intensity of care

Table 2. Number of inpatients with influenza-like illness who received specialized care at KP medical center, San Diego, California, USA, during October–December 2009*

Specialized care	No. patients	Total LOS, d	Mean LOS
Intensive care unit	7	126	18
Vasopressors	5	26	5.2
Ventilated	6	88	14.7
BiPAP/CPAP	12	26	2.2
Telemetry	17	161	9.5
Oxygen	51	ND	ND
Chronic hemodialysis	4	6	ND
Acute hemodialysis	5	51	10.2
Oseltamivir	87	ND	ND
Peramivir	1	ND	ND
Corticosteroids	12	ND	ND

*All 7 patients admitted to the ICU had positive PCR results for influenza A(H1N1)pdm09. Bipap, bilevel positive airway pressure; CPAP, continuous positive airway pressure; KP, Kaiser Permanente; LOS, length of stay; ND, no data available.

Table 3. Number of KP health plan members with ILI/influenza diagnosis only versus those with ILI and pneumonia, San Diego, California, USA, October–December 2009*

Characteristics	October	November	December
KP health plan population, San Diego*	495,718	494,911	494,947
Admitted to KP San Diego Medical Center, n = 90	34	52	4
Pneumonia diagnosis upon discharge	13	27	1
ILI/influenza	25	28	3
ILI/influenza, mean hospital LOS, d	4	3.5	2.7
Pneumonia based on chest radiograph	9	24	1
Pneumonia, mean hospital LOS, d	12.7	8.5	4
Admitted to non-KP hospital, n = 81	28	33	20
ILI/influenza	15	10	0
ILI/influenza, mean hospital LOS, d	2.5	3.2	2
Pneumonia	13	23	20
Pneumonia, mean hospital LOS, d	4.01	4.95	5.85

*Mean KP San Diego member population for October–December was 495,192. ILI, influenza-like illness; KP, Kaiser Permanente; LOS, length of stay.

rendered were recorded. In addition, tests for influenza A and the antimicrobial regimens were logged. This combination of data provided a comprehensive profile of these patients with influenza/ILI. However, neither the number of patients in this population with influenza A(H1N1) pdm09 nor the attack rate could be determined.

This study accepts an estimated attack rate of ≈20%. Estimates such as these are useful when planning for pandemic influenza; however, I found no studies that logged the number of visits to outpatient healthcare in a specific population, as this study does. For a monthly health plan population of ≈495,000, an attack rate of 20% would have resulted in ≈99,000 cases of influenza A(H1N1)pdm09 per month, but only a small percentage of the estimated number of infected persons accessed medical care. During October, November, and December, 2,432 (2.5% of estimated infected), 3,202 (3.2% of estimated infected), and 1,038 (1.0% of estimated infected) outpatient visits were recorded, respectively (Table 1).

As part of this study, I reviewed the demand versus supply of antimicrobial agents prescribed to outpatients. During October–December 2009, a total of 6,672 KP patients with ILI accessed outpatient care; 219 had diagnoses of and were treated for pneumonia. Of those 0–18 years of age, 64 received azithromycin. The hospital's 1-day par level (minimum in-stock quantity) was adequate for 117 patient-courses of 100 mg/5 mL, 361 patient-courses of 200 mg/5 mL, and 2,990 of 250 mg. Of those age ≥19 years of age, 16 received azithromycin 500 mg, requiring 96 tablets, versus a 1-day par level adequate for 1,309 patient-courses. Of those ≥19 years of age, 10 received amoxicillin, requiring 10 days or 100 tablets; the 1-day par level as adequate for 3,117 patient-courses of 250 mg and 17,503 of 500 mg. Par levels given are from 2014, when monthly population was approximately the same as in 2009.

The rates of ILI admissions per 100,000 KP members during October, November, and December 2009 were 12.5, 17.2, and 4.8, respectively. These rates represent 2.5%, 2.6%, and 2.3% of outpatient visits, respectively (Table 1).

In 2005, the CDC published the FluSurge 2.0 software program, which is a tool for projecting the number of hospitalizations, ICU admissions, patients requiring ventilation, and an estimated mortality rate that might be anticipated in medical facilities during a pandemic (http://www.cdc.gov/flu/pandemic-resources/tools/flusurge.htm [*3*]). In November 2009, the CDC published the FluSurge Special Edition, specifically tailored to the 2009 influenza A(H1N1) pandemic (http://www.cdc.gov/h1n1flu/tools/flusurge/). These programs project admissions for 3 scenarios during an influenza A pandemic: minimum, likely, and maximum.

For the San Diego KP membership, rounded to 500,000, Technical Appendix Table 10 contains the data for the most likely scenarios projected by FluSurge2 (designated FluSurge05) for an attack rate of 15% and the FluSurge Special Edition (designated FluSurge09). The data include the distribution of patients admitted to a hospital, treated in ICU, and ventilated and those who died per week predicted by these programs. A comparison of the FluSurge predictions by these programs for the number of admissions to the hospital for the KP population versus the actual number of members admitted to the hospital shows that the minimum estimated number of admissions/week by the FluSurge05 program (attack rate 15%) and the most likely estimated number of admissions by the FluSurge09 program was approximately the same as the actual number of admissions/week in this study at an attack rate of 20% (Figure). If one accepts the San Diego KP population as a fair approximation of the general population in San Diego County (online Technical Appendix Table 1), the FluSurge09 program demonstrates substantial improvement in the ability to predict the number of admissions to the hospital over FluSurge05. However, for the data in this study, the most likely scenario projected by the FluSurge09 program overestimates the number of patients projected to require ICU care by 1.6 times and ventilation by 1.3 times, although these estimates are still an improvement on those from FluSurge2 (attack rate

15%), which overestimates the number requiring ICU care by 3.5–7-fold and the number projected to need ventilation and admission by ≈3-fold (data not shown). Baker et al. also found that the FluSurge2 most likely scenario overestimated the number of persons projected to require admission, ICU care, and ventilation (*4*). The FluSurge2 projection of the number of patients on ventilators was a factor that influenced KP California to buy additional ventilators for stockpiling (Kaiser Permanente, unpub. data). The number stockpiled may have been fewer if it were not for the FluSurge2 projection. However, so that adequate surge policy is adopted and adequate supplies stockpiled, it may be beneficial for the estimates to be higher than that required, although over-stockpiling may not be cost-effective.

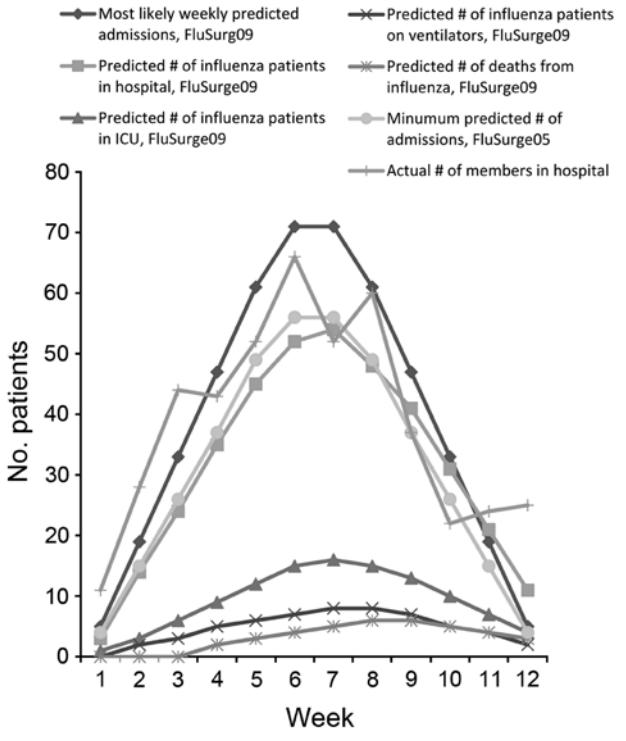

Figure. Hospital admissions per week for a predicted Kaiser Permanente health plan population of ≈500,000 members versus actual numbers of inpatients admitted to Kaiser Permanente San Diego Medical Center during the influenza A(H1N1) pandemic, San Diego, California, USA, October–December 2009. Predictions were compiled by using FluSurge2 (FluSurge05) and FluSurge Special Edition (FluSurge09) (http://www.cdc.gov/flu/pandemic-resources/tools/flusurge.htm) software Assumptions for FluSurge2: average length of non-ICU hospital stay for influenza-related illness, 5 d; average length of ICU stay for influenza-related illness, 10 d; average length of ventilator usage for influenza-related illness, 10 d; average proportion of admitted influenza patients who will need ICU care, 15%; average proportion of admitted influenza patients who will need ventilators, 7.5%; average proportion of influenza deaths assumed to be hospitalized patients, 70%; daily percentage increase in cases arriving compared to preceding day, 3%; attack rate, 15%; total no. hospital beds 392, ICU beds 34, ventilators 40. Unable to find assumptions for FluSurge Special Edition. ICU, intensive care unit.

One death occurred among the 90 inpatients during the 3-month study period. A previous study of a selected population of 108 patients categorized as having moderate to intermediate illness related to diagnoses of influenza A(H1N1)pdm09 found no deaths, although that study excluded patients requiring ventilation (*5*). In a United States study, Skarbinski et al. reported a death rate of 8% among 255 inpatients in 45 states (*6*). For a 15% attack rate, the FluSurge2 program predicts 8 and 31 deaths, respectively, in weeks 4 and 8 during an influenza pandemic, and the FluSurge Special Edition predicts 2 deaths at week 4 and 6 at week 8.

In this study, 47 inpatients received ceftriaxone, the most frequent antibacterial agent ordered. The daily par level supply (2,014) was ≈350 1-g bags and 150 2-g bags of ceftriaxone, which is more than enough for a single daily dose of 1 or 2 g for the 47 inpatients

A total of 7 patients in this study were admitted to ICU at the KP San Diego Medical Center, from a population of ≈495,192 members. This number does not include patients hospitalized in non-KP facilities, patients on which complete information was not available. Assuming that the number of patients admitted to the ICU/total admissions to the hospital for ILI would be about the same for the non-KP facilities as for the KP San Diego Medical Center, since there were 81 hospitalizations for ILI in non-KP facilities, there would have been ≈6 patients admitted to non-KP ICUs during October–December 2009. Combined, these numbers would result in an estimated 13 ICU admissions/495,192 population or 26 ICU admissions/1,000,000 population. In The Australian and New Zealand Intensive Care (ANZIC) study, from June 1–August 31, 2009, a total of 28.7 cases/1 million inhabitants were admitted to the ICUs of Australia and New Zealand (*7*). A population study in Denmark found 9 (5.69%) of 158 patients were admitted to a hospital ICU during the second wave of the 2009 influenza pandemic (*8*), compared with ≈7.6% (≈13/171) in this study.

The median LOS in ICU was 18 days (mean 18) in this study versus 7 days in the ANZIC study (*7*) and 22 days in the Orsted study (*8*). To exceed the KP San Diego Medical Center ICU bed capacity of 34 with just influenza A patients, assuming an LOS of 18, as noted above, 2 patients per day would need to be admitted for 18 consecutive days; >2 patients per day would result in exceeding the ICU bed capacity sooner. At 18 days, 36 patients would have been admitted compared to the 7 admitted in this study. Thus, a much higher attack rate would be necessary, or the number with severe disease greater, to exceed the 34-bed ICU capacity.

In this study, 56.7% patients received oxygen, 13.3% BiPAP/CPAP, and 6.7% (6/90, but 6/7 in ICU) ventilation in the KP San Diego Medical Center. If an estimate is made of patients ventilated in non-KP facilities in the same manner as that used above, an additional 5 patients would have been ventilated, for a total of 11 ventilated patients/495,192 population or ≈22 ventilated patients/1 million population. For comparison, the rate in the ANZIC study was 18 ventilated patients/1 million population (*7*). The median LOS on a ventilator in this study was 13 (mean 14.7) days, compared with 8 days in the ANZIC study (*7*). In a study of critically ill patients with influenza A(H1N1)pdm09, Kumar et al. found that 81% of patients required ventilation; the median LOS on a ventilator in that study was 12 days (*9*). In the Orsted study, the median LOS on a ventilator was 17 days (*8*). Regarding vasopressors, in this study, 6 (85.7%) of 7 patients admitted to the ICU required vasopressors for a median duration of 3 days. In the ANZIC study, 498/722 (≈69%) were provided vasopressor support (*7*).

Table 3 shows data on inpatients with pneumonia, sorted by month and facility. The complete data of clinical diagnosis of pneumonia versus a radiologist's diagnosis of pneumonia on the basis of chest radiograph was not available for patients cared for outside the KP network. Data extracted from the KP San Diego Medical Center charts is sorted by ILI diagnosis, clinical diagnosis of pneumonia, and a radiologist's diagnosis of pneumonia on the basis of chest radiograph. The mean monthly LOS for patients with ILI was 4, 3.5, and 2.7 days and that for a radiologist's diagnosis of pneumonia on the basis of chest radiograph was 12.7, 8.5, and 4 for the months of October, November, and December, respectively.

Conclusions

In conclusion, this study of a stable population during the second wave of the 2009 influenza pandemic provides good estimates of the number of patients who accessed outpatient care for ILI and those admitted to the hospital. Outpatient treatment data includes antimicrobial therapy of those with and without pneumonia. Inpatient treatment data includes the treatment of those with and without pneumonia, and the level of care (medical bed, telemetry bed, ICU), respiratory therapy (oxygen, BIPAP/CPAP, ventilation), antimicrobial therapy, vasopressors, and hemodialysis. The comparisons made with data from this and other studies are surprisingly similar. This data can be used to improve epidemiologic models, although it is anticipated that these models will need revision over time, just as the FluSurge program has been revised, to account for anticipated changes in characteristics of influenza A, population demographics, and medical therapeutics.

Acknowledgment

I thank Elizabeth Le for data extraction.

A subcommittee of the Kaiser Permanente Southern California Institutional Review Board reviewed and approved this study.

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Epidemiology of Serotype 1 Invasive Pneumococcal Disease, South Africa, 2003–2013

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In South Africa, 7-valent pneumococcal conjugate vaccine (PCV) was introduced in April 2009 and replaced with 13-valent PCV in April 2011. We describe the epidemiology of serotype 1 *Streptococcus pneumoniae* disease during the pre- and post-PCV eras (2003–2013). Using laboratory-based invasive pneumococcal disease (IPD) surveillance, we calculated annual incidences, identified IPD clusters, and determined serotype 1–associated factors. Of 46,483 IPD cases, 4,544 (10%) were caused by serotype 1. Two clusters of serotype 1 infection were detected during 2003–2004 and 2008–2012, but incidence decreased after 2011. Among children <5 years of age, those who had non–serotype 1 IPD had shorter hospital stays, fewer cases of penicillin-nonsusceptible disease, and lower HIV prevalence and in-hospital death rates than did those with serotype 1 IPD; similar factors were noted for older patients. Serotype 1 IPD had distinctive clinical features in South Africa, and annual incidences fluctuated, with decreases noted after the introduction of PCV13.

Streptococcus pneumoniae serotype 1 is highly invasive and rarely carried asymptomatically (*1*). The incidence of serotype 1 invasive pneumococcal disease (IPD) fluctuates year to year; disease is associated with outbreaks in closed communities and hospitals and, in Africa, with communitywide meningitis outbreaks (*2*). Compared with other *S. pneumoniae* serotypes, serotype 1 tends to cause fewer cases of fatal disease, and antibiotic-resistant cases are unusual (*1*).

IPD is common in children with underlying diseases, especially HIV. A study conducted among children <18 years of age in Israel before introduction of 7-valent pneumococcal conjugate vaccine (PCV7) showed that, compared with other common serotypes, serotype 1 caused more bacteremic pneumonia and peritonitis, occurred in older children and certain ethnic groups, and affected otherwise healthy children (*3*). After PCV7 introduction, infections caused by serotypes included in the vaccine declined, but other pneumococcal serotypes (e.g., serotype 1, which was later included in 13-valent vaccine [PCV13]) became relatively more common (*4–6*); serotype 1 ranked among the top 4 serotypes infecting children <5 years of age (*7*). Although PCV7 use may have contributed to the relative increase in serotype 1 infections, some studies showed no correlation between the vaccine and serotype 1 disease incidence (*8*). Lack of correlation is likely due to the epidemic-prone nature of serotype 1 disease and annual fluctuations in disease incidence (*9*). In addition, replacement disease is mainly due to common colonizing serotypes. An indirect cohort analysis using data from the United Kingdom Health Protection Agency (now Public Health England) surveillance program could not demonstrate significant protection against serotype 1 IPD by PCV13, although the point estimate suggested protection (vaccine effectiveness 62% [95% CI –112% to 92%]) (*10*). Two trials of a 9-valent vaccine showed waning protection against serotype 1 in the absence of a booster vaccine dose in the second year of life; vaccine failures clustered in children >18 months of age (*11*,*12*).

In South Africa, PCV7 was introduced into the national immunization schedule in April 2009 as a 3-dose

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DOI: http://dx.doi.org/10.3201/eid2202.150967

regimen for infants 6 weeks, 14 weeks, and 9 months of age; in April 2011, the vaccine was replaced with PCV13. Among children <1 year of age, reported coverage for the third dose of PCV improved from 10% in 2009 to 81% in 2012 but declined to 62% in 2013 (*13*). In 2012, after PCV13 introduction, serotype 1 IPD incidence showed a temporally associated decline in children <2 years of age (–57%, 95% CI –79% to –16%) and adults 25–44 years of age (–33%, 95% CI –46% to –17%) compared with incidence in 2005–2008 (*14*).

Information regarding *S. pneumonia*e serotype 1 epidemiology in Africa is limited. We compared serotype 1 disease epidemiology in South Africa with that of other serotypes over an 11-year period, before and after introduction of PCV7 and PCV13. We also explored whether temporal or spatial clusters of serotype 1 disease occurred during the study period.

Methods

Study Design and Setting

Persons of any age were included in the study if they were hospitalized in South Africa during 2003–2013 for laboratory-confirmed IPD and had an available *S. pneumoniae* serotype result for an isolate from a normally sterile site. Patients were identified through an active national, laboratory-based surveillance program for *S. pneumoniae*. Data were contributed by >200 hospital-based diagnostic laboratories that submitted pneumococcal isolates to the National Institute for Communicable Diseases, Johannesburg, South Africa. Most laboratories were nonenhanced sites where only isolates and accompanying laboratory report forms with patient age, sex, date and source of the specimen were submitted. However, 24 sites (primarily tertiary hospitals) implemented enhanced surveillance, in which dedicated surveillance officers collected additional clinical information on identified patients; at least 1 site was located in each South Africa province, giving national representation (*14*). Enhanced sites were chosen on the basis of convenience, interest from site investigators, and number of isolates submitted each year; thus, some differences existed between enhanced and nonenhanced sites (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/2/15-0967-Techapp1.pdf). Annual audits conducted by using a laboratory-based information system were used to identify unreported cases, which were included and used in incidence calculations.

Participants identified from enhanced and nonenhanced sites were included for determining incidence rates and cluster mapping. For the analyses of factors associated with serotype 1 pneumococcal disease and fatality, only participants from enhanced sites with detailed clinical information and known in-hospital outcomes were included.

Approval was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg (M081117), and other hospital or provincial ethics committees, as required. Informed consent was obtained for all patients.

Definitions

IPD cases were defined as disease in persons with *S. pneumoniae* detected in cultures of specimens from normally sterile sites or persons with culture-negative samples that were positive by latex agglutination and/or Gram stain microscopy or *lytA* PCR (*15*). Pneumococci were serotyped by the Quellung method (Statens Serum Institut, Copenhagen, Denmark).

Serotype 1 clusters were defined as an increase in serotype 1 IPD numbers relative to other serotype numbers in a specific geographic area and time. Cluster location was based on hospital district where cases were diagnosed; actual geographic location was considered to be the centroid of the district polygon. Other definitions are provided in the online Technical Appendix.

Incidence Estimations

We calculated annual incidence of serotype 1 disease per 100,000 population during 2003–2013 by using data for participants in defined age groups. We divided the number of age-specific, culture-positive serotype 1 IPD cases reported each year by age-specific midyear population estimates. Incidences for non–serotype 1 disease were similarly calculated. Serotype data for cases without serotype results from culture (including cases with only PCR serotype results) were imputed by age and year to obtain final incidence rates. Missing data were assumed to be random among different serotypes. Midyear population denominators were obtained from Statistics South Africa (http://www.statssa.gov.za/). To show differences in serotype incidences between prevaccine and postvaccine years, we compared an average incidence from prevaccine years (2003–2008) to 1 postvaccine year (2013). As a baseline for comparison, we included the average for years without clusters (2005–2007). CIs were calculated by using Poisson distribution for incidence rates.

Factors Associated with Serotype 1 IPD and Case-Fatality Rates

For the analyses of factors associated with serotype 1 IPD, we included only participants with culture- and PCR-positive results from enhanced sites during 2003–2013. Patients were stratified into 2 age groups (<5 and ≥5 years), and disease-associated factors in those with serotype 1 IPD were compared with those in patients with non–serotype 1 IPD by using a multivariable logistic regression model. A

second model to assess in-hospital fatalities restricted the analysis to serotype 1 IPD cases.

For both models, we assessed all variables considered significant ($p<0.2$) on univariate analysis and removed nonsignificant factors ($p\geq0.05$) by manual backward elimination. Patients with missing data for included variables were excluded. Statistical analysis was implemented by using Stata version 13.1 (StataCorp LP, College Station, TX, USA).

Spatiotemporal Analysis for Detection of Serotype 1 IPD Clusters

We conducted a space–time scan analysis to detect serotype 1 clusters by aggregating IPD cases with available serotype results from January 2003–December 2013 by month and district. To minimize potential biases introduced by temporal and geographic differences in specimen-collecting practices, healthcare-seeking behavior, or surveillance system improvements over time, we compared cases (serotype 1 IPD cases) with controls (non–serotype 1 IPD cases) from the same geographic area and time period; a Bernoulli model (*16*,*17*) was used for the comparison.

To account for control number reductions after PCV7 introduction, we adjusted (increased) observed control numbers by the percent reduction from the prevaccine period (*14*). To obtain estimated monthly numbers of controls, assuming no PCV introduction, we linearly interpolated estimated annual proportional reductions from June to June of consecutive years from 2009 through 2013. Because the percentage of reduction in the control numbers may have differed by geographic area due to locality differences in PCV7 uptake over time, we obtained monthly adjustment factors for each province. This adjustment would decrease the likelihood of detecting a cluster if, in fact, a cluster did not occur (null hypothesis).

To identify spatial clusters, we used an elliptical area of search that was allowed to vary in size, shape, and direction. Significance was assessed at $p<0.05$ over 999 replications. Space-time analysis was conducted by using SaTScan version 9.3.1 (http://www.satscan.org/); maps were generated by using ArcGIS version 9.2 (http://www.esri.com/). To calculate relative risks for districts, we divided observed number of cases by expected number of cases in each district.

Results

During 2003–2013, a total of 46,483 persons with IPD were enrolled in the study; 32,841 (71%) had viable isolates and known *S. pneumoniae* serotype, and 1,204 (3%) had serotype determination by PCR. Of the 46,483 persons, 20,564 (44%) were enrolled from enhanced sites; of these 6,211 (30%) were <5 years of age, 14,004 (68%) were ≥5 years of age, and 349 (2%) had unknown age (Figure 1). Of the 4,985 patients who died, 68% (3,365) did so within 3 days of admission. Of the 12,013 patients who recovered, 14% (1,673) were hospitalized for ≤3 days, 62% (7,427) for 4–14 days, and 24% (2,913) for >2 weeks. In the pre-PCV7 period (2003–2008), serotype 1 was the sixth most common *S. pneumoniae* serotype among children <5 years of age, but by 2013, it was eleventh. In contrast, among persons ≥5 years of age, serotype 1 was the most common serotype across all years, although case numbers decreased after PCV13 introduction.

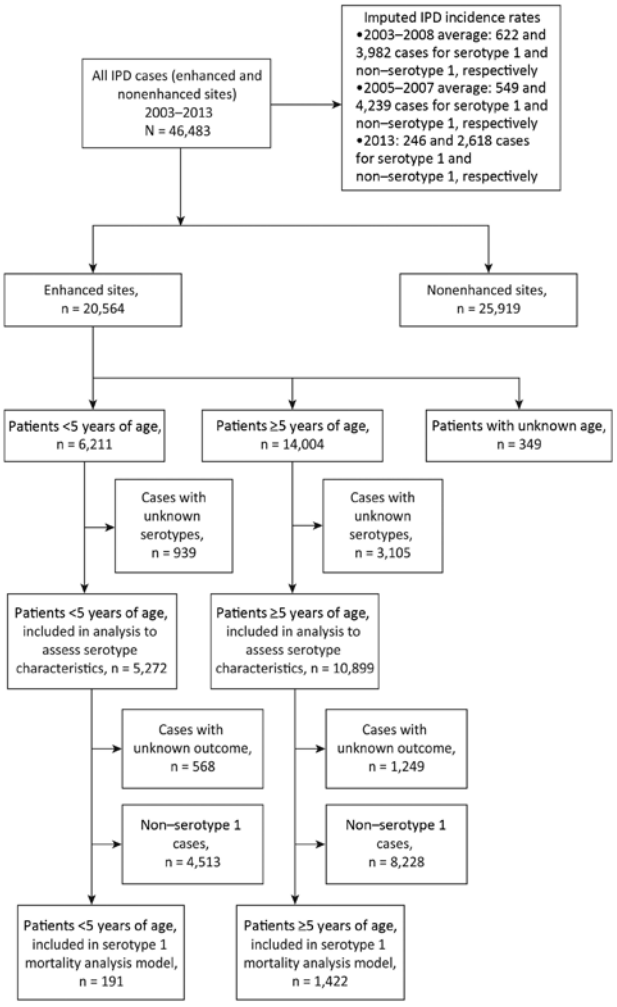

Figure 1. Selection flowchart for study of invasive *Streptococcus pneumoniae* disease (IPD) cases in South Africa, 2003–2013. Cases were reported by Group for Enteric Respiratory and Meningeal Disease Surveillance sites (GERMS-SA). Years indicate prevaccine (2003–2008), baseline (2005–2007), and postvaccine (2013) periods. Nonenhanced sites only submitted isolates and accompanying laboratory report forms, which included patient age and sex and the date and source of the specimen; enhanced sites (primarily tertiary hospitals) implemented enhanced surveillance, in which dedicated surveillance officers collected additional clinical information on identified patients.

Comparison of Enhanced and Nonenhanced Sites

Characteristics of enhanced and nonenhanced sites differed (online Technical Appendix Table 1). Compared with nonenhanced sites, enhanced sites had a higher proportion of cases among younger children, more cases from certain provinces, fewer cases in 2012–2013, more penicillin-nonsusceptible cases, more blood culture results, and fewer serotype 1 IPD cases.

Incidence of Serotype-Specific IPD in Different Age Groups

During the prevaccine era (2003–2008), serotype 1 incidence per 100,000 population was highest among persons <1 (1.8 cases), 5–9 (1.6 cases), and 25–44 (1.8 cases) years of age (Figure 2, panel A). Serotype 1 incidence did not differ significantly for 2003–2008 compared with 2005–2007, when there were no clusters. In 2013, serotype 1 incidence was highest among persons 5–9 (0.7 cases) and 25–44 (0.6 cases) years of age; reductions were significant ($p<0.001$) in all age groups except the >64-year-old age group ($p = 0.07$).

For all other serotypes during 2003–2008, the highest incidence rates per 100,000 population were among persons <1 (71.8 cases), 1–4 (13.9 cases), and 25–44 (10.1 cases) years of age (Figure 2, panel B). In 2013, the highest incidence rates were among persons <1 (27.3 cases) and >25 (>5.0 cases) years of age. Reductions in incidence among persons <5 and 25–44 years of age were significant ($p<0.001$).

The incidence of serotype 1 IPD fluctuated over the 11-year period (online Technical Appendix Figure 1). For the <5-year-old age group, incidence rates were significantly reduced in 2006 ($p = 0.01$), 2007 ($p = 0.03$), 2010 ($p = 0.006$), and 2012–2013 ($p<0.001$) compared with rates in 2005. In the ≥5-year-old age group, incidence rates were significantly higher in 2003 ($p = 0.001$) and 2004 ($p = 0.002$) compared with 2005 but lower during 2006–2008 and 2010–2013 ($p<0.001$).

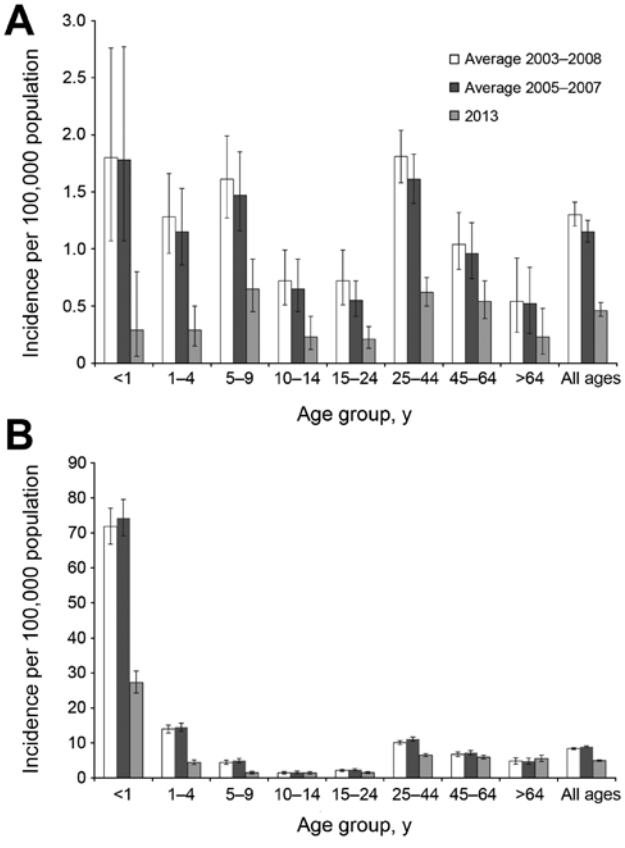

Figure 2. Incidence of serotype 1 and non–serotype 1 invasive pneumococcal disease (IPD) by age group, South Africa, 2003–2013. Years indicate prevaccine (2003–2008), baseline without clusters (2005–2007), and postvaccine (2013) periods. A) Serotype 1 IPD incidence by age group during prevaccine (no. cases = 622), baseline (no. cases = 549), and postvaccine (no. cases = 246) years. B) Non–serotype 1 IPD incidence by age group during prevaccine (no. cases = 3,982), baseline (no. cases = 4,239), and postvaccine years (no. cases = 2,618). Error bars indicate 95% CIs.

Factors Associated with Serotype 1 IPD

After adjustment for geographic location (province), year (based on prominent serotype 1 fluctuations), and clinical syndrome, we saw a difference among patients at enhanced sites who had IPD caused by serotype 1 versus other serotypes. Multivariable analysis showed a difference in disease distribution by province, year, and age among children <5 years of age; these difference were more apparent in children >3 than <1 years of age. Compared with children with non–serotype 1 IPD, those with serotype 1 disease had significantly shorter hospitalizations (≤3 days vs. 4–14 days [odds ratio (OR) 0.58, 95% CI 0.33–1.02] or ≥15 days [OR 0.44, 95% CI 0.23–0.85]) and were less likely to have HIV disease (OR 0.19, 95% CI 0.12–0.31), to die while hospitalized (OR 0.38, 95% CI 0.19–0.76), or to have penicillin-nonsusceptible disease (OR 0.02, 95 CI 0.01–0.05) (Table 1).

Among persons ≥5 years of age, serotype 1 IPD (compared with non–serotype 1 IPD) was significantly associated with province, year, and patient age: compared with persons >64 years of age, ORs (95% CIs) were 13.48 (5.53–32.82) for children 5–9 years of age; 8.02 (3.15–20.43) for children 10–14 years of age; 5.65 (2.31–13.82) for persons 15–24 years of age; 3.67 (1.53–8.76) for persons 25–44 years of age; and 2.57 (1.06–6.23) for persons 45–64 years of age (online Technical Appendix Table 2). Compared with persons with non–serotype 1 IPD, those with serotype 1 disease had significantly shorter hospitalization (≤3 days vs. 4–14 days [OR 0.86, 95% CI 0.68–1.09] and vs. ≥15 days [OR 0.64, 95% CI 0.48–0.86]) and lower rates of previous admissions (OR 0.45, 95% CI 0.35–0.57) and tuberculosis treatment (OR 0.73, 95% CI 0.57–0.95).

Table 1. Characteristics of 5,272 patients <5 years of age with invasive pneumococcal disease caused by serotype 1 or non–serotype 1 *Streptococcus pneumoniae*, South Africa, 2003–2013*

Variable	No. cases/no. total (%)		Univariate analysis†		Multivariable analysis†	
	Serotype 1	Non–serotype 1	OR (95% CI)	p value	aOR (95% CI)	p value
Age, y						
<1	63/211 (30)	2,754/5,061 (54)	Reference	<0.001	Reference	<0.001
1	35/211 (17)	1,155/5,061 (23)	1.32 (0.87–2.01)		2.36 (1.31–4.26)	
2	43/211 (20)	519/5,061 (10)	3.62 (2.43–5.40)		6.91 (3.78–12.64)	
3	37/211 (18)	355/5,061 (7)	4.56 (2.99–6.94)		12.03 (6.12–23.64)	
4	33/211 (16)	278/5,061 (5)	5.19 (3.35–8.05)		7.13 (3.60–14.13)	
Province						
Gauteng	95/211 (45)	2,067/5,061 (41)	Reference	<0.001	Reference	<0.001
Western Cape	11/211 (5)	1,158/5,061 (23)	0.21 (0.11–0.39)		0.11 (0.04–0.26)	
KwaZulu-Natal	46/211 (22)	957/5,061 (19)	1.05 (0.73–1.50)		1.04 (0.59–1.84)	
Eastern Cape	15/211 (7)	152/5,061 (3)	2.15 (1.22–3.79)		1.98 (0.74–5.28)	
Free State	25/211 (12)	383/5,061 (8)	1.42 (0.90–2.24)		1.06 (0.56–2.00)	
Mpumalanga	4/211 (2)	104/5,061 (2)	0.84 (0.30–2.32)		0.58 (0.07–4.86)	
North-West	5/211 (2)	46/5,061 (1)	2.36 (0.92–6.09)		5.65 (1.33–24.05)	
Limpopo	4/211 (2)	48/5,061 (1)	1.81 (0.64–5.13)		1.79 (0.41–7.90)	
Northern Cape	6/211 (3)	146/5,061 (3)	0.89 (0.39–2.08)		0.50 (0.15–1.64)	
Year of specimen collection						
2003	31/211 (15)	544/5,061 (11)	1.20 (0.72–1.99)	0.004	1.10 (0.49–2.49)	0.05
2004	26/211 (12)	699/5,061 (14)	0.78 (0.46–1.32)		0.58 (0.25–1.34)	
2005	32/211 (15)	672/5,061 (13)	Reference		Reference	
2006	21/211 (10)	551/5,061 (11)	0.80 (0.46–1.40)		0.77 (0.34–1.72)	
2007	15/211 (7)	547/5,061 (11)	0.58 (0.31–1.07)		0.67 (0.26–1.75)	
2008	10/211 (5)	542/5,061 (11)	0.39 (0.19–0.80)		0.40 (0.15–1.03)	
2009	23/211 (11)	494/5,061 (10)	0.98 (0.57–1.69)		1.43 (0.63–3.24)	
2010	19/211 (9)	361/5,061 (7)	1.11 (0.62–1.98)		0.82 (0.33–2.08)	
2011	19/211 (9)	240/5,061 (5)	1.66 (0.92–2.99)		1.04 (0.44–2.44)	
2012	12/211 (6)	190/5,061 (4)	1.33 (0.67–2.63)		0.49 (0.18–1.33)	
2013	3/211 (1)	221/5,061 (4)	0.29 (0.09–0.94)		0.12 (0.02–0.59)	
Medical conditions/treatment						
Length of hospital stay, d						
≤3	57/186 (31)	1,238/4,489 (28)	Reference	0.09	Reference	0.04
4–14	96/186 (52)	2,138/4,489 (48)	0.98 (0.70–1.36)		0.58 (0.33–1.02)	
≥15	33/186 (18)	1,113/4,489 (25)	0.64 (0.42–1.00)		0.44 (0.23–0.85)	
Previously hospitalized	39/164 (24)	1,676/4,110 (41)	0.45 (0.31–0.65)	<0.001		
Underlying medical condition‡	27/114 (24)	1,321/3,371 (39)	0.48 (0.31–0.75)	0.001		
Antimicrobial drug use in previous 2 mo§	10/147 (7)	742/3,549 (21)	0.28 (0.14–0.53)	<0.001		
HIV infected	43/132 (33)	2,125/3,539 (60)	0.32 (0.22–0.47)	<0.001	0.19 (0.12–0.31)	<0.001
TB treatment in previous 3 mo	11/161 (7)	570/3,928 (15)	0.43 (0.23–0.80)	0.008		
Malnourished¶	24/95 (25)	1,109/2,619 (42)	0.46 (0.29–0.74)	0.001		
Died during hospitalization	24/191 (13)	1,105/4,513 (24)	0.44 (0.29–0.68)	<0.001	0.38 (0.19–0.76)	0.006
Pneumococcal isolate characteristics						
Penicillin nonsusceptible#	4/203 (2)	2,580/4,950 (52)	0.02 (0.01–0.05)	<0.001	0.02 (0.01–0.05)	<0.001
Previous invasive pneumococcal disease**	2/211 (1)	356/5,061 (7)	0.13 (0.03–0.51)	0.004		
Clinical syndrome††						
Meningitis	59/198 (30)	1,668/4,736 (35)	Reference	0.001		
Pneumonia	124/198 (63)	2,358/4,736 (50)	1.49 (1.08–2.04)			
Bacteremia	15/198 (8)	710/4,736 (15)	0.60 (0.34–1.06)			

*All patients were reported from the enhanced Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) surveillance sites. aOR, adjusted odds ratio; OR, odds ratio; TB, tuberculosis.

†Only variables significant on univariate and multivariable analysis are shown. Variables not included are sex, race, Pitt bacteremia score, prematurity, antimicrobial drug use in previous 24 h, viable culture, and specimen type.

‡Includes asplenia or sickle cell anemia; chronic illness (i.e., chronic lung, renal, liver, cardiac disease, and diabetes); other immunocompromising conditions (i.e. including organ transplant, primary immunodeficiency, immunotherapy, and malignancy, but excluding HIV); and other risk factors (i.e., head injury with possible cerebral spinal fluid leak, neurologic disorders, burns, and chromosomal abnormalities). Excludes malnutrition.

§Use of any antimicrobial drug in 2 mo prior to admission.

¶Malnutrition was classified as a weight-for-age *z*-score of less than −2 (World Health Organization child growth standards 2009) (*18*), nutritional edema, or both.

#Considered penicillin nonsusceptible at MIC ≥0.12 μg/mL; intermediately resistant and resistant groups were combined into a nonsusceptible group.

**Invasive pneumococcal disease diagnosis >21 d before this episode.

††Clinical diagnoses were made on the basis of documented discharge diagnoses in patient medical records; clinical syndrome were separated into 3 groups: meningitis, bacteremic pneumonia, and bacteremia without focus or other diagnosis (e.g., septic arthritis, endopthalmitis, peritonitis, pericarditis).

Persons ≥5 years of age with serotype 1 disease were also significantly less likely to have HIV (OR 0.39, 95% CI 0.31–0.49) or penicillin-nonsusceptible disease (OR 0.02, 95% CI 0.01–0.04), and they were more likely than those with non–serotype 1 IPD to receive a diagnosis of pneumonia (OR 1.28, 95% CI 1.03–1.58) or bacteremia (OR 1.76, 95% CI 1.22–2.55) rather than meningitis. In-hospital death compared with recovery was not significant in the ≥5 year age group.

Factors Associated with In-Hospital Deaths among Patients with Serotype 1 IPD

We conducted multivariable analysis to explore factors associated with death in children <5 years of age with serotype 1 IPD (Table 2). Compared with 4-year-old children, those <1 year of age were more likely to die (OR 12.06, 95% CI 1.45–100.26), as were children with underlying medical conditions than those without. Odds of death were also increased among children with HIV (OR 2.82, 95% CI 1.36–5.84) or meningitis versus those with pneumonia or bacteremia. Duration of hospitalization was shorter among persons who died compared with those who recovered (<3 days vs. 4–14 days [OR 0.06, 95% CI 0.03–0.15] or ≥15 days [OR 0.02, 95% CI 0.01–0.07]).

Similar factors were associated with increased odds of death in persons ≥5 years of age with serotype 1 IPD (online Technical Appendix Table 3). In addition, death was more likely among persons who had received tuberculosis treatment in the previous 3 months (OR 1.75, 95% CI 1.25–2.45) and among severely ill persons (OR 5.26, 95% CI 3.53–7.84 for patients with a Pitt bacteremia score ≥4). No difference was seen in the odds of death by HIV status. Compared with children 5–9 years of age, persons >25 years of age had incrementally increased odds of death by age group: 25–44 years of age, OR 5.07 (95% CI 2.74–9.38); 45–64 years of age, OR 9.00 (95% CI 4.66–17.35); and >64 years of age, OR 10.13 (95% CI 4.46–23.00).

Detection of Serotype 1 IPD Clusters

Of the 46,483 IPD cases, 34,032 (73%) had available data (i.e., date of specimen collection, geographic location of patient, and serotype results) and were included in the space–time scan analysis. Of these 34,032 cases, 4,544 (13%) were caused by serotype 1 IPD. Two clusters of serotype 1 were detected. The first (713 cases) occurred during May 2003–December 2004 and affected Gauteng Province and adjacent districts of Mpumalanga, Limpopo, and North-West Provinces (Figure 3, panel A; online Technical Appendix Table 4). The second cluster (718 cases) occurred during September 2008–April 2012 and affected KwaZulu-Natal and Free State Provinces and adjacent districts of Gauteng, North-West, Mpumalanga, and Eastern Cape Provinces (Figure 3, panel B; online Technical Appendix Table 4). We also assessed clustering of disease caused by 2 other epidemic-prone serotypes (serotypes 5 and 8); neither showed significant increases in case numbers compared with numbers in 2005.

Discussion

In South Africa, serotype 1 pneumococcal disease had a number of distinct features. Children <5 years of age with serotype 1 IPD were less likely to die than were children with disease caused by other serotypes; this association between serotype 1 and death was not seen in older children and adults. Patients with serotype 1 IPD had fewer cases of penicillin-nonsusceptible disease, a lower prevalence of HIV, and less severe disease than patients with non–serotype 1 IPD. However, pneumonia and bacteremia occurred more commonly in patients with serotype 1 IPD than in patients with IPD caused by other serotypes.

Serotype 1 IPD incidence differed by geographic area and year, reflecting its epidemic potential (*1*). In older children and adults, serotype 1 was the most common serotype over the entire study period, even though numbers were lower after PCV13 introduction. Before PCV7 introduction, serotype 1 was the sixth most common serotype in children <5 years of age; by 2013, it no longer ranked in the top 10 serotypes in this age group.

IPD is common in children with underlying diseases, including HIV. Compared with infections caused by other common pneumococcal serotypes, serotype 1 IPD was associated with more bacteremic pneumonia and peritonitis, occurred in older children and specific ethnic groups, and affected otherwise healthy children (*3*).

Serotype 1 IPD has marked temporal variability (*19*) and is associated with outbreaks (*20*,*21*). In our study, we noted fluctuations in incidence rates for serotype 1 IPD, especially among young children before PCV introduction. Incidence of serotype 1 IPD decreased in all age groups after 2011, likely due to the effect of PCV13, and serotype 1 disease nearly disappeared among the youngest children by 2013, two years after PCV13 introduction (*10*). We cannot exclude that other factors (e.g., improvements in access to antiretroviral treatment and programs for the prevention of mother-to-child HIV transmission) may have contributed to this decrease (*14*,*22*). We identified 2 large clusters that were not recognized prospectively because of the difficulty in identifying communitywide clusters in real time, especially using laboratory-based surveillance.

Our findings showed differences in the geographic distribution of serotype 1 and non–serotype 1 disease. Serotype 1 has been described to occur more frequently in underprivileged populations in developing countries (*19*); in our study, differences in specimen collection practices between provinces may have

Table 2. Factors associated with death in patients <5 years of age with serotype 1 invasive pneumococcal disease, South Africa, 2003–2013*

Variable	Univariate analysis			Multivariable analysis	
	No. deaths/no. cases (%)	OR (95% CI)	p value	aOR (95% CI)	p value
Age group, y					
<1	102/355 (29)	11.49 (2.75–47.95)	<0.001	12.06 (1.45–100.26)	0.02
1	22/154 (14)	4.75 (1.08–20.88)		3.83 (0.41–35.35)	
2	11/94 (12)	3.78 (0.81–17.69)		1.30 (0.12–14.34)	
3	6/73 (8)	2.55 (0.49–13.14)		1.40 (0.12–15.82)	
4	2/59 (3)	Reference		Reference	
Province					
Gauteng	53/327 (16)	Reference	0.001		
Western Cape	15/111 (14)	0.81 (0.44–1.50)			
KwaZulu-Natal	26/111 (23)	1.58 (0.93–2.68)			
Eastern Cape	12/44 (27)	1.94 (0.94–4.01)			
Free State	11/62 (18)	1.11 (0.55–2.28)			
Mpumalanga	7/19 (37)	3.02 (1.13–8.01)			
North-West	11/23 (48)	4.74 (1.99–11.30)			
Limpopo	7/21 (33)	2.58 (1.00–6.71)			
Northern Cape	1/17 (6)	0.32 (0.04–2.49)			
Medical condition/treatment					
Length of hospital stay, d					
≤3	94/209 (45)	Reference	<0.001	Reference	<0.001
4–14	36/354 (10)	0.14 (0.09–0.21)		0.06 (0.03–0.15)	
≥15	10/160 (6)	0.08 (0.04–0.16)		0.02 (0.01–0.07)	
Pitt bacteremia score†					
0–3	102/608 (17)	Reference	<0.001		
≥4	16/28 (58)	6.61 (3.04–14.40)			
Underlying medical condition‡					
No	55/343 (16)	Reference	0.19	Reference	0.003
Yes	33/158 (21)	1.38 (0.86–2.23)		3.21 (1.49–6.91)	
Antimicrobial drug use in 24 h before admission					
No	82/504 (16)	Reference	0.05		
Yes	15/56 (26)	1.88 (1.00–3.56)			
HIV status					
HIV-uninfected	37/252 (15)	Reference	0.13	Reference	0.005
HIV-infected	52/263 (20)	1.43 (0.90–2.27)		2.82 (1.36–5.84)	
Malnourished§					
No	44/277 (16)	Reference	0.03		
Yes	43/176 (24)	1.71 (1.07–2.74)			
Clinical syndrome/specimen type					
Specimen type					
CSF	59/166 (36)	Reference	<0.001		
Blood	83/530 (16)	0.34 (0.23–0.50)			
Other	1/39 (3)	0.05 (0.01–0.36)			
Clinical syndrome¶					
Meningitis	74/209 (35)	Reference	<0.001	Reference	0.0003
Pneumonia	50/410 (12)	0.25 (0.17–0.38)		0.25 (0.11–0.54)	
Bacteremia	18/111 (16)	0.35 (0.20–0.63)		0.11 (0.03–0.42)	

*All patients were reported from the enhanced Group for Enteric, Respiratory, and Meningeal Disease Surveillance in South Africa (GERMS-SA) surveillance sites. Only variables significant on univariate and multivariable analysis are shown. Variables not included in table are sex, year, previous hospital admission, prematurity, antimicrobial drug in previous 2 mo, and penicillin nonsusceptible invasive pneumococcal disease. aOR, adjusted odds ratio; OR, odds ratio.
†Pitt bacteremia score calculated using temperature, hypotension, mechanical ventilation, cardiac arrest and mental status. Severe disease defined as score of ≥4 points.
‡Includes asplenia or sickle cell anemia; chronic illness (i.e., chronic lung, renal, liver, cardiac disease, and diabetes); other immunocompromising conditions (i.e., organ transplant, primary immunodeficiency, immunotherapy, and malignancy, but excluding HIV); and other risk factors (i.e., head injury with possible cerebral spinal fluid leak, neurologic disorders, burns, and chromosomal abnormalities). Excludes malnutrition.
§Children with weight-for-age *z*-score of less than −2 (World Health Organization child growth standards 2009) (*18*), nutritional edema, or both.
¶Clinical diagnoses were made on the basis of documented discharge diagnoses in patient medical records, with clinical syndrome separated into 3 groups: meningitis, bacteremic pneumonia, and bacteremia without focus or other diagnosis (e.g., septic arthritis, endopthalmitis, peritonitis, pericarditis)

contributed to differences seen in disease distribution, as shown in other studies (*23*). Similar to findings by others (*24,25*), we found a difference in serotype distribution by age: serotype 1 IPD incidence was proportionally similar among older children and adults compared with that among children <1 year of age, whereas other serotypes predominated in the youngest age group and showed only a small peak in young adults. A number of factors may contribute to these age-associated differences (*25*). Compared with other serotypes, serotypes 1 and 5 are rarely carried by healthy persons; a short duration of carriage results in less opportunity for recombination events and

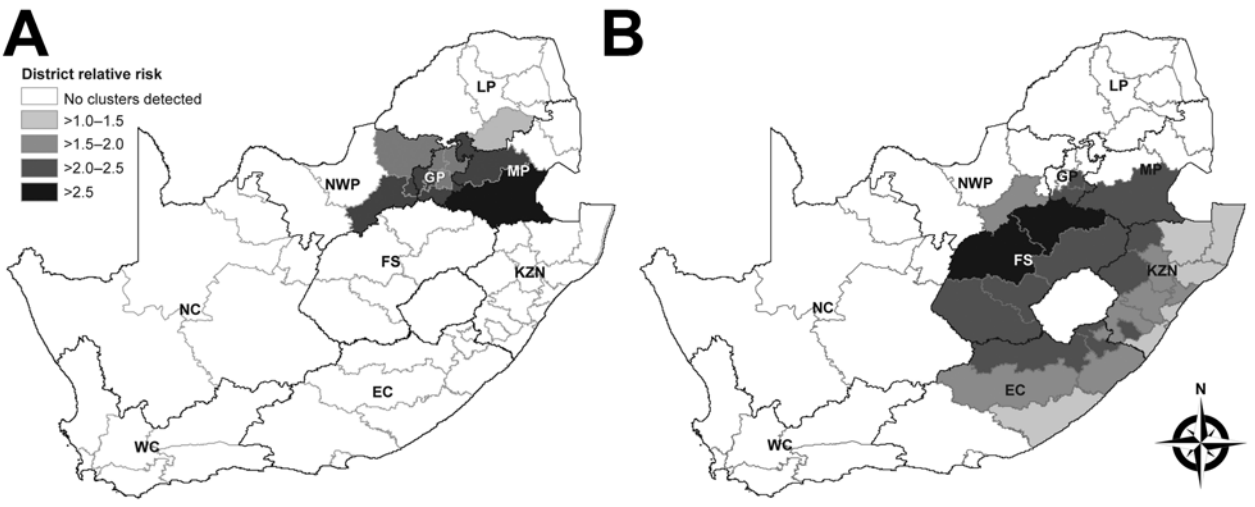

Figure 3. Serotype 1 invasive pneumococcal disease clusters by district, South Africa. A) May 2003–December 2004. B) September 2008–April 2012. Gray borders indicate district boundaries; black borders indicate provincial boundaries. Provinces: EC, Eastern Cape; FS, Free State; GP, Gauteng; KZN, KwaZulu-Natal; LP, Limpopo; MP, Mpumalanga; NC, Northern Cape; NWP, North-West; WC, Western Cape. District relative risk was calculated by dividing the observed number of cases per district by the number of cases expected by district (as determined on the basis of numbers in control groups).

less antibiotic selection pressure, resulting in reduced antibiotic nonsusceptibility in serotype 1 isolates (*26*).

Similar to findings in other studies (*3*), we found that, compared with other pneumococcal serotypes, serotype 1 caused more bacteremic pneumonia than meningitis. In addition, among HIV-uninfected children, serotype 1 IPD made up a larger proportion of disease than in HIV-infected children (*27*,*28*), suggesting that serotype 1 is more invasive and virulent, thus affecting otherwise healthy persons (*29*,*30*). Among children <5 years of age, those with serotype 1 disease were less likely to die than those with disease caused by other serotypes (*31*), and those most at risk of death were the very young (<1 year of age) and those HIV infected. In older persons, no association was found between serotype 1 disease and death when compared with other serotypes. Another analysis from the prevaccine era showed an increased risk of death among adults with serotype 1 disease compared with those with serotype 4 disease (*32*); this increased risk has been shown in few other studies (*33*).

Our study had several limitations. First, we included only patients who sought care at healthcare facilities with laboratories that submitted pneumococcal isolates to the National Institute for Communicable Diseases and who had specimens collected; patients with mild clinical pneumococcal disease treated in the community were not included. Second, we were able to map serotype 1 IPD incidence only at district level, so minor changes in incidence and clusters at the individual healthcare facility level may have been missed. Third, because of the small number of patients in the <5-year-old age group, we did not show clusters by age. We expect that reported clusters would have been similar for all ages. Fourth, we did not collect details regarding duration of symptoms before admission and thus could not assess whether intensity of symptoms when healthcare was sought affected case-fatality rates. Fifth, PCR serotype results from samples with a *lytA* cycle threshold (C_t) of ≥35 may not be accurate. We did not use PCR results in the trend analysis, and the proportion of *lytA* samples with high C_t values was low in the surveillance program (*34*), so the C_t accuracy is unlikely to have affected our results. Sixth, we used non–serotype 1 cases as our comparison group in the descriptive factor analysis; although this group changed over the study period, PCV13 serotypes (excluding serotype 1) made up >50% of this group until 2012 and 40% in 2013. Last, our study covered only a short period of observation after PCV13 introduction, making it difficult to determine whether reductions in serotype 1 IPD were due to introduction of this vaccine.

In conclusion, compared with IPD caused by other serotypes, IPD caused by serotype 1 in South Africa was characterized by shorter hospital stays, fewer cases of resistant disease, fewer in-hospital fatalities in children <5 years of age, and lower prevalence among HIV-infected persons. Serotype 1 caused disease in all age groups, although prevalence peaked in older children and young adults. Temporal reductions in serotype 1 IPD have been observed within 2 years of PCV13 introduction in South Africa; this observation must be corroborated by ongoing surveillance over an extended period of time.

Acknowledgments

We thank all persons, and their caregivers, who kindly agreed to be included in this study. We acknowledge all GERMS-SA surveillance officers for their hard work in enrolling participants into the study and obtaining vaccination histories; the GERMS-SA coordinators for assisting the surveillance officers and clinical and intellectual input for the surveillance; laboratory staff throughout the country for submitting isolates to NICD; and staff at the NICD laboratory, Centre for Respiratory Diseases and Meningitis, for their efforts in processing and characterizing these isolates.

This study was supported by NICD/National Health Laboratory Service, South Africa; PEPFAR (President's Emergency Plan for AIDS Relief) through the Centers for Disease Control and Prevention (cooperative agreement No. 5U2GPS001328); and the Global Alliance for Vaccines and Immunisation, Accelerated Vaccine Introduction Initiative Special Studies Team.

C.vM. has received honoraria from Pfizer. A.vG. has received research funding from Pfizer. S.A.M. has received honoraria from GlaxoSmithKline, Pfizer, and Sanofi Pasteur, and research funding from GlaxoSmithKline, Pfizer, and Novartis. H.D. has received honoraria from Novartis, Pfizer, Merck & Co., Inc. and a travel grant from Mylan. C.C. has received research funding from Pfizer and Sanofi Pasteur.

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Dogs and Opossums Positive for Vaccinia Virus during Outbreak Affecting Cattle and Humans, São Paulo State, Brazil

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During a vaccinia virus (VACV) outbreak in São Paulo State, Brazil, blood samples were collected from cows, humans, other domestic animals, and wild mammals. Samples from 3 dogs and 3 opossums were positive for VACV by PCR. Results of gene sequencing yielded major questions regarding other mammalian species acting as reservoirs of VACV.

Since the first vaccinia virus (VACV) outbreak in Brazil in 1999, researchers have speculated on the origins and possible reservoirs of VACV (*1*,*2*). Wild and peridomestic rodents are known to be reservoirs of cowpox in Europe (*3*), but in Brazil, their involvement as VACV reservoirs is unclear. Although studies have reported experimental transmission of VACV between rodents and cows (*4*), this finding was not confirmed during outbreaks in Brazil.

The isolation and characterization of a VACV isolate in a peridomestic rodent has been described on a farm in Minas Gerais State, which raised questions about the role of rodents in VACV maintenance in Brazil (*5*). However, a recent serologic study on VACV reservoirs suggested that wild rodents might have a secondary role in VACV maintenance in São Paulo State (*6*).

Despite the absence of reports of clinical signs in dogs and other domestic or wild mammals during VACV outbreaks, in this report, we describe 3 dogs (*Canis lupus familiaris*) and 3 opossums (*Didelphis albiventris*) without clinical signs that were obtained during from a VACV outbreak. Blood samples from these animals were positive by PCR for VACV.

The Study

This study was approved by the Ethical Committee of Animals Uses in Veterinary Medicine and Animal Production of São Paulo State University. The capture of wild animals was authorized by the Brazilian Institute of Environment and Natural Resources Renewable.

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DOI: http://dx.doi.org/10.3201/eid2202.140747

In October 2012, the veterinary service of Itatinga County (23°6′7″S, 48°36′57″W) in São Paulo State, Brazil, reported an outbreak similar to that of bovine smallpox that affected 2 small dairy farms. The Veterinary Medical and Animal Husbandry School of São Paulo State University were contacted, and a team of veterinarians visited the 2 dairy farms to collect samples for diagnosis and relevant information by using an epidemiologic questionnaire.

Both farms (1.5 km apart) used manual milking systems and had similar sanitary management of herds. The animals were vaccinated against foot and mouth disease, brucellosis, and clostridiosis. Ivermectin or albamectin were used for control of endoparasites. Animals were raised in pastures, and there were no changes in management or introduction of new animals on both farms. There were no previous outbreaks on these farms.

Blood and scab samples from udders of cows or nostrils of calves were collected from 31 affected animals. Serum and whole blood were collected from 2 humans who worked as milkers and had lesions on their hands and arms (Figure 1). Blood samples were also collected from 6 dogs, 6 pigs, 2 horses, 2 rams, 3 opossums, 1 coati (*Nasua nasua*), and 2 wild rodents (*Akodon montensis* and *Nectomys squamipes*). Both domestic and wild mammals were evaluated at the time of collection for characteristic clinical signs of VACV, such as vesicles, scabs, or crusts.

Opossums and the coati were captured by using a trap (Tomahawk Live Trap, Hazelhurst, WI, USA) containing

Figure 1. Lesion on arm of a human who worked as a milker, São Paulo State, Brazil. The lesion was determined to be caused by infection with vaccinia virus.

thigh or drumstick chicken as bait. These animals were anesthetized with tiletamine and zolazepam by using the recommended dose for each species (*7*). Wild rodents were captured by using a trap (H.B. Sherman Traps, Inc., Tallahassee, FL, USA) containing peanuts, cornmeal, oats, and canned sardines as bait. These animals were anesthetized in an autoclavable bag containing gauze soaked in ethyl ether and then euthanized by using deep anesthesia. Five trap nights were required to capture animals in the native forest areas surrounding both farms.

Virus DNA was extracted from scabs and total blood samples by using the Invisorb Spin Tissue Mini Kit and the Invisorb Spin Blood Mini Kit (Stratec Molecular, Berlin, Germany), respectively. A seminested PCR was conducted for amplification of the *A56R* gene of VACV (*8*), and positive samples were submitted for gene sequencing at the Federal University of Minas Gerais State (Belo Horizonte, Brazil).

Responses to the epidemiologic questionnaire showed that no factors could be correlated with the 2 outbreaks. No domestic or wild mammals had clinical signs at the time of blood sample collection. However, 3 dogs and 3 opossums were positive by PCR for VACV. Gene sequences obtained (GenBank accession nos. KJ741387.1, KJ741388.1, KJ741389.1, KJ741390.1, KJ741391.1, and KJ741392.1) showed that VACV isolated in this study had the same deletion of six amino acids at position 251 that is found in group I VACVs from Brazil, such as Cantagalo virus, Araçatuba virus, Passatempo virus, and Guarani P2 virus (*4*,*9*,*10*) (Figure 2).

Conclusions

The fact that the dogs and opossums positive by PCR for VACV did not have clinical signs of infection might indicate only a subclinical infection or may be related to the dichotomy of VACVs from Brazil, which is not only genetic but also biologic. Ferreira et al. (*4*) showed that although there was no difference in severity of lesions in humans and cows affected by group I and II VACVs, BALB/c mice that were intranasally inoculated with group I VACVs did not have clinical signs. In contrast, mice inoculated with group II VACS, such as Belo Horizonte virus and Gurani P1 virus, had acute respiratory illness, followed by death.

More than 1 mammalian species, either wild or domestic, might be acting as a reservoir of group I VACVs from Brazil, possibly acquiring and transmitting the virus without showing clinical signs. This assumption corroborates the findings of Peres et al. (*6*), which showed a high seroprevalence in dogs without clinical signs, questioning their possible role as a reservoir and suggesting more studies to confirm these findings.

Genetic and epidemiologic analysis showed that VACV circulating in this region are members of group I VACVs in Brazil. These findings might support the absence of clinical signs and raise major questions about the potential for >1 mammalian species, either wild or domestic, to act as a reservoir. More studies are needed to further elucidate this ecologic situation.

Figure 2. Phylogenetic tree of A56R genes of orthopoxviruses, São Paulo State, Brazil. Tree was constructed by using the neighbor-joining method, the Tamura-3 model of nucleotide substitutions, and 1,000 bootstrap replicates in MEGA 4.0 (http://www.megasoftware.net/mega4/mega.html). Black circles indicate group 1 vaccinia virus (VACV) isolates from this study. Numbers along branches are bootstrap values. ID 5 (KJ741390.1), ID 7 (KJ741391.1), and ID 13 (KJ741392.1) are from opossum (*Didelphis albiventris*) blood samples; ID 5 and ID 7 are from the first farm sampled; ID 13 is from the second farm sampled; ID 4 (KJ741387.1), ID 9 (KJ741388.1), and ID 10 (KJ741389.1) are from canine blood samples; and ID 4, ID 9, and ID 10 are from the first farm sampled. MURV, Muriaé virus; CTGV, Cantagalo virus; ARAV, Araçatuba virus; MARV, Mariana virus; PSTV, Passatempo virus; GP2V, Guarani P2 virus; HSPV, horsepox virus; MPXV, monkeypox virus; VARV, variola virus; CPXV, cowpox virus. Scale bar indicates nucleotide substitutions per site.

Acknowledgment

We thank Jonatas S. Abrahão for performing gene sequencing assays.

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Hemorrhagic Fever with Renal Syndrome, Zibo City, China, 2006–2014

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Analysis of hemorrhagic fever with renal syndrome cases in Zibo City, China, during 2006–2014 showed that it occurred year-round. Peaks in spring and fall/winter were caused by Hantaan and Seoul viruses, respectively. Rodent hosts were the striped field mouse for Hantaan virus and the brown rat and house mouse for Seoul virus.

Hemorrhagic fever with renal syndrome (HFRS) is caused by hantavirus and transmitted primary by rodents (*1*,*2*). HFRS occurs worldwide, but ≈90% of HFRS cases have been reported in China (*3*). During 2006–2012, a total of 77,558 HFRS cases, including 866 deaths, were reported in China (*4*). Zibo City in Shandong Province, located in eastern China, has a high incidence rate of HFRS (*5*,*6*). We analyzed the clinical data for HFRS cases and surveyed the prevalence of hantaviruses in rodent populations in rural area of Zibo City during 2006–2014.

The Study

Zibo City is a prefecture-level city located at 36°47′N 118°3′E (Figure 1). The city consists of 6 districts and 3 counties distributed over 5,938 km^2 of land; the total population during the 2010 census was 4.53 million, of whom 900,000 persons were farmers. The city is ≈42% mid-sized mountains in the south, 30% hills in the center, and 28% plains in the north (http://115.238.252.51:9001/html/2007/09/13/20070913141750.html).

We obtained data for HFRS patients from the Zibo Center for Disease Control and Prevention and analyzed these data with Excel 2007 (Microsoft, Redmond, WA, USA) and SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) and χ^2 test for statistical analysis. The research protocol was approved by the human bioethics committee of the Zibo Center for Disease Control and Prevention, and all participants provided written informed consent. HFRS cases were confirmed by detection of hantavirus IgM in patients' serum with an ELISA kit (Beijing Wantai, Beijing, China).

During 2006–2014, a total of 782 HFRS cases were reported in Zibo City; 9 (1.2%) persons died. The incidence rate of HFRS varied from 0.74 to 3.65 per 100,000 persons annually (average 1.96 cases/100,000 persons) (Table). HFRS patients were from rural areas in all 9 districts and counties in Zibo City, but most (73.3%) were from hilled areas in the central and southern parts of the city, including Yiyuan County (278 cases), Zichun District (161 cases), and Boshan District (134 cases) (Figure 1). During 2006–2010, HFRS patients were predominantly from Zichuan and Boshan Districts (51.8%) in the central part of the city. During 2011–2014, Yiyuan County became the major source of HFRS cases, accounting for 51.3% of all cases during this period.

HFRS cases peaked twice each year. The spring peak occurred during March–May, and the fall/winter peak occurred during October–December (Figure 2). Serum samples from 36 patients from 2006 through 2008 were typed by focus reduction neutralization test with Hantaan virus (HTNV) strain 76-128 and Seoul virus (SEOV) strain UR in Vero cells. Reducing 80% of plaques was considered a positive result (*7*). Focus reduction neutralization test showed that 93.4% (15/16) patients from the spring peak were infected with SEOV, and 85% (17/20) from the fall/winter peak were infected with HTNV.

The incidence for the fall/winter peak was higher than that for the spring peak for the entire city (Figures 1, 2). However, the seasonal distribution of HFRS cases varied from place to place. For example, Yiyuan County and Boshan District had a substantially higher fall/winter peak of HFRS but did not have an obvious spring peak, whereas in Zichuan District, the spring peak was higher than the fall/winter peak. The number of HFRS cases in the spring peak and fall/winter peak in the 3 high-incidence areas differed significantly ($\chi^2 = 74.40$, $p<0.0001$).

The seasonal distribution of HFRS also varied in different years (Table; Figure 1, 2). In 2006, the spring peak was higher than the fall/winter peak, and 41.0% (64/156) of cases reported during that year occurred during March–May. In 2012, one primary peak occurred in which 73.6% (67/91) of cases occurred during the winter. In 2007 and 2010, the peak was not obvious in the spring or the fall. In 2009 and 2014, the spring peak was predominant, and the fall/winter peak was not obvious. In 2008, 2011, and 2013, the epidemic mainly occurred in the fall/winter, when incidence was much higher than for the spring peak.

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DOI: http://dx.doi.org/10.3201/eid2202.151516

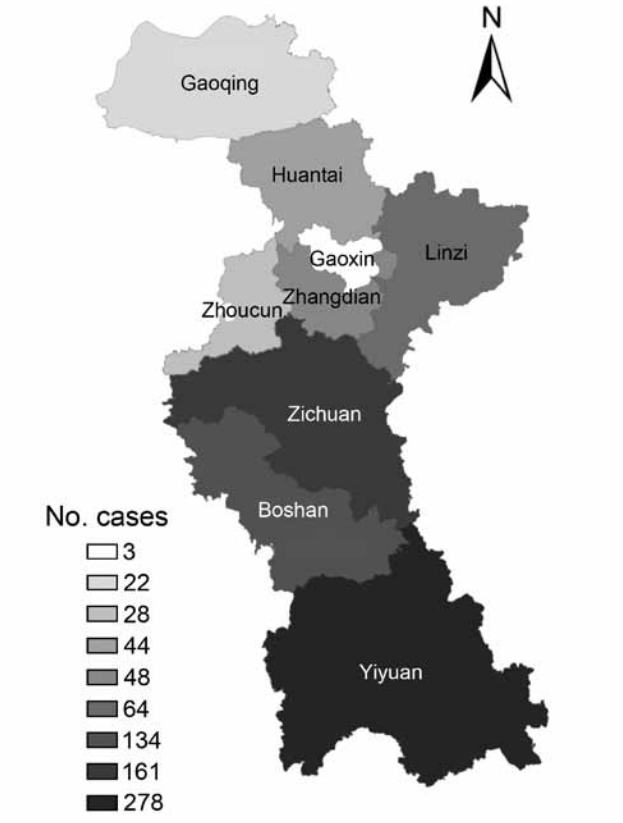

Figure 1. Geographic distribution of cases of hemorrhagic fever with renal syndrome among districts and counties, Zibo City, China, 2006–2014.

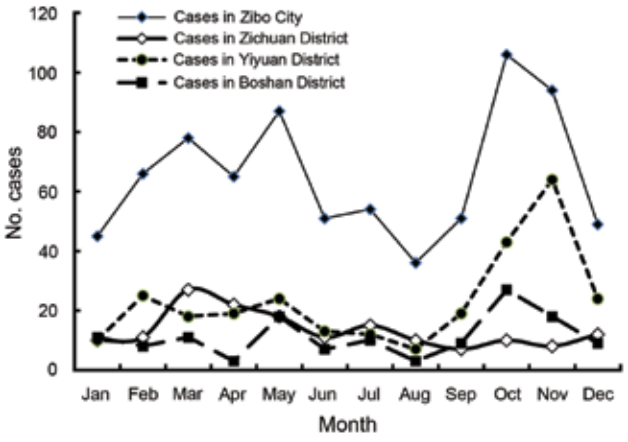

Figure 2. Monthly distribution of cases of hemorrhagic fever with renal syndrome, Zibo City, China, 2006–2014.

The number of HFRS cases differed significantly between the spring season and fall season each year ($\chi^2 = 38.01$, $p<0.0001$).

Ages of the 782 HFRS patients ranged from 7 to 85 years (median 46 years). Most (73.8%) patients were 30–65 years of age, and more than half (51.4%) were 40–50 years of age. Most (68.4%) patients were farmers, and most (87.6%, 685/782) lived in the countryside. The male:female ratio was 2.2:1.

Of the 9 persons who died, 4 died in October, 3 died in November, and 1 each died in August and September. Five decedents were male. Decedent ages ranged from 33 to 60 years; 3 were 30–49 years of age, and 6 were 50–59 years of age. HFRS was diagnosed 3–8 days (average 5.3 days) after illness onset. The patients died 4–8 days (average 5.9 days) after illness onset.

During 2006–2014, we trapped 559 rodents with 21,384 snap-traps in residential area (inside and outside farmhouse) and in fields. The rodents' lungs were tested for hantavirus antigen with monoclonal antibody (Shanghai Institute of Biologic Products, Shanghai, China) by direct immunofluorescence assay (*8*,*9*). We detected hantavirus antigens in 29 (5.2%) rodents. In residential areas, the brown rat (*Rattus norvegicus*) was the predominant rodent species and had the highest infection rate (5.3%, 21/393). The house mouse (*Mus musculus*) was next in both population and infection rate (2.4%, 1/42). Other residential rodents were all negative for hantavirus, including 16 Chinese hamsters (*Cricetulus griseus*) and 1 striped field mouse (*Apodemus agrarius*). Among field rodents, the striped field mouse was the most common species and had the highest hantavirus infection rate (11.9%, 7/59). Other field rodent species were all negative to hantavirus, including 27 brown rats, 18 buff-breasted rats (*R. flavipectus*), and 3 house mice. Infection rates for the striped field mouse, brown rat, and house

Table. Characteristics of hemorrhagic fever with renal syndrome, Zibo City, China, 2006–2014

Year	No. cases	Incidence rate, cases/100,000 population	No. deaths (%)	Cases in spring, no. (%)	Cases in fall/winter, no. (%)
2006	156	3.65	0	64 (41.0)	24 (15.4)
2007	90	2.10	0	31 (34.4)	24 (26.7)
2008	69	1.60	1 (1.45)	19 (27.5)	29 (42.0)
2009	35	0.81	1 (2.86)	14 (40)	5 (14.3)
2010	32	0.74	1 (3.13)	6 (18.8)	7 (21.9)
2011	42	0.93	0	5 (11.9)	18 (42.9)
2012	91	2.00	4 (4.40)	12 (13.2)	67 (73.6)
2013	136	2.97	2 (1.53)	35 (25.7)	45 (33.1)
2014	131	2.85	0	44 (33.6)	30 (22.9)
Total	782	1.96	9 (1.15)	230 (29.4)	249 (31.8)

mouse did not differ significantly ($\chi^2 = 5.50$, $p = 0.06$). The hantavirus antigen–positive rodents were further tested by reverse transcription PCR amplification of hantavirus M segment (*10*) from lung tissue, and 19 rodents were PCR positive. BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that 13 sequences belonged to SEOV and 6 belonged to HTNV. SEOV-positive rodents were all brown rats from residential areas, and HTNV-positive rodents were all striped field mice from field areas. The reverse transcription PCR–positive rate was 3.3% (13/393) for SEOV in brown rats from residential areas and 10.2% (6/59) for HTNV from field areas.

Conclusions

HFRS consistently occurred in Zibo City and peaked twice: in the spring and fall/winter. The spring peak was caused mainly by SEOV and the fall/winter peak mainly by HTNV. The major animal host of HTNV was the striped field mouse, and the major animal hosts of SEOV were the residential brown rat and house mouse. The house mouse is not considered the animal host of HTNV or SEOV, but this study and recent studies (*11–13*) have detected both HTNV and SEOV in this rodent. The role of the house mouse as the animal host of HTNV and SEOV needs further investigation.

This study was supported by grants from Shandong Province Science and Technology Development Program (2014GSF121004), National Natural Science Funds of China (31570167, 81401368), National Natural Science Foundation of China (grant no. 81102171), and Shandong Medical Science and Technology Development Program (grant no. 2011HZ055).

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African Buffalo Movement and Zoonotic Disease Risk across Transfrontier Conservation Areas, Southern Africa

Alexandre Caron, Daniel Cornelis, Chris Foggin, Markus Hofmeyr, Michel de Garine-Wichatitsky

We report on the long-distance movements of subadult female buffalo within a Transfrontier Conservation Area in Africa. Our observations confirm that bovine tuberculosis and other diseases can spread between buffalo populations across national parks, community land, and countries, thus posing a risk to animal and human health in surrounding wildlife areas.

Since the early 2000s in southern Africa, Transfrontier Conservation Areas (TFCAs) have been created to promote biodiversity conservation and local development (*1*). Increased connectivity between protected areas is designed to promote wildlife movement, ecosystem functioning, and genetic exchange and lead to increased wildlife populations, which should benefit communities living in these areas (e.g., through tourism and sustainable use of natural resources). Small-scale crop and livestock production are the main livelihood options for poor farmers living in communal lands in TFCAs. The extensive wildlife–livestock–human interface areas in TFCAs potentially result in human–wildlife conflicts, including crop destruction by wildlife, competition for resources between wild and domestic ungulates, livestock predation by wild carnivores, and poaching of wildlife; these conflicts are likely to increase as wildlife populations expand (*2*). The potential for the emergence and spread of infectious diseases is also of concern because of increased contact between wild and domestic hosts (*3*,*4*).

The Great Limpopo TFCA (GLTFCA) was created in 2002 and straddles Mozambique, South Africa, and Zimbabwe. It includes the Limpopo, Kruger, and Gonarezhou National Parks (NPs) and other land-use types surrounding the parks (Figure 1). The African buffalo (*Syncerus caffer caffer*) population in Kruger NP is known to maintain animal diseases, including zoonoses such as bovine tuberculosis (bTB) and brucellosis. Buffalo are also suspected of playing a role in the epidemiology of Rift Valley fever (*5*). In 2009, a bTB strain related to the strain occurring in buffalo in northern Kruger NP was detected in buffalo in Gonarezhou NP, suggesting a recent spread from Kruger NP in South Africa to Gonarezhou NP in Zimbabwe (*6*). Although possible explanations were proposed for this transfrontier spread, including direct transmission from buffalo to buffalo or from an unidentified wild or domestic ungulate species to buffalo (*6*), these modes of transmission were not supported by firm data. We report preliminary results from telemetry studies and visual observations of individually identified African buffalo within the GLTFCA.

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DOI: http://dx.doi.org/10.3201/eid2202.140864

The Study

During 2008–2013, a total of 68 satellite or global positioning system radio collars were deployed on African buffalo captured in southern Gonarezhou NP; in northern Kruger NP, south of the Limpopo River; and in Zimbabwe, north of Limpopo River (on Sengwe communal land). Of the 68 buffalo, 47 were adult females, selected because their behavior is representative of core herd movements. Two adult males were also equipped with global positioning system devices because males are believed to move between herds (*7*); however, these devices failed after a few weeks because the collars fell off. Nineteen subadult female buffalo 2.5–4.5 years of age (age determined by teeth eruption) were also selected because individuals from this group are believed to disperse from their native herds (*8*; R. Bengis, pers. comm.). During chemical immobilizations of buffalo, blood samples were taken and stored appropriately for disease screening, and individually numbered ear-tags were applied.

During the study, extraordinarily long-distance movements for 3 subadult females were plotted by satellite telemetry readings (Figure 1). In January 2014, a 2.5-year-old female buffalo collared in South Africa walked a maximum direct distance of 95 km. In 6 days, she crossed into Zimbabwe, then into Mozambique, and into Zimbabwe again to enter Gonarezhou NP, with localizations within the home range of the buffalo herd in which bTB was first diagnosed in a female buffalo in 2008 (*6*). This subadult buffalo later left the park and visited a commercial farm area before reentering Gonarezhou NP. In February 2014, another

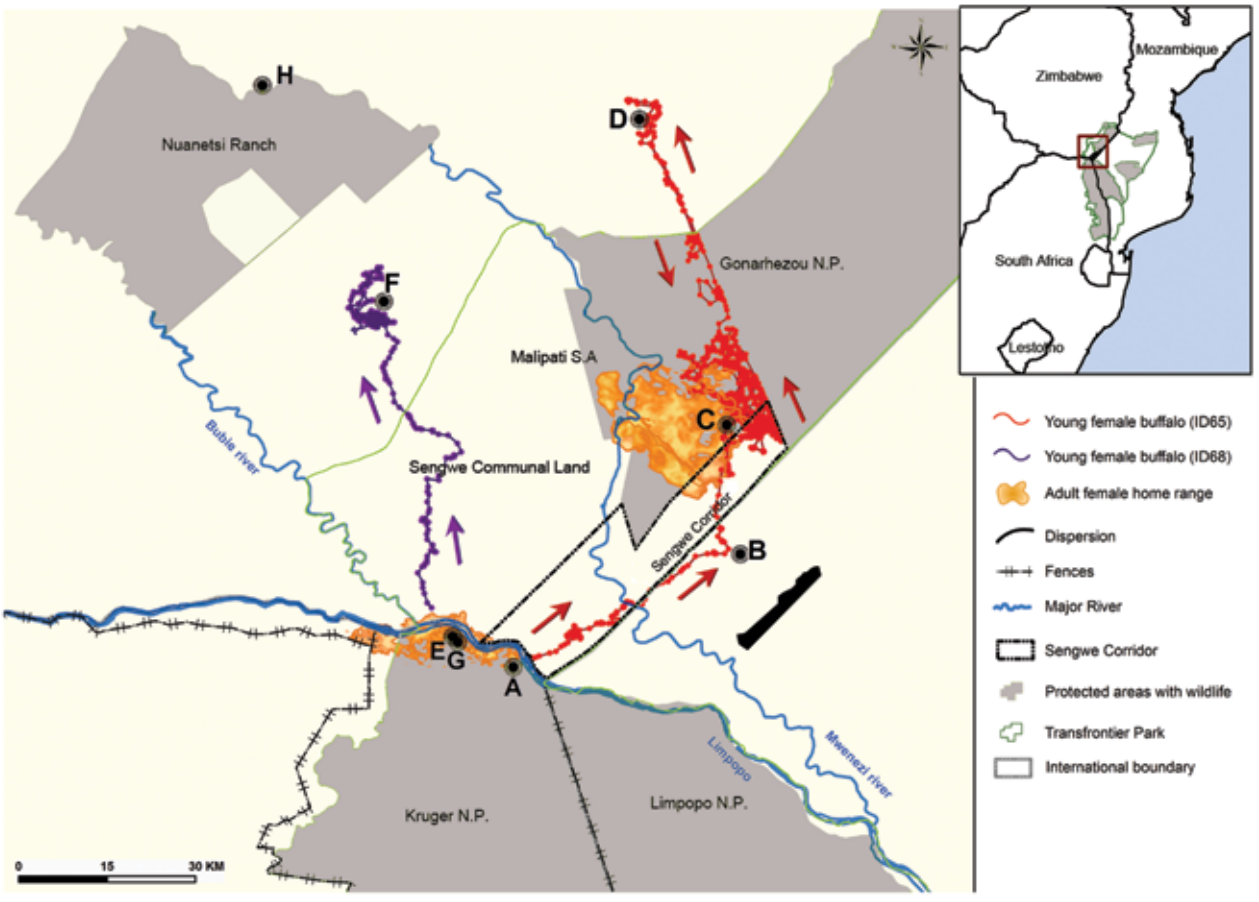

Figure 1. Study area encompassing part of Mozambique, South Africa, and Zimbabwe. Inset map shows location of the Great Limpopo Transfrontier Conservation Area within southern Africa. Orange/yellow shaded areas represent the home ranges of 5 satellite collar–equipped adult female African buffaloes, representative of the 5 herds followed for the study in Kruger National Park (NP) (n = 3) and Gonarezhou NP (n = 2). Because of overlap among the herds, boundaries for the 5 herds cannot be seen. Data for the other adult female buffalo in the study are not represented. The home range of Kruger NP herds span the Limpopo River between South Africa and Zimbabwe. Long-distance movements of 3 subadult female buffalo are shown. Arrows indicate the direction of movements for 2 buffalo; sites of capture and resighting are shown for the third buffalo. A complete description of the movements of these 3 buffalo is provided in the expanded figure legend online (http://wwwnc.cdc.gov/EID/article/22/2/14-0864-F1.htm).

4-year-old female buffalo walked a direct distance of 64 km in 8 days. Finally, in March 2013, a 4.5-year-old female captured in July 2011 was sighted in a location deep into communal land at a distance of 96 km from her capture site. In contrast to these young female buffalo, no adult females collared in this study moved such long distances outside their home range during 2008–2014. The long-distance travel of these 3 subadult females occurred over a few days during the rainy season (Figure 2) and included movements outside the GLTFCA boundary.

Conclusions

Our findings strengthen the hypothesis that bTB was spread from Kruger NP to Gonarezhou NP through buffalo-to-buffalo transmission by subadult females that dispersed from their native herds. Buffalo populations in Kruger and Gonarezhou NPs are connected through long-distance movements of individuals, specifically prebreeding heifers. Although this movement is important for buffalo conservation in TFCAs, it could also facilitate the spread of animal diseases, including zoonoses, across borders. In 2010 and 2011, bTB, Rift Valley fever, and brucellosis were detected in Kruger NP buffaloes, although a previous study failed to detect brucellosis in the Gonarezhou NP population (*9*) (Table). Buffalo ID65, which was initially captured in Kruger NP, was seen among a breeding herd in Gonarezhou NP, indicating the possibility of direct, buffalo-to-buffalo transmission of bTB by dispersing infected individuals, without the need for bridge hosts (e.g., other wild or domestic ungulate species) (*10*).

Additional ecological information on buffalo dispersion is needed: frequency of dispersion events; size, age, and sex composition of the dispersing groups; and information about whether dispersed individuals later return to their

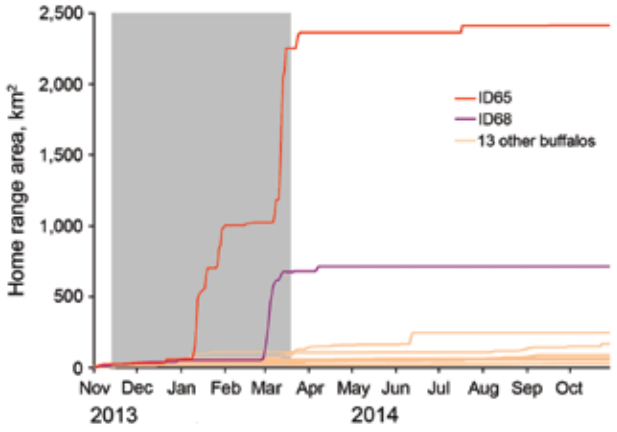

Figure 2. Cumulative home-range area of 15 buffalo collared with global positioning system devices in Kruger National Park, South Africa, in November 2013. Only buffalo with collars that functioned for an entire year are displayed. Data for 2 subadult female buffalo (paths displayed in Figure 1) are shown (ID65 and ID68) separately from data for the 13 other buffalo (subadult and adult females). Gray shading indicates the rainy season (generally when movement began).

home ranges. Subadult females appear to be particularly prone to dispersing behavior, unlike adult females, and we speculate that they may do so in small groups of individuals that are approximately the same age (*8*). No record exists of subadult female buffalo mixing with male bachelor groups, which are also known to connect to adjacent herds (*7*). So far, the drivers of such movement patterns are unclear. One possible explanation may be an out-breeding mechanism (*11*) that occurs before the start of reproduction; subadult females may leave their native herd to begin their reproduction in a distant herd to minimize in-breeding. Furthermore, abundant resources (i.e., water and grazing areas) available during the rainy season maximize the probability of success of such behavior.

We found that subadult females were infected with bTB, brucellosis, and Rift Valley fever (Table), diseases with different mechanisms of transmission. Age and social position in the herd may influence individuals' rate of exposure to pathogenic infections and consequently may affect the dynamics of infection within and between herds. Our results indicate that subadult female buffalo could play a role in the spread of diseases among distant populations, across protected areas and international borders, and during the rainy season. This seasonal pattern contrasts with the timing of most wildlife and livestock contact between adult females, which has been observed to occur predominantly during the dry season in the study area (*4*). Buffalo have been observed far outside the boundaries of protected areas, even outside the GLTFCA, in communal land where livestock farming is the main livelihood; these observations considerably widen the wildlife–livestock interface area where disease spread can occur (*12*). Wildlife–livestock interfaces can encompass large areas, rather than being a fence or strip of land at the edge of protected areas. These data should assist in refining disease modeling by showing the importance of temporal and spatial considerations and by redefining variables (e.g., age and sex) involved in risk for pathogen spillover or emergence (i.e., identifying super-spreaders) (*13*).

Our results suggest that the spillover of bTB and other zoonoses at the wildlife–livestock–human interface constitutes a risk to animal and human health in the GLTFCA (*9*,*14*). The health issue in TFCAs cannot be overlooked and must be part of any management decision. Combining ecological and epidemiologic knowledge is necessary to understand disease dynamics in these complex agro-ecosystems.

Acknowledgments

We thank the Department of National Parks and Wildlife Management Authority of Zimbabwe for permission to operate in Gonarezhou National Park; the South African National Parks for permission to conduct our research in Kruger National Park; and the veterinary services of both countries for permits and efficient field support. We also thank Hugo ven der Westhuizen for an inflight sighting of buffalo ID65 and Marie Gely for designing the map.

This work was conducted within the frameworks of the Production and Conservation in Partnership Research Platform and the Animal and Human Health for the Environment and

Table. Diagnostic results for bovine tuberculosis, brucellosis, and Rift Valley fever in African buffalo populations sampled in Kruger National Park, South Africa, and on the northern bank of the Limpopo River, Sengwe Communal Land, Zimbabwe, 2010 and 2011*

	No. positive/no. total						
	Calf		Subadult		Adult		
Type of infection tested for†	M	F	M	F	M	F	All animals‡
Bovine tuberculosis	0/4	0/5	2/11	4/25	0/9	2/23	8/77 (4/38)
Brucellosis	0/4	1/5	1/12	4/28	2/9	5/25	13/83 (0/38)§
Rift Valley fever	0/4	0/5	0/12	1/31	1/9	3/28	5/89 (2/38)

*Individuals and herds belong to the same population (see Figure 1, adult female home range). Calf, <2.5 y of age; subadult, 2.5–4.5 y; adult, >4.5 y.
†Laboratory tests used: for bovine tuberculosis, Gamma-interferon test and isolation; for brucellosis, Rose Bengal and Complement fixation tests; for Rift Valley fever, indirect ELISA test.
‡Published results (*9*) for the same diseases tested in the Gonarezhou National Park buffalo population in Zimbabwe are shown in parentheses.
§Difference between Kruger and Gonarezhou population results for brucellosis was significant by Fisher exact test for equality of proportion for small samples ($p<0.02$).

Development. The project was funded by CIRAD; South African National Parks; Veterinary Services in South Africa's Department of Agriculture; and the Ministère Français des Affaires Etrangères through the French Embassy in Zimbabwe.

Dr. Caron is a disease ecologist who was based in Zimbabwe from 2006 and relocated to Mozambique in 2015. His main research interest is the ecology of infectious transmission at interfaces of wild and domestic animals. He has worked on avian influenza transmission between wild and domestic birds and on pathogens shared at wildlife and cattle interfaces in Africa, including rinderpest, bovine tuberculosis, and foot and mouth disease.

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Anaplasmataceae-Specific PCR for Diagnosis and Therapeutic Guidance for Symptomatic Neoehrlichiosis in Immunocompetent Host

Michael Schwameis, Julia Auer, Dieter Mitteregger, Ingrid Simonitsch-Klupp, Michael Ramharter, Heinz Burgmann, Heimo Lagler

Candidatus Neoehrlichia is increasingly being recognized worldwide as a tickborne pathogen. We report a case of symptomatic neoehrlichiosis in an immunocompetent Austria resident who had recently returned from travel in Tanzania. The use of *Anaplasmataceae*-specific PCR to determine the duration of antimicrobial therapy seems reasonable to avert recrudescence.

Human neoehrlichiosis is an infectious disease that primarily affects immunocompromised persons and persons with severe concurrent medical conditions (*1*–*5*). We describe symptomatic *Candidatus* Neoehrlichia infection in an otherwise healthy woman who had returned from a 28-day vacation in Tanzania, and we illustrate the applicability of *Anaplasmataceae*-specific PCR for diagnosis and therapeutic guidance.

The Study

In January 2013, a 30-year-old white woman with no relevant medical history was admitted to the Division of Infectious Diseases and Tropical Medicine, General Hospital of Vienna, in Vienna, Austria, because of a 3-week history of high fevers (up to 39.9°C), chills, and night sweats accompanied by headache, muscle pain, and malaise. Four weeks before hospitalization, the woman had returned from a 28-day vacation in Tanzania. She had not taken antimalarial prophylaxis drugs while in Tanzania; instead she carried atovaquone/proguanil tablets as a standby medication. The woman felt well during the entire stay in Tanzania, so she did not take the atovaquone/proguanil.

Spiking fevers began 5 days after her return to Vienna. She visited the outpatient clinic at the General Hospital of Vienna, where malaria, Dengue virus fever, and typhoid fever were ruled out as causative diseases. During her first days in Tanzania, the woman had voluntary skin contact with a prosimian, but she recalled no recent tick bites or exposures to animals. Blood samples were obtained; multiple cultures were negative. However, over the next 10 days, persistent fever and a deteriorating general condition led to hospitalization for further evaluation. Clinical data at admission and results of primary diagnostic tests are provided in the Table.

During the first days of hospitalization, the woman had fever (up to 39°C) accompanied by general discomfort and headache. Giemsa-stained thick and thin blood smears showed no evidence of malaria. Likewise, serologic and PCR test results for *Plasmodium* spp. were negative.

On hospitalization day 5, a peripheral blood sample was tested by using a 16S rRNA gene–based eubacterial broad range PCR (SepsiTest; Molzym GmbH & Co. KG, Bremen, Germany); results were positive. The amplification products (300 bp) were sequenced (GenBank accession no. KT895260) and compared, using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), with known sequences in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) database. The sequence showed 98% (294/300 bp) homology with *Candidatus* Neoehrlichia lotoris (GenBank accession no. EF633744.1; only 1 database entry was available) and 97% (293/301 bp) homology with *Candidatus* Neoehrlichia mikurensis (GenBank accession no. KF155504.1; several database entries were available and showed a reproducible single base deletion at position 225). These findings were confirmed by *Anaplasmataceae*-specific 16S ribosomal RNA gene–based PCR. Primer pairs EHR16SD (5′-GGT ACC YAC AGA AGA AGT CC-3′) and EHR16SR (5′-TAG CAC TCA TCG TTT ACA GC-3′) were chosen to amplify a 345-bp fragment (*6*). The protocol was adjusted to that in the manual for High-Fidelity PCR enzyme mix (Thermo Scientific, Waltham, MA, USA) and to that of Brown et al. (*7*). Bidirectional sequencing of the 345-bp amplicon showed a sequence of 243 bp corresponding to the cDNA strand (GenBank accession no. KT953340) and yielded similar results: 97% (235/243 bp) sequence homology was shared with *Candidatus* Neoehrlichia lotoris (GenBank accession no. EF633744.1), and 96% (235/244 bp) sequence homology was shared with *Candidatus* Neoehrlichia mikurensis (GenBank accession no. JQ359046.1). Because the percentages of shared homologies were not sufficient to attribute the identified microbial agent to an official species, we tentatively named the agent *Candidatus* Neoehrlichia Tanzania. In addition, a microscopy review of Giemsa-stained blood smears obtained within the first days of admission

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DOI: http://dx.doi.org/10.3201/eid2202.141762

Table. Clinical data at admission and primary diagnostic test results for a patient with *Candidatus* Neoehrlichia infection, Austria, 2013*

Clinical variable	Finding/value
Subjective symptoms	Malaise, diffuse muscle pain, dull headache (without signs of meningism), and tenderness in the left upper abdominal quadrant
Tympanic temperature	37.8°C, while taking acetaminophen
Heart auscultation	Systolic murmur (right sternal border), tachycardia (125 beats per minute)
Condition of skin	No rash or signs of cutaneous exposure to arthropods
Laboratory testing†	
C-reactive protein	5 mg/dL (<0.5)
Procalcitonin	0.14 ng/mL (<0.5)
Leukocyte count	3.9×10^9/L (4–10)
Neutrophils	53% (50–75)
Lymphocytes	27% (25–40)
Monocytes	16% (0–12)
Fibrinogen	480 mg/dL (180–390)
Serum amyloid A	164 mg/L (<5)
γ-globulins	26.2% (11.1–18.8)
Erythrocyte sedimentation rate	70 mm/h (<15)
Platelet count	121×10^9/L (150–350)
Hemoglobin	9 g/dL (12–16)
Chest radiography	No consolidations, no opacities
Abdominal ultrasonography	Splenomegaly of 15.5 x 6.7 cm
Transesophageal echocardiography	Normal cardiac function and valves, no evidence of vegetations
Cranial computed tomography	Parasagittal meningioma, otherwise normal
Ophthalmologic examination	Bilateral papilloedema
Cerebrospinal fluid	Clear and colorless; absolute cell count 4/μL protein, glucose, and lactate levels within reference range
Abdominal ultrasonography	Splenomegaly, 15.5 × 6.7 cm
Urinary dip stick and urinary cultures	No growth
Blood cultures	No growth
Serologic testing	
HIV	Negative
Hepatitis B and C viruses	Negative
Epstein-Barr virus	Negative
Cytomegalovirus	Negative
Mycoplasma spp.	Negative
Adenovirus	Negative
Enterovirus	Negative
Coxsackievirus	Negative
Influenza A,B, and C viruses	Negative
Parainfluenza virus	Negative
Anaplasma spp.	Negative
Rickettsia spp.	Negative
Tuberculous mycobacteria	Negative
Plasmodium spp.	Negative
Syphilis (VDRL, TPPA)	Negative
PCR testing	
Leishmania spp.	Negative
Trypanosoma spp.	Negative
Plasmodium spp.	Negative
Giemsa-stained thin and thick blood smears	
Plasmodium spp.	Negative

*TPPA, *Treponema pallidum* particle agglutination assay; VDRL, Venereal Disease Research Laboratory test.
†Laboratory data are given as absolute number or percentage (reference range).

showed structures possibly equivalent to microbial pathogens within leukocytes (Figure 1).

Antimicrobial treatment with oral doxycycline (300 mg per day) was subsequently initiated, resulting in improvement in the patient's overall condition within 2 days and in a continuous decrease of all inflammation markers, normalization of platelet counts, and abatement of fever (Figure 2). However, serum Neoehrlichia DNA remained detectable at high levels. To provide the optimal duration of antibiotic treatment, we performed daily *Anaplasmataceae*-specific 16S PCR measurements of blood samples. Over the next 10 days of therapy, the DNA signal intensity continuously diminished. Doxycycline was stopped 1 day after disappearance of Neoehrlichia serum DNA.

In contrast with patients in previously published reports of human neoehrlichiosis, the patient described in our report was a healthy young woman without concurrent medical conditions. She had signs and symptoms of disease

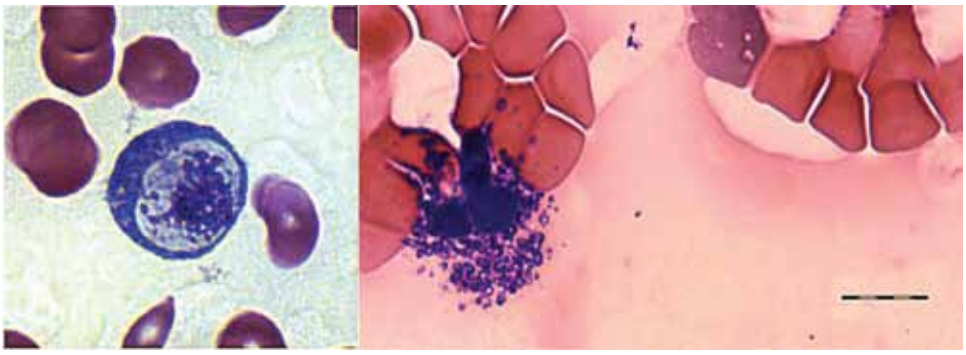

Figure 1. Giemsa-stained blood smear from an immunocompetent patient with *Candidatus* Neoehrlichia infection, Austria, 2013. The blood smear shows possible microbial pathogens within leukocytes. Scale bar indicates 10 μm.

for 4 weeks without any symptomatic improvement before therapy was initiated. Treatment led to a rapid clinical response and rapid clearance of serum Neoehrlichia DNA, which may be attributable to her otherwise good medical condition but may also reflect high antimicrobial efficiency of the high-dose therapeutic regimen applied.

Because symptomatic neoehrlichiosis usually occurs in patients with immunosuppression, we examined the patient for an underlying malignancy or autoimmune disorder. These conditions were largely ruled out by negative test results for HIV and mycobacteria and by a normal finding on 18F-FDG-PET/CT (18F-fluordeoxyglucose-positron emission tomography/computed tomography) examination (except for enhanced splenic FDG uptake). The patient had moderate disease with nonspecific symptoms partly resembling those of human anaplasmosis. The splenomegaly was attributed to polyclonal B cell activity (indicated by hypergammaglobulinemia), but it could also have resulted from direct infection of splenic sinusoidal cells, as found in Neoehrlichia-infected Wistar rats (*8*). However, spleen size decreased over the course of antimicrobial treatment and reached a normal diameter by a 3-week follow-up examination.

No evidence exists regarding the exact incubation period of human neoehrlichiosis, but it probably approximates that of human granulocytic anaplasmosis, suggesting that the patient in our study acquired neoehrlichiosis in Tanzania. Nonetheless, several tickborne diseases are highly endemic in Austria. Glatz et al. (*9*) recently reported a 4.2% prevalence of *Candidatus* Neoehrlichia in *Ixodes ricinus* ticks in Austria. However, in the 5-day period between returning home from Tanzania and fever onset, the patient in our study had stayed in the urban area of Vienna; thus, the possibility that she may have been

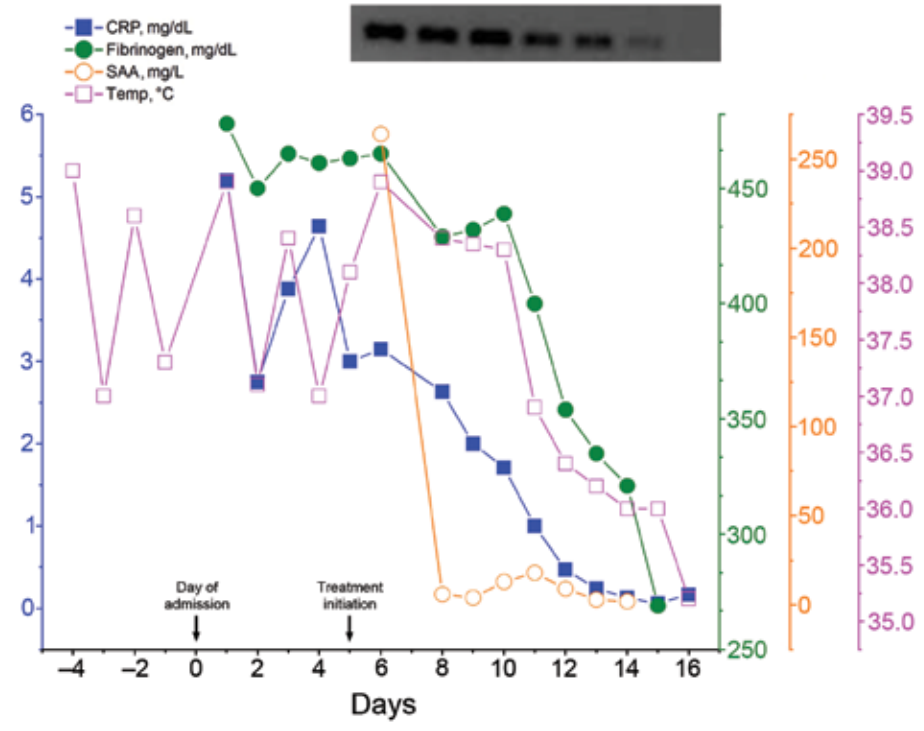

Figure 2. Body temperature and markers of inflammation over the course of hospitalization for a patient with *Candidatus* Neoehrlichia infection, Austria, 2013. Day 0 indicates time of admission. Antimicrobial therapy with doxycycline (300 mg per day) was begun on day 5 and led to a rapid resolution of clinical symptoms and a progressive decrease of all inflammatory parameters. Daily *Anaplasmataceae*-specific PCR measurements guided therapy, which was safely stopped 1 day after disappearance of serum *Candidatus* Neoehrlichia DNA. Upper right shows 1.5% agarose gel electrophoresis analysis. The intensity of the 345-bp DNA band amplified from blood samples progressively decreased over the course of treatment. CRP, C-reactive protein; SAA, serum amyloid A; Temp, tympanic temperature.

exposed to ticks in Austria is limited but not excluded. Furthermore, the patient returned to Vienna at the height of winter, making the possible transmission of *Candidatus* Neoehrlichia by a domestic tick even less plausible. On the other hand, no epidemiologic data are available on the prevalence of *Candidatus* Neoehrlichia in ticks in Tanzania, but *Candidatus* Neoehrlichia mikurensis was recently found in ticks of 2 species collected in Nigeria (*10*). Thus, the presence of *Candidatus* Neoehrlichia in ticks in Tanzania and the risk for transmission from ticks to humans seem conceivable. Because a 16S rDNA sequence difference of >2% is arbitrarily considered as indicative for delineation at the species level, it seems possible that a new *Candidatus* Neoehrlichia agent was detected in the patient in our study.

Conclusions

This case demonstrates that *Candidatus* Neoehrlichia can affect healthy persons who have no underlying hematologic or autoimmune disorders. Neoehrlichiosis should be considered in the differential diagnosis for patients with appropriate symptoms, independent of concurrent conditions and immune status. As long as no evidence-based recommendations regarding treatment of human neoehrlichiosis exist, it seems reasonable to use *Anaplasmataceae*-specific PCR to monitor treatment response and determine the duration of antimicrobial therapy to avert recrudescence.

Acknowledgments

We thank the patient in our study for giving consent to publish the data, Wolfgang Barousch for providing assistance with GenBank, and Albert Lalremruata for performing the *Plasmodium* spp. PCR.

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Letters

Letters commenting on recent articles as well as letters reporting cases, outbreaks, or original research are welcome. Letters commenting on articles should contain no more than 300 words and 5 references; they are more likely to be published if submitted within 4 weeks of the original article's publication. Letters reporting cases, outbreaks, or original research should contain no more than 800 words and 10 references. They may have 1 Figure or Table and should not be divided into sections. All letters should contain material not previously published and include a word count.

Candidatus Coxiella massiliensis Infection

Emmanouil Angelakis, Oleg Mediannikov, Sarah-Lyne Jos, Jean-Michel Berenger, Philippe Parola, Didier Raoult

Bacteria genetically related to *Coxiella burnetii* have been found in ticks. Using molecular techniques, we detected *Coxiella*-like bacteria, here named *Candidatus* Coxiella massiliensis, in skin biopsy samples and ticks removed from patients with an eschar. This organism may be a common agent of scalp eschar and neck lymphadenopathy after tick bite.

Only 1 species of bacteria in the genus *Coxiella* is officially recognized: *Coxiella burnetii* (*1*). However, in recent decades, many genetically related bacteria have been found in hard and soft ticks (*2*). These *Coxiella*-like bacteria genetically cluster with *C. burnetii* and share some degree of identity with these bacteria but not enough to be considered the same species (*2*,*3*). Genotypes of *Coxiella*-like bacteria vary among ticks of different species (*3*); however, bacteria with different genotypes have not been isolated, and whether there is a tick reservoir is not known. *Coxiella*-like bacteria have been associated with infection in birds (*4*,*5*). To explore pathogenicity to humans, we used molecular techniques targeting *Coxiella*-like bacteria to retrospectively analyze skin biopsy samples and ticks collected from patients with eschars. We also evaluated serologic tests for *Candidatus* Coxiella massiliensis diagnosis.

The Study

During 2011–2014, we identified patients in hospitals throughout France, who had eschars after tick bite. We retrospectively tested skin biopsy or swab samples of the eschars, serum samples when possible, and ticks from the patients. Ticks were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonics, Billerica, USA) (*6*). All human samples and ticks were tested for *Rickettsia*, *Borrelia*, *Bartonella*, and *Ehrlichia* spp.; *Francisella tularensis*; *Staphylococcus aureus*; and *Coxiella burnetii* by quantitative PCR (qPCR) (*2*,*7*). On the basis of the aligned *rrs* gene sequences of *Coxiella*-like bacteria, we developed a specific qPCR to detect the DNA of all *Coxiella* species and degenerated primers aimed to amplify a 659-bps long portion of the *groEL* gene of *Coxiella* spp. (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/15-0106-Techapp1.pdf). Skin biopsy samples were also tested by universal eubacteria 16S rRNA gene amplification and sequencing (*7*).

Five spawns of *Rhipicephalus sanguineus* ticks infected with *Candidatus* C. massiliensis were used for antigen production (*8*). Molecular assays indicated that these spawns were negative for *Rickettsia*, *Borrelia*, *Bartonella*, *Ehrlichia* spp.; *F. tularensis*; *S. aureus*; and *C. burnetii*. We used spawns of *R. sanguineus* ticks without *Candidatus* C. massiliensis infection as negative controls to confirm that their antigens did not react with serum from the patients. To confirm the presence of *Candidatus* C. massiliensis, we used qPCR and transmission electron microscopy to visualize the bacteria (online Technical Appendix Figure). To determine the specificity of our immunofluorescence assay (IFA), we used healthy blood donors as negative controls; to determine if there was cross-reactivity with *C. burnetii*, we used serum from patients with Q fever.

Patients were considered infected with *Coxiella*-like bacteria when a skin biopsy sample was positive by qPCR and there was no evidence of infection with another agent. Patients were considered possibly infected if they had clinical signs (fever, skin eschar, local lymph node enlargement) and if a removed tick was positive for *Coxiella*-like bacteria according to qPCR but no skin biopsy was sampled or when serologic results were positive. For data comparison, we used Epi Info 6.0 (https://wwwn.cdc.gov/epiinfo/html/ei6_downloads.htm). We considered a p value of 0.05 to be significant.

Phylogenetic analysis based on *groEL* confirmed that *Coxiella* spp. from ticks of different species are genetically very distant (*9*). On the basis of phylogenetic clustering, epidemiologic role, and the fact that we used its antigens for the diagnostic of human infection, we propose the name for the *Coxiella*-like bacteria associated with *R. sanguineus*, *R. turanicus*, and *H. pusillus* ticks to be *Candidatus* C. massiliensis (Figure 1).

A total of 57 ticks removed from 55 patients were available for testing. Of these, 20 (35%) ticks from 19 patients were infected with *Coxiella*-like bacteria only: 11 (55%) *Dermacentor marginatus*, 7 (35%) *R. sanguineus*, 1 (5%) *R. bursa*, and 1 (5%) *Ixodes ricinus* ticks. *Coxiella*-like bacteria were found significantly less commonly in *I. ricinus* ticks ($p = 0.002$, relative risk = 0.5).

We tested convalescent-phase serum from 5 patients. Total immunoglobulin titers of 1:400 against *Candidatus* C. massiliensis were detected for 1 patient and >1:800 for 2 patients (Figure 2). All IgG titers obtained were identical. These results indicated an infection caused by *Candidatus*

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DOI: http://dx.doi.org/10.3201/eid2202.150106

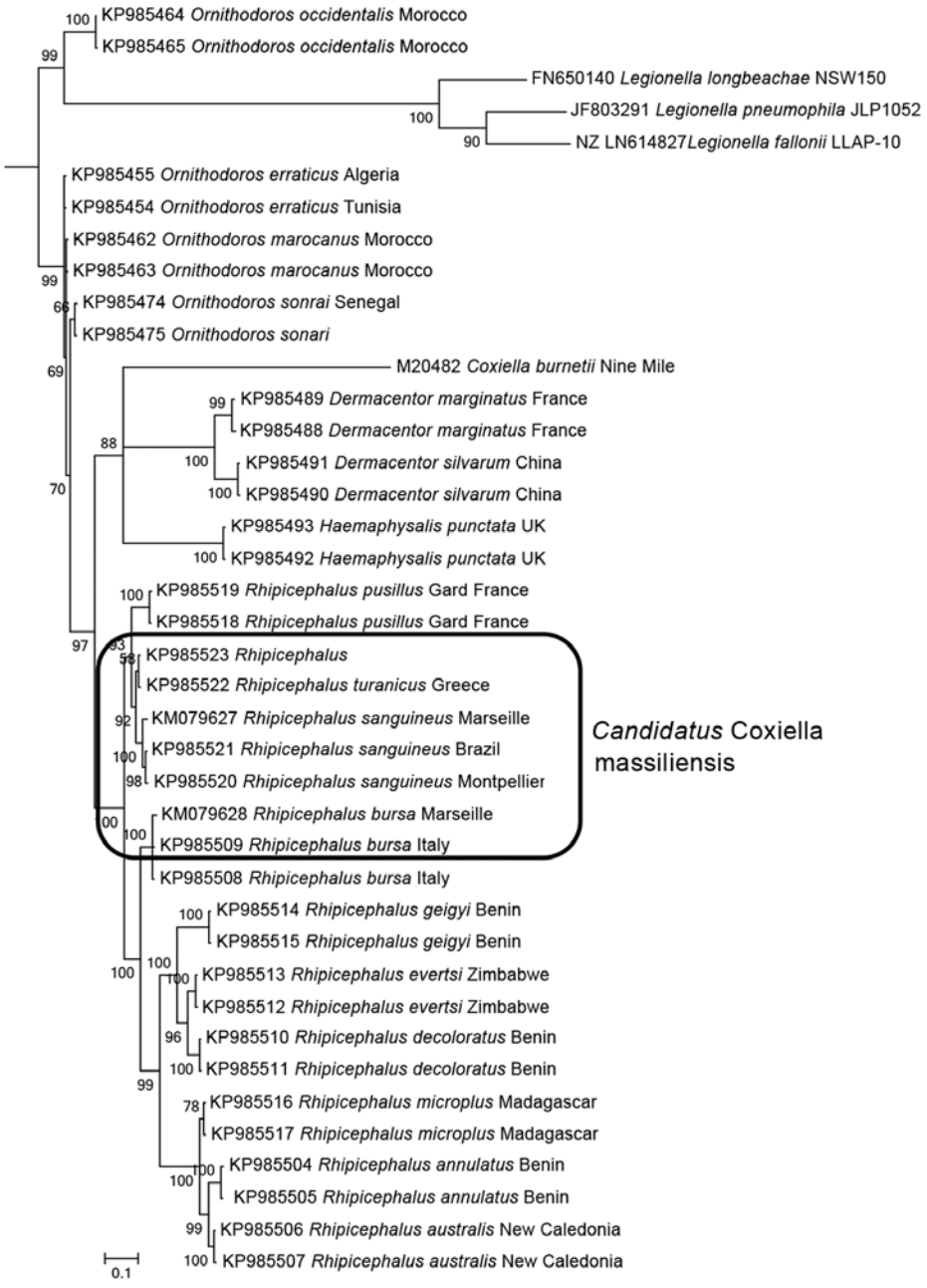

Figure 1. Phylogenetic tree based on *GroEL* sequences including *Coxiella*-like strains of bacteria from ticks, *Coxiella burnetii* reference strains, and bacterial outgroups. *GroEL* gene sequences (online Technical Appendix Table 2, http://wwwnc.cdc.gov/EID/article/22/2/15-0106-Techapp1.pdf) were aligned by using ClustalW (http://www.ebi.ac.uk/Tools/msa/), and phylogenetic inferences were obtained by using Bayesian phylogenetic analysis with TOPALi 2.5 software (http:// www.topali.org/) and the integrated MrBayes (http://mrbayes.sourceforge.net/) application with the HKY+Γ (Hasegawa-Kishino-Yano plus gamma) substitution model for the first and third codons and the JC model for the second codon. GenBank accession numbers are indicated first, followed by the tick host. Numbers at nodes are bootstrap values obtained by repeating the analysis 100 times to generate a majority consensus tree.The final dataset contained 576 positions. Scale bar indicates 10% nucleotide sequence divergence.

C. massiliensis. IFA results indicated that all patients were negative for *Rickettsia* spp., *C. burnetii*, *F. tularensis*, and *Bartonella* spp. Among serum samples from 40 blood donors, total immunoglobulins titer was 1:200 for 6 donors and IgG titer was 1:400 for 1. Receiver operating characteristic curves, defined by the true-positive rate (serum from patients infected with *Candidatus* C. massiliensis) as a function of the false-positive rate (serum from blood donors) demonstrated that for a total immunoglobulin cutoff of >1:400, sensitivity was 60% and specificity was 100%, and for an IgG cutoff of >1:400, sensitivity was 60% and specificity was 98%. Moreover, among 13 patients with acute Q fever, 12 with Q fever endocarditis, and 5 who had had Q fever in the past, serum was positive for *Candidatus* C. massiliensis, indicating the cross-reactivity of our IFA with *C. burnetii*.

Of 465 skin biopsy samples from 465 patients, cell culture results (*10*) were negative for all. However, samples from 8 (2%) patients were positive for *Candidatus* C. massiliensis, and a possible infection was considered for another 7. Of these 15 patients, 8 (53%) were female, 8 had recently participated in outdoor activities in France, and 2 had recently traveled to Algeria and Israel. Three cases occurred during winter, 9 during spring, and 4 during

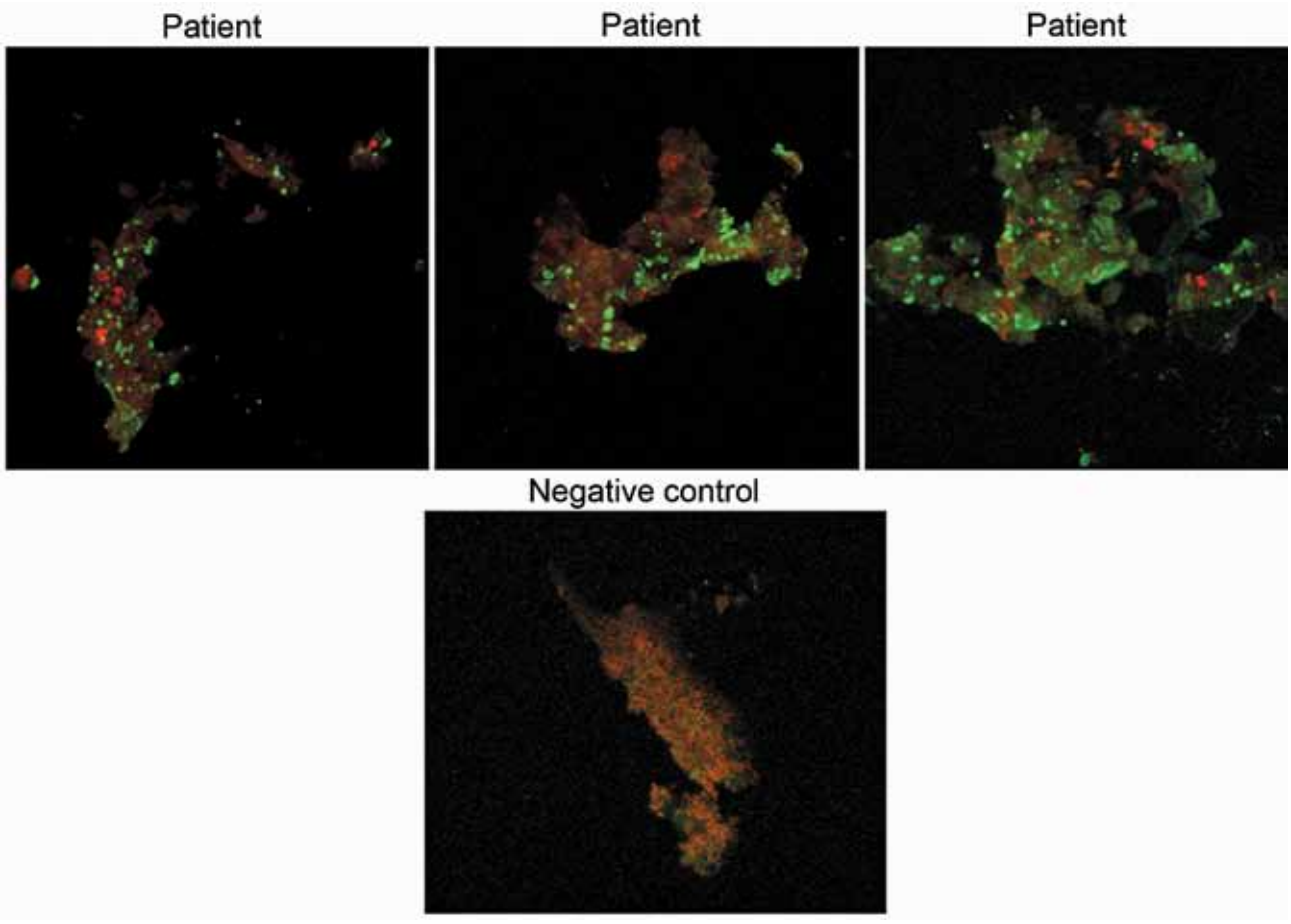

Figure 2. Immunofluorescence assay results of samples from 3 *Candidatus* Coxiella massiliensis–infected patients and 1 noninfected person (negative control). Original magnification ×63. Original magnification ×630.

summer. All patients had an eschar, regardless whether they had lymphadenopathy (Table). A scalp eschar with cervical lymphadenopathy was common (40%). Other common findings were fever (40%), increased C-reactive protein (60%), and thrombocytopenia (40%). Most patients received oral doxycycline, 2 with macrolides and 2 with a β-lactam. Symptoms resolved for all patients.

Conclusions

We determined that *Candidatus* C. massiliensis is an etiologic agent of human infections. For our molecular assay, we routinely included large numbers of negative controls that were processed identically to the test samples. Only *Coxiella*-like bacteria DNA was present in the samples, indicating that these bacteria were the only infectious agents. Supplementary serologic testing was used to confirm the molecular results, and results were validated with samples from blood donors; specificity was good but sensitivity was low. The fact that all serum tested demonstrated IgG only does not eliminate the possibility of previous exposure (patients and blood donors) because these bacteria are common in ticks (*9*). Although *Candidatus* C. massiliensis IFA results were cross-reactive with *C. burnetii*, all patients were negative for *C. burnetii*.

A study limitation was that our assays were specific for *Candidatus* C. massiliensis only. In addition, many of the ticks that bit the patients were not available for examination, and the level of serologic cross-reactivity among *Candidatus* C. massiliensis and other *Coxiella*-like bacteria is unknown. Thus, the patients reported here may have been infected not exclusively with *Candidatus* C. massiliensis but also with another *Coxiella* species associated with ticks.

Most patients had a scalp eschar and cervical lymphadenopathy, reminiscent of a recently proposed clinical entity named SENLAT (scalp eschar and neck lymphadenopathy after tick bite) (*11*). All symptoms arising from *Candidatus* C. massiliensis infection can be easily attributed to other infectious agents transmitted by ticks (*12*,*13*) and can easily go unrecognized because of the absence of systematic research on these bacteria and the nonspecific clinical manifestations of diseases caused by them. Most patients in our study were empirically prescribed

Table. Characteristics of *Candidatus* Coxiella massiliensis–infected patients, France, 2011–2014

Characteristic	No. (%) cases
Male sex	7 (47)
Tick species collected from patient	
Dermacentor marginatus	6 (55)
Rhipicephalus sanguineus	3 (27)
Ixodes ricinus	1 (9)
Rhipicephalus bursa	1 (9)
Not collected	4
Eschar location	
Scalp	8 (53)
Leg	3 (20)
Shoulder	2 (13)
Back	1 (7)
Ear	1 (7)
Fever	6 (40)
Lymphadenopathy	
Cervical	6 (55)
Axillary	1 (9)
Inguinal	2 (18)
Thrombocytopenia	6 (40)
Increased liver enzyme levels	2 (13)
Increased C-reactive protein level	9 (60)
Treatment	
Doxycycline	11 (73)
Oher	4 (27)

doxycycline, the treatment of choice for many tick-transmitted infections (*14*). We illustrated the value of systematically testing for *Candidatus* C. massiliensis in skin biopsy samples. *Candidatus* C. massiliensis may be a common agent of SENLAT, which may go unrecognized or misdiagnosed as a sign of another tick-transmitted infection.

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Ebola Virus Persistence in Semen Ex Vivo

Robert J. Fischer, Seth Judson, Kerri Miazgowicz, Trent Bushmaker, Vincent J. Munster

On March 20, 2015, a case of Ebola virus disease was identified in Liberia that most likely was transmitted through sexual contact. We assessed the efficiency of detecting Ebola virus in semen samples by molecular diagnostics and the stability of Ebola virus in ex vivo semen under simulated tropical conditions.

On March 20, 2015, an isolated Ebola virus disease (EVD) case was diagnosed in Liberia, 30 days after confirmation of the previous EVD case (the incubation period for Ebola virus [EBOV] infection is 4–21 days). The patient had no history of travel to areas with reported EVD, no interaction with visitors from Sierra Leone or Guinea, no funeral attendance, and no contact with a patient with EVD symptoms (*1*).

The patient, a 44-year-old woman, reportedly had unprotected sex with a male survivor of EVD (*1*). His semen was positive for EBOV by real-time quantitative reverse transcription PCR (qRT-PCR) 199 days after symptom onset; his cycle threshold (C_t) value was 32, seven days after EBOV was confirmed in the woman (*1*).

Among the criteria for declaring an end to the Ebola outbreak in West Africa, the World Health Organization includes testing of semen of convalescent men until 2 samples are negative (*2*). Most of these specimens will be analyzed by qRT-PCR. Therefore, during May–September 2015, we analyzed the stability of EBOV in semen by qRT-PCR and titration. Because most of the EVD diagnostic laboratories are more familiar with values obtained from blood, we compared standard curves of C_t values with the 50% tissue culture infectious dose ($TCID_{50}$) per mL in semen, blood, and tissue culture medium.

The Study

Human semen and blood were obtained from Lee Biosolutions (St. Louis, MO, USA). All assays were consistent with the procedures used at the Centers for Disease Control and Prevention/National Institutes of Health laboratory at the Eternal Love Winning Africa campus in Monrovia, Liberia, to diagnose EVD in the 44-year-old woman. RNA was extracted by using a QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA) following the manufacturer's protocol, with an additional wash step of wash buffer 1. qRT-PCR was conducted by using Roche LightCycler 480 RNA Master Hydrolysis Probes (Roche, Indianapolis IN, USA) reagents with primers and probes targeting the L gene of EBOV on the SmartCycler (Cepheid, Sunnyvale, CA, USA) platform (*3*).

Ebolavirus/H.sapiens-tc/GUI/2014/Makona-WPGC07 was used in all experiments. To enable comparison among the C_t values of EBOV in semen with samples routinely analyzed during the current outbreak, we constructed standard curves of EBOV in semen, blood, and medium. Matrices were spiked to 10^6 $TCID_{50}$/mL, then serially diluted 10 times. Five biologic replicates were used to construct the curves (Figure 1, panel A). The dynamic range of the assay extends down to 10° for blood and semen. The PCR efficiency determined from the slope of the standard curve was nearly 100% for each of the matrices; the C_t value was 1.2 times higher on average for semen than for blood.

We tested the stability of EBOV in human semen in the liquid (bulk) and dry states during an 8-day period (27°C, 80% relative humidity [RH]). EBOV was diluted in triplicate in semen to 1×10^6 and 1×10^3 $TCID_{50}$/mL; 50-μL aliquots of semen were removed daily and placed into 450 μL of DMEM. Additional aliquots were obtained for qRT-PCR. To assess the stability in dried semen, 50 μL of spiked semen was spread onto the bottom of each well of a 24-well plate and recovered by resuspending in 500 μL of DMEM. To assess the viability of EBOV in condoms, 700 μL of semen spiked with 1×10^3 $TCID_{50}$/mL EBOV was placed in condoms (Durex Extra Sensitive; Reckitt Benckiser Group, Slough, UK) in triplicate, stored at 27°C and 80% RH, and sampled on alternate days. All samples were stored at –80°C until titration. We previously had determined no significant effect on EBOV titers by a single freeze/thaw step (*4*).

Titrations were performed on Vero E6 cells in a 48-well format. $TCID_{50}$ per milliliter was calculated by using the Spearman-Karber method (*4*,*5*). Statistical analysis were performed with GraphPad 6.05 (GraphPad Software, San Diego, CA, USA).

Standard curves for EBOV in semen and blood did not significantly differ (analysis of covariance, $p = 0.8965$) between the slopes of the standard, indicating that the PCR efficiency is similar between the 3 matrices; however, differences in C_t value between semen and blood were significant (analysis of covariance, $p<0.0001$) (Figure 1, panel A) (*6*). No linear correlation was observed between the C_t value and the dilution factor, together with the additional

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DOI: http://dx.doi.org/10.3201/eid2202.151278

wash step used during the extraction procedure; this finding suggests that the differences were not due to presence of inhibitors but rather to the efficiency of extraction (Figure 1, panel B). The decrease in extraction efficiency could be related to the nonhomogeneous nature of semen. SE within undiluted samples was larger in semen than in blood (Figure 1, panel B). This finding was confirmed by similar SE variation for the housekeeping gene B2M (semen C_t value 28.34 ± 3.35 and blood C_t value 20.78 ± 0.81).

We tested he stability of EBOV in dry and bulk semen for 8 days under tropical conditions (27°C, 80% RH). Under bulk conditions, EBOV was viable for all 8 days at 1×10^6, but at 1×10^3, viable virus was recovered only to day 6. EBOV viability was greatly reduced in dried semen: viable virus was detected to days 4 and 1 at 1×10^6 and 1×10^3, respectively (Figure 2). Viable virus was recovered from semen spiked with 1×10^3 $TCID_{50}$/mL EBOV stored in condoms to day 6, whereas the C_t value remained stable throughout the experiment. This finding highlights the importance of the proper disposal of condoms used by EVD

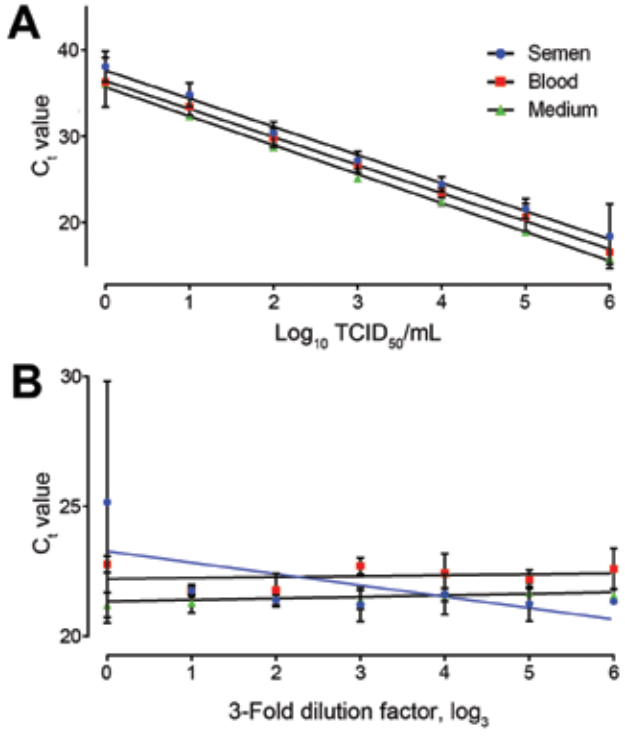

Figure 1. A) Standard curves of Ebola virus spiked into 3 matrices: semen, blood, and tissue culture medium. Samples were analyzed on the basis of 5 biologic replicates. PCR efficiency was from 98% in cell culture medium, 102% in semen, and 103% in blood. Analysis of covariance showed no significant difference ($p<0.05$) between the slopes of the linear regressions of blood and semen. B) Matrix dilution in which semen, blood, and tissue culture medium were 3-fold serially diluted in sterile physiologic saline solution and spiked with 10^4 $TCID_{50}$/mL Ebola virus. The slopes for blood and medium did not differ significantly from 0. If the semen sample data are analyzed disregarding the undiluted sample, the resulting slope also does not differ significantly from 0. C_t, cycle threshold; $TCID_{50}$, 50% tissue culture infectious dose. Error bars represent the mean ± SEM of 5 quantitative PCR analyses.

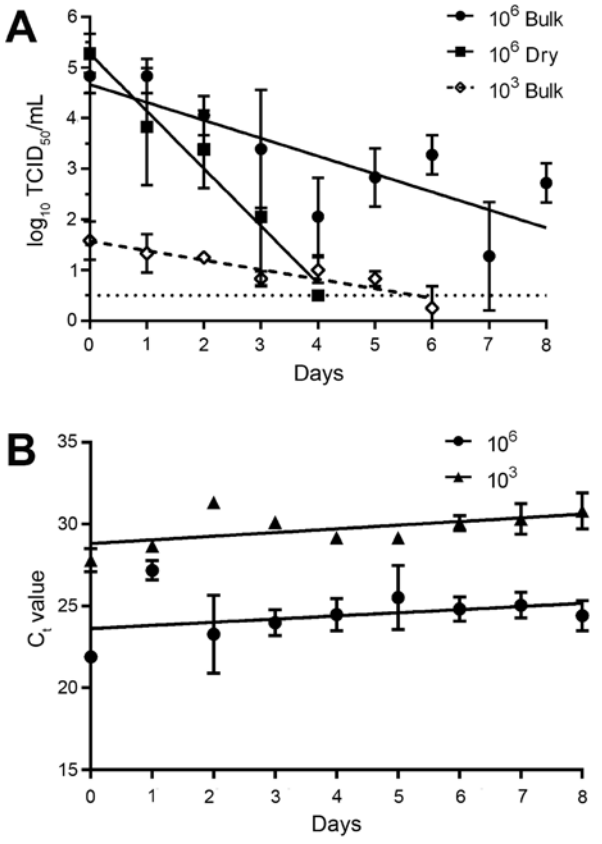

Figure 2. Linear regression model showing stability of Ebola virus (EBOV) and EBOV RNA in semen at 27°C and 80% relative humidity over 8 days. A) EBOV in bulk (liquid) semen versus dry semen at initial titers of 106 $TCID_{50}$/mL and 103 $TCID_{50}$/mL. The higher titer 1 × 106 $TCID_{50}$/mL was used to provide a comparison with EBOV in blood, and the lower titer 1×10^3 $TCID_{50}$/mL was derived from Ct values reported in semen samples. Viable virus was reduced significantly faster ($p<0.0001$) in dry semen than in bulk semen. The goodness-of-fit for the linear regression represented as the r^2 value is 0.53 for bulk semen and 0.82 for dry semen with an initial titer of 106 $TCID_{50}$/mL, respectively, and 0.65 for bulk semen with an initial titer of 103 $TCID_{50}$/mL. No curve is shown for the initial titer 10^3 $TCID_{50}$/mL in the dry semen because no viable virus was recovered after day 1. The titer on day 1 was 1.1 log10 $TCID_{50}$/mL. In all cases except the high-titer bulk semen sample, the final data point was followed by 2 consecutive days of no recovered virus. B) C_t values produced by analysis of bulk semen samples analyzed by real-time quantitative reverse transcription PCR. The data did not fit a linear regression model ($r^2 = 0.08964$), but the RNA clearly remained stable during the entire experiment. Three biologic replicates were analyzed at each time point. Error bars represent mean ± SEM virus titer. Dashed line indicates the limit of detection for the titration assay. An analysis of covariance was used to compare linear regression models and determine differences in virus reduction rates. C_t, cycle threshold; $TCID_{50}$, 50% tissue culture infectious dose.

convalescent men. EBOV RNA was detectable in semen for all 8 days with no decrease in C_t values, suggesting that RNA can be detected in semen samples obtained from convalescent men over an extended time, even if the cold chain is interrupted.

Before the EVD case in the woman reported here, EBOV was known to persist for an extended period in semen (*7*). In November 2014, a man returning to India from Liberia after recovery from EVD produced a positive semen sample while testing negative for EBOV in blood, saliva, and urine by qRT-PCR (*8*,*9*). Isolation of EBOV from semen samples collected during prior outbreaks has been reported. In 1 case, EBOV was isolated from the semen of a convalescent patient 82 days after symptom onset (*10*). EBOV has been detected at 101 days from symptom onset by qRT-PCR (*11*). Sexual transmission has been implicated in Marburg virus infection, but until now only equivocal evidence existed of EBOV transmission through sexual contact (*12*,*13*).

Our study has several limitations. First is our use of semen spiked with EBOV rather than naturally infected by EBOV. If EBOV is cell associated in a natural infection, the results of the experiment might be altered, although no difference was observed with blood previously (*4*). Second, the starting titers of EBOV in the semen might not represent naturally occurring levels of viable virus in semen because reported C_t values cannot be inferred toward viable virus or infectiousness. Third, the absolute C_t values presented directly apply to the instruments and reagents used in these experiments. Other systems might yield different C_t values and PCR efficiencies.

Conclusions

Because of the potential for sexual transmission, the World Health Organization and Centers for Disease Control and Prevention have recommended measures to prevent transmission by sexual contact, including semen screening for survivors and use of condoms (*14*,*15*) and safe handling and disposal of condoms (*2*). In a region where organized waste management is almost nonexistent and availability of condoms is limited, to the extent that persons may wash and reuse condoms, this recommendation might not be strictly adhered to. The prolonged viability of EBOV in semen ex vivo supports the WHO recommendation for safe handling and disposal of condoms (*2*).

Acknowledgments

We thank Anita Mora and the Visual Medical Arts Section at Rocky Mountain Laboratories for assistance with the figures.

This work was supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Ebola Virus RNA Stability in Human Blood and Urine in West Africa's Environmental Conditions

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We evaluated RNA stability of Ebola virus in EDTA blood and urine samples collected from infected patients and stored in West Africa's environmental conditions. In blood, RNA was stable for at least 18 days when initial cycle threshold values were <30, but in urine, RNA degradation occurred more quickly.

Real-time reverse transcription PCR (rRT-PCR) has become the standard diagnostic tool for patients infected by Ebola virus (EBOV) (*1,2*). Control of the current outbreak and proper management of patients cannot be achieved without laboratory testing. In the field in West Africa and notably in Guinea, most Ebola treatment units are located near (often in the same compound) a laboratory that can process collected samples within hours. However, most healthcare centers or dispensaries lack this diagnostic capability and cannot ensure rapid testing of samples from patients suspected of having Ebola.

Few laboratory studies have investigated Ebola virus RNA stability over time in collected samples. The period of stability has been estimated to be 5–14 days for virus suspensions on solid surfaces kept in darkness or for virus-spiked human blood or naturally infected, nonhuman primate blood stored under simulated tropical conditions (*3–5*). A recently published study found that viral RNA was consistently detectable in blood of cynomolgus macaques until 3 weeks after euthanasia (*6*). According to an anecdotal report, Ebola virus was isolated in blood samples stored for a month at room temperature (P.E. Rollin, pers. comm.). Such reports of sample stability must be considered for organizing collection and movement of samples in the field. In addition, quantification (copies/mL) or cycle threshold (C_t) determination of EBOV RNA for estimating viral load has been shown to be a major prognosis marker in affected patients (*1,7,8*). C_t value, which can be used to perform RNA semiquantification, is not an absolute quantification, which necessitates use of a calibration curve. Moreover, C_t value does not reflect viral viability but only presence of RNA in samples. By using C_t determination, we attempted to assess the stability of EBOV RNA in EDTA plasma (widely used in the field) and in urine from infected patients in the environmental conditions (i.e., air temperature and humidity) of West Africa.

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DOI: http://dx.doi.org/10.3201/eid2202.151395

The Study

Our study was conducted in the Laboratory of the Centre de Traitement des Soignants in Conakry, Guinea, during the Ebola outbreak in Guinea during January–March 2015. Establishing a Biosafety Level 4 (BSL-4) laboratory in Guinea was not possible. To enable biologists to work safely, a BSL-3 laboratory with a class-3 biologic-safety cabinet and single-use personal protective equipment were used (*9*).

We measured EBOV RNA stability in blood and urine samples from 7 case-patients with laboratory-confirmed Ebola. Twelve blood samples from the 7 case-patients were obtained by venipuncture by using Vacutainer tubes containing EDTA (BD Vacutainer; Becton Dickinson, Franklin Lakes, NJ, USA). Thirteen urine samples from the 7 case-patients were collected in individual sterile receptacles (BD Vacutainer Urine Collection Cup; Becton Dickinson).

Samples were immediately transferred to the laboratory. Upon arrival, samples were processed by centrifugation (at 4,000 rpm for 15 min) and stored at room temperature (22°C–29°C; 50%–80% humidity) for 18–30 days in a BSL-3 laboratory. At regular intervals (i.e., generally every other day), viral RNA was extracted from 100 µL of undiluted initial plasma or urine (QIAmp Viral RNA Mini Kit; QIAGEN, Valencia, CA, USA). To manage the limited volume of initial sample, our protocol used 100 µL of sample, less than manufacturer's recommendations of 140 µL. We therefore modified the volume of lysis buffer (Buffer AVL; QIAGEN) to 400 µL to maintain the manufacturer's recommended ratio of 1:4. We performed 40 cycles of rRT-PCR tests for detecting EBOV by using a commercially available kit (RealStar Filovirus Screen RT-PCR Kit 1.0; Altona Diagnostic GmbHh, Hamburg, Germany) (*10*).

Results were expressed as C_t values, which are inversely proportional to the quantity of viral RNA in samples.

For each sample, extraction control and amplification of the positive control gave expected values; no inhibition of amplification occurred. At admission, all 7 case-patients had blood C_t values <30.0 (mean 21.0, 95% CI 19.5–22.5). For the 12 blood samples studied (Figure, panel A), C_t values ranged from 18.3 to 35.0. When the initial C_t value was <30.0, C_t values were stable for up to 18 days after collection (day 0 mean 20.95, 95% CI 19.1–22.7; day 18 mean 21.9, 95% CI 19.9–23.8). For 2 samples with initial C_t values of 24.0 and 25.0, RNA was undetectable by days 28 and 22, respectively. For 3 samples collected from convalescent patients with C_t values ranging from 30.0 to 32.0, RNA was detected until day 4. For samples with C_t >32.0, RNA was detected on the initial test only.

In the 13 urine samples (Figure, panel B), initial C_t values had a range of 18.2–35.5 (mean 28.6, 95% CI 25.7–31.5). RNA in urine was undetectable by days 10 and 14 for initial C_t counts of 19.0 and 18.0, respectively. For equivalent C_t values for blood, RNA was detectable for at least 18 days.

For urine samples with initial C_t values >30.0, rRT-PCR results were negative within 2–4 days. However, urine results were inconsistent; 3 urine samples from the same patient had initial C_t values of 27.0–28.0 and were detectable for 24–30 days.

These results from human samples taken during the Ebola outbreak in Guinea are similar to reported data for postmortem samples from cynomolgus macaques; for those samples, RNA was detectable for a few weeks after death (*6*). Our results indicate that EBOV RNA is stable in EDTA plasma samples collected and tested in the environmental conditions of West Africa. In the early phase of Ebola disease, blood sampling is probably more sensitive and reliable than oral swabbing and should be used whenever possible (*11,12*). Plasma samples can be tested for up to 18 days after collection, even if stored at ambient temperature, and positive results can still be reported. However, negative results may be false negative (in case of samples with low viral load) and should be reported as invalid; retesting should be performed on a new blood sample.

Urine does not seem useful for initial diagnosis of Ebola disease. Even if collection is easy, detectable viral RNA appears in the urine of infected patients at a later stage of the disease than in blood, and C_t values are higher (*13,14*). The presence of protease, RNase, or bacteria and the absence of proteins that stabilize the virus and RNA in urine may explain the rapid degradation of viral RNA and thus the limited usefulness of this type of biologic sample.

This study is subject to limitations. First, our analysis was performed with samples that were centrifuged (i.e., plasma), according to manufacturer's instructions and our laboratory protocol. No analysis was performed with noncentrifuged blood. For samples collected in facilities with no laboratory nearby, centrifugation before transport would not be possible and stability of viral RNA in these samples could have slightly different results than those found in this study. In such scenarios, for accurate comparisons of the stability of viral RNA over time, all tests would need to be performed on noncentrifuged blood, not on plasma. Second, the limited volume of collected samples did not enable us to perform tests in duplicate or at a frequency of every other day for 30 days for every sample.

Conclusions

Our study was performed in real conditions (i.e., with samples from infected patients during the Ebola outbreak in Guinea in an Ebola treatment center laboratory there) and

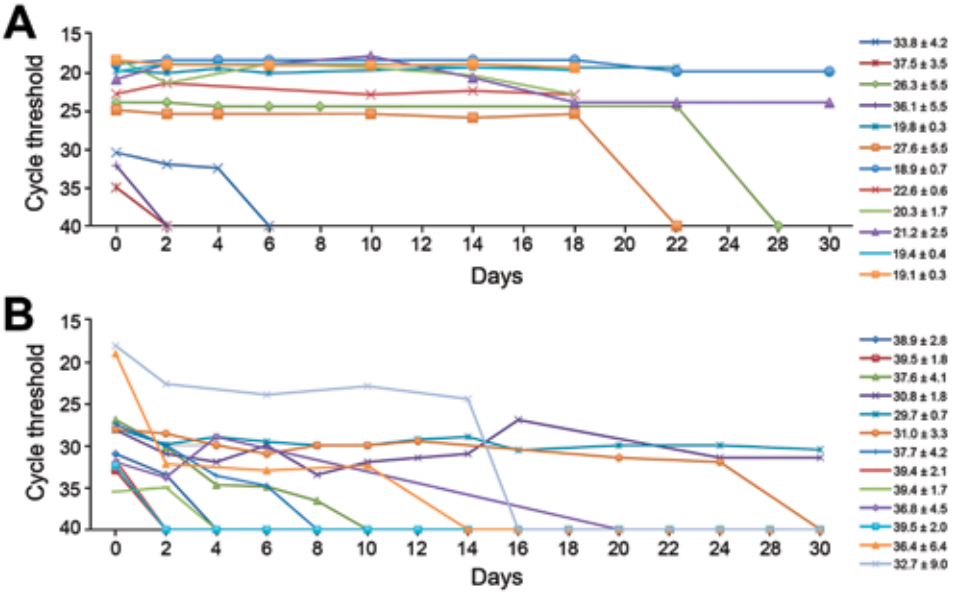

Figure. Stability of Ebola virus RNA in A) EDTA plasma and B) urine samples from patients in Guinea, as measured by real-time reverse transcription PCR. EDTA plasma and urine were processed immediately after receipt at a laboratory and left at room temperature (22°C–29°C; 50%–80% relative humidity) for various periods before the PCR analysis. Average cycle threshold values ± SD for individual samples are shown.

shows that EBOV RNA is stable in EDTA plasma. The development of practices for safe collection in the field and transport within a few hours or days to a local or national reference laboratory equipped with rRT-PCR capabilities seems feasible and will offer a more sustainable strategy for laboratory diagnosis and surveillance during and after the current outbreak.

Acknowledgments

We thank Julie Top, Lucie Queval, Cécile Vigier, and Sophie Gorbatch for laboratory support.

This study was supported by the French Military Health Service.

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Uveitis and Systemic Inflammatory Markers in Convalescent Phase of Ebola Virus Disease

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We report a case of probable Zaire Ebola virus–related ophthalmologic complications in a physician from the United States who contracted Ebola virus disease in Liberia. Uveitis, immune activation, and nonspecific increase in antibody titers developed during convalescence. This case highlights immune phenomena that could complicate management of Ebola virus disease–related uveitis during convalescence.

The Case-Patient

Fever developed in a physician providing health care in Liberia on August 29th, 2014 (day 0). The physician was positive for EBOV by reverse transcription PCR (RT-PCR) of plasma and was evacuated to the United States. Details of his acute phase clinical course and management were recently described (*1*).

The patient was given investigational drug TKM-100-802 siRNA LNP (Tekmira Pharmaceuticals, Burnaby, British Columbia, Canada). He also received convalescent-phase plasma from a survivor of EVD on day 9. During hospitalization, bilateral conjunctivitis (*1*) developed, but it resolved. He did not undergo formal ophthalmologic examination or report ocular symptoms and was discharged on day 26.

The patient came to the UMass Memorial Medical Center (Worcester, MA, USA) 37 days after onset of EVD with a 2-day history of nonproductive cough, low-grade fever, and generalized weakness. He was given azithromycin for suspected pneumonia. The patient reported irritation and redness of the left eye and was given topical polymyxin B sulfate/trimethoprim for presumed conjunctivitis. Blood cultures and nasal wash and swab specimens were negative for respiratory pathogens (online Technical Appendix, http://wwnc.cdc.gov/EID/article/22/2/15-1416-Techapp1.pdf). RT-PCR result for a plasma specimen was negative for EBOV RNA.

The patient came to the UMass Memorial Eye Center on October 7, 2014 (day 40), with a 1-day history of painful vision loss, redness, and photophobia of the left eye. Results of review of other systems were negative. His medical history included treated latent tuberculosis and presumed acute Lyme disease treated in June 2014 with doxycycline.

He reported no history of ocular problems. Best corrected visual acuity was 20/25 in the right eye and 20/70 in the left eye. Intraocular pressures were 20 mm Hg in the right eye and 8 mm Hg in the left eye. Results of examination of the right eye were not remarkable. Slit lamp examination of the left eye showed diffuse blood vessel injection, mild corneal edema with fine inferior keratic precipitates, fibrin reaction, and leukocytes in the anterior chamber without hypopyon (Figure 1). The anterior vitreous humor was clear. The left fundus viewed by indirect ophthalmoscopy was hazy because of anterior segment findings but showed a grossly normal posterior segment. The patient was initially given topical 1% topical prednisolone acetate (every hour while awake) and 1% homatropine (2×/d). These drugs were gradually tapered over several weeks as he showed clinical improvement.

EBOV transmission was a concern because of reports of prolonged viral shedding on the ocular surface (*2–4*). After consent was obtained, the patient remained in home isolation pending results of conjunctival swab specimen testing. On day 42, one dry conjunctival swab specimen and 1 conjunctival swab specimen (in viral transport medium) from the inferior fornix of each eye were collected (online Technical Appendix). Specimens were shipped to the Centers for Disease and Prevention (CDC; Atlanta, GA, USA), and all showed negative results by RT-PCR for EBOV RNA.

On day 50, the patient had worsened best corrected visual acuity in the left eye (20/200) and increased floaters despite improved anterior chamber findings. Fundus examination demonstrated vitreous haze (standardization of uveitis nomenclature [*5*] classification grade 2–3 and classification grade 6 of Davis et al. [*6*]). No choroidal or retinal lesions were noted. Spectral domain ocular coherence tomography (Heidelberg Engineering, Carlsbad, CA, USA) showed diffuse vitreous opacity and vitreous adhesions that appeared as small particles in a line of vitreous strands (Figure 2). In addition, In addition, spectral domain–optical coherence

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DOI: http://dx.doi.org/10.3201/eid2202.151416

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Figure 1. Slit lamp examination of the left eye of a physician from the United States who contracted Ebola virus disease in Liberia and had eye inflammation develop during convalescence. Image shows diffuse conjunctival injection, mild corneal edema with fine inferior keratic precipitates, fibrin reaction, and leukocytes in the anterior chamber without hypopyon. Used with permission of the patient.

tomography imaging showed cystoid macular edema and vitreous adhesions tethered to the optic disc.

Diagnostic studies for etiologies of uveitis were performed on day 54. Results were positive for human leukocyte antigen (HLA)–B27 haplotype. We found increased levels of IgM and IgG against cytomegalovirus, Epstein-Barr virus, and varicella zoster virus. Lyme disease screening with confirmatory Western blot showed IgM against *Borrelia burgdorferi* (IgG Western blot result was negative). The perinuclear–antineutrophil cytoplasmic antibody titer was 1:80, and the erythrocyte sedimentation rate (48 mm/h) was increased. Complete laboratory data are shown in the online Technical Appendix. The patient showed persistence of EBOV RNA virus in semen during convalescence (CDC, unpub. data).

The patient was given prednisone (60 mg/d), and posterior segment inflammation had improved at follow-up 4 days later. Intraocular fluid sampling was considered for identifying EBOV RNA in aqueous or vitreous humors pending progress of the clinical course of the patient, but because his ocular inflammation responded well to medical therapy, this sampling was deferred.

Tapering of prednisone was initiated 1 week after treatment. Seven weeks (day 89) after initial presentation, left eye visual acuity increased to 20/25. As of March 2015, the patient was asymptomatic and his visual acuity was 20/20. The posterior segment was clear, and repeat spectral domain ocular coherence tomography confirmed normal macular structure (Figure 2).

We implemented universal precautions during examinations and designated 1 examination room and a set of equipment for office visits. Personal protective equipment used is detailed in the o nline Technical Appendix. Signs or symptoms of illness did not develop in any staff who cared for the patient.

Conclusions

EVD evolved in December 2013 from a regional outbreak in West Africa to a major global health concern (*7*). As the number of survivors of Ebola increases, evaluation of

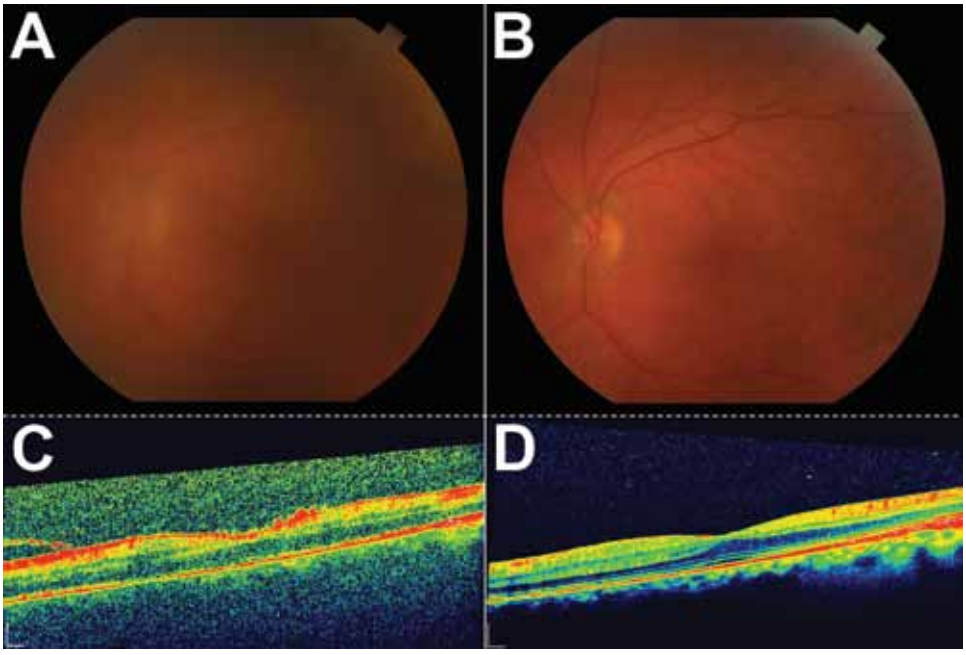

Figure 2. Color fundus and optical coherence tomography (OCT) images during active uveitis and after resolution for a physician from the United States who contracted Ebola virus disease in Liberia and had eye inflammation develop during convalescence. A) Color fundus image of the left eye showing a hazy view to the posterior pole during active uveitis (standardization of uveitis nomenclature classification grade 2–3). B) Color fundus image of the left eye showing a clear view to the posterior pole after resolution of uveitis. C) OCT of macula showing vitreous debris and small particles in a line of vitreous strands, consistent with inflammatory debris. D) OCT of macula showing resolution of vitreous and inflammatory debris. Scale bars indicate 200 μm.

ocular disease, particularly uveitis, will be a major component of patient management. Evaluation of non-EVD causes of uveitis in this patient showed notable results. First, he was positive for HLA-B27. This major histocompatibility class I allele has a well-recognized association with anterior uveitis (*8*). It is unclear whether this HLA status contributed to development of uveitis in the context of recent EVD. Second, there was evidence of global immune activation (increased erythrocyte sedimentation rate) and dysregulation of antibody production given the broad spectrum of positive serologic results, all of which fully returned to reference values on repeat testing after corticosteroid treatment (online Technical Appendix).

Lymphocyte responses have been described in humans with EVD, and sustained responses have been noted up to 60 days after symptom onset (*9*). Prolonged presence of activated virus-specific lymphocytes might be caused by retained viral antigen despite undetected EBOV viral load. Antibody-producing plasmablasts levels increased during acute EVD, and measures of EBOV-specific responses suggested polyclonal expansion, including cells with other specificities (*9*). The pronounced increase in levels of serologic markers observed for this patient suggests that dysregulation of antibody production might contribute to immunopathogenesis and provides supporting evidence of a robust inflammatory response during EVD. Health care providers should be aware that the usual laboratory workup for uveitis might be confounding in the setting of recent EBOV infection.

We considered the possibility of an immune reaction secondary to recent treatment with TKM-100-802 siRNA LNP and convalescent-phase plasma from survivors of EVD. However, little data are available regarding these treatments and immune potentiation or uveitis. Larger trials would be necessary to evaluate these potential associations.

Similar to results of Varkey et al. (*10*), we did not detect EBOV RNA in conjunctival samples. However, the findings of viable EBOV persisting in aqueous humor during convalescence (*10*) and EBOV in semen in convalescent-phase samples (*2*,*11*) provides evidence that EBOV is harbored in immune-privileged organs. EBOV RNA was detected in semen specimens from our patient, and testing has yielded positive results (CDC, unpub. data). It remains unclear whether EVD-associated uveitis is caused by cytopathic effect of the virus or immune response, but early use of systemic corticosteroids appears to be beneficial, and sampling of intraocular fluid might not be necessary in patients who clinically improve with medical therapy.

Acknowledgments

We thank Deborah Mack, the Diagnostic Imaging and Clinical Staff of the UMass Memorial Eye Center, the Massachusetts Department of Public Health, and Angela Hewlett for their contributions to the study and Amy Schuh for performing RT-PCRs for conjunctival specimens.

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Louseborne Relapsing Fever among East African Refugees, Italy, 2015

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During June 9–September 30, 2015, five cases of louseborne relapsing fever were identified in Turin, Italy. All 5 cases were in young refugees from Somalia, 2 of whom had lived in Italy since 2011. Our report seems to confirm the possibility of local transmission of louse-borne relapsing fever.

Louseborne relapsing fever (LRF) was once widely distributed in all geographic areas, including Europe and North America, occurring in association with poverty and overcrowding. In Europe, it virtually disappeared after World War I in parallel with improved living conditions that led to substantially decreased body lice infestations in humans (*1*). Currently, LRF is reported mostly from Ethiopia and surrounding countries, where it is endemic (*2*): in this region, it is an extremely common infection with substantial mortality. The causative agent is the spirochete bacterium *Borrelia recurrentis*. In nature, the only relevant vector is the body louse, which feeds only on humans; no other reservoir for this infection is known (*1*,*3*). The incubation period is 3–12 days. We report 5 cases of LRF in refugees to Italy from East Africa that occurred during 2015.

The Cases

All 5 patients were young men from Somalia (Table). Patients 1, 4, and 5 had recently arrived in Italy after traveling from Somalia through Kenya, Uganda, and Sudan to Libya, where they boarded a boat to Sicily (Figure 1). Patients 2 and 3 had resided in Italy since 2011, and both denied any travel outside Europe in the past 4 years. These 2 men lived in the same building in Turin, occupied by ≈600 refugees of different nationalities, most of them from Somalia. Patients 4 and 5 also reported a short stay in the same building.

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DOI: http://dx.doi.org/10.3201/eid2202.151768

All patients sought care at one of the city emergency departments (EDs), reporting a 2–4-day history of fever with chills and headache. Other common symptoms included vomiting, myalgia, and abdominal pain. One patient had diarrhea.

Routine blood exams, performed on all 5 men, showed marked thrombocytopenia (22,000–48,000/μL) and elevated C-reactive protein (values 16–115 times the upper reference value). Procalcitonin, measured in 3 patients, was markedly increased (11.4–21 ng/mL [reference <0.5 ng/mL]). Liver function tests and bilirubin were either normal or slightly elevated.

All patients were transferred to the Infectious Disease Hospital in Torino for further assessment. Giemsa-stained thin and thick blood smears were negative for malaria parasites but showed spirochetes (Figure 2). LRF was suspected, and the patients were treated with either doxycycline alone or doxycycline plus ceftriaxone. Patients 1 and 5 showed an acute febrile reaction after the first antimicrobial dose: symptoms were compatible with a Jarisch-Herxheimer reaction (JHR).

No lice were found on the patients or on their clothes; patient 5 had skin lesions caused by scratching. None of the patients had rash or bleeding as described in the literature (*3*). Hepatomegaly was not observed; only patient 4 showed an enlarged spleen at ultrasound. Tachycardia was common, but alterations of cardiac function were not observed (no murmurs, corrected QT interval in normal range). A low systolic blood pressure (<90 mm Hg) was observed only in patient 4.

Bacterial DNA was extracted from 200 μL of blood from each of the 5 patients by using the QIAmp Mini Kit (QIAGEN, Hilden, Germany) and was detected amplifying the 16S rRNA. Nucleotide sequences of PCR products were determined. Sequences were identified by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). *B. recurrentis* was identified in all patients, showing 100% identity with sequences of *B. recurrentis* reference strain A1 (GenBank accession nos. NR074866 and CP000993).

Italy has recently received large numbers of refugees from East Africa, particularly from Somalia. These refugees come from and travel through countries where *B. recurrentis* is endemic; along the way, they are often sheltered in crowded conditions with very poor hygienic facilities. Two of the patients reported here indicated that, while staying in Libya, they were held with many other persons in a close environment, and all refugees housed together reported severe itching.

Table. Characteristics of louseborne relapsing fever among East African refugees, Italy, 2015*

Characteristic	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Time from arrival in Italy	3 d	4 y	3 y	10 d	10 d
Age, y	20	27	31	20	26
Date of admission	Jun 9	Jul 28	Sept 5	Oct 1	Sept 29
Date of symptom onset	Jun 7	Jul 24	Sept 1	Sept 27	Sept 26
Symptom					
Fever	Yes	Yes	Yes	Yes	Yes
Abdominal pain	No	No	Yes	Yes	No
Vomiting	Yes	Yes	Yes	Yes	No
Diarrhea	No	No	Yes	No	No
Headache	Yes	Yes	Yes	Yes	Yes
Myalgia	No	Yes	No	No	Yes
Other	None	Lumbar pain	None	Cough	Chest pain, itching
Laboratory test (reference)					
Platelets (>150,000/μL)	32,000	47,000	22,000	41,000	2,9000
Bilirubin (<1.2 mg/dL)	2.3	1.8	3.9	4.2	1.8
Liver function†	Normal	Normal	AST ×4; ALT ×3	AST ×3; ALT normal	Normal
Prothrombin time (>70%)	78%	66%	68%	100%	70%
CRP†	×16	×56	×95	×115	×59
Procalcitonin (<0.5)	11.36	NA	21	11.9	NA
QTc (<440 msec)	428	361	423	391	414
PCR for *Borrelia recurrentis*	Positive	Positive	Positive	Positive	Positive
Treatment	Doxycicline	Doxycicline	Doxycicline	Doxicicline + ceftriaxone	Doxycicline, switched to ceftriaxone
Jarisch-Herxheimer reaction	Yes, mild	Not observed	Not observed	Not observed	Yes, moderate

*ALT, alanine aminotransferase; AST, aspartate aminotransferase; CRP, C-reactive protein; NA, not available; QTc, corrected QT interval.
†Indicated as normal (within reference value) or × upper reference value.

Many of these refugees enter Italy through Sicily, from where they are sent to reception centers throughout the country. Some of these reception centers have grown to substantial size and now house a more stable population, with continuous input of new arrivals. In these conditions, local transmission can occur with a possible risk for epidemics: 2 of the 5 patients reported here were long-term residents in Italy, and they denied recent travel to Africa, so they probably acquired the infection while being housed in the same facilities as the newly arrived refugees. Although it is possible that they denied recent travel for fear of legal consequences, they are unlikely to have had the opportunity to travel out of Europe for economic reasons.

We did not find any louse on the body or in the clothing of these patients. However, we identified *B. recurrentis* by gene sequencing, and the dynamic of

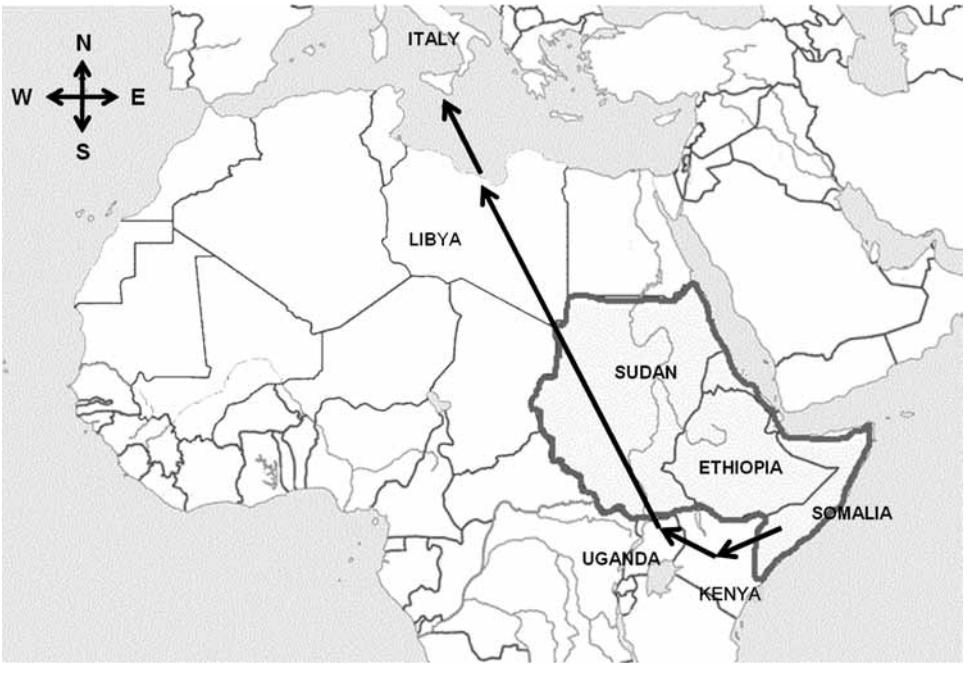

Figure 1. Route (arrows) followed by refugees from Somalia to Libya, where they boarded a boat to Sicily. Gray shading indicates *Borrelia recurrentis*–endemic countries.

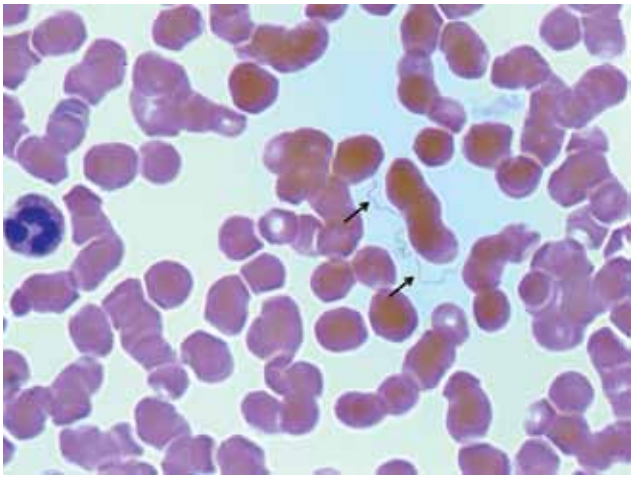

Figure 2. Giemsa-stained thin blood smear showing spirochetes (arrows) in a 27-year-old male refugee from Somalia (patient 2) with louseborne relapsing fever, Italy, 2015. Original magnification ×1,000.

transmission we postulate in our cases fits more closely the model of louse transmission (overcrowding, poor hygienic conditions, migration) than that of tick transmission (*1*,*3*,*4*). Lice are relatively short-lived and remain infected throughout their lives but cannot transmit borreliae to their progeny. They do not inject borreliae directly while feeding (infection takes place when they are crushed on skin) (*1*): infected lice can survive on uninfected persons but, when moved to other persons, give rise to infection. Thus, identifying a direct chain of transmission is often challenging. The presence of lice has been reported as a growing problem in western Europe homeless persons with no history of travel, threatening an alarming scenario (*4*,*5*).

All 5 patients reported here sought care at one of the EDs in Turn for fever associated with other nonspecific symptoms; all 5 diagnoses were made as occasional observations of spirochetes on thin and thick blood smears conducted to search for malaria parasites. These 5 patients might represent a minority of persons who are actually affected by relapsing fever. In fact, in many instances, persons seen at an ED for fever, especially persons without a history of recent travel to malaria-endemic countries, would not be investigated for malaria and would probably be treated empirically with antimicrobial drugs, leading to resolution of symptoms without further investigations.

Two of the patients we report had symptoms suggestive of a JHR. JHR is an acute, febrile reaction, potentially fatal, occurring shortly after starting antimicrobial drugs, probably attributable to the release of cytokines associated with clearance of borreliae from blood (*2*,*6*). A higher frequency of JHR has been reported in the literature, up to 80% (*6*,*7*), with some differences depending on the antimicrobial drug used (*7*,*8*). However, most observations were performed in LRF-endemic countries. These patients were all hospitalized and were receiving supportive treatment (intravenous rehydration and paracetamol, either intravenously or orally, to control headache, myalgia, and abdominal pain), which might have played a role in avoiding JHRs or in reducing its symptoms. Several studies have been conducted on possible preventive options to avoid the arousal of JHR, but results have not been consistent (*9*). Some clue of a possible action of paracetamol on the development of JHR has been reported (*8*).

Conclusions

In summary, we identified *B. recurrentis* infection in 5 patients in Italy who were refugees from East Africa; 2 of these patients had not traveled outside Italy for several years. Beginning in July 2015, several reports from countries in Europe have described relapsing fever in refugees from East Africa (*10*–*13*). In some of these cases, transmission might have occurred during transit through Italy (*12*). Our findings confirm the possibility of local transmission of LRF caused by *B. recurrentis*.

Dr. Lucchini is an infectious disease specialist at the Hospital for Infectious Diseases in Turin. Her primary research interests include sexually transmitted infections, HIV, and tropical diseases in travelers.

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Mediterranean Fin Whales (*Balaenoptera physalus*) Threatened by Dolphin MorbilliVirus

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During 2011–2013, dolphin morbillivirus was molecularly identified in 4 stranded fin whales from the Mediterranean Sea. Nucleoprotein, phosphoprotein, and hemagglutinin gene sequences of the identified strain were highly homologous with those of a morbillivirus that caused a 2006–2007 epidemic in the Mediterranean. Dolphin morbillivirus represents a serious threat for fin whales.

Fin whales (*Balaenoptera physalus*) living in the Mediterranean Sea belong to a population that is part of the Atlantic stock (*1*). For feeding purposes, these whales tend to concentrate in specific areas, one of which is Pelagos Sanctuary, the widest protected marine area for sea mammals in the Mediterranean Basin between Italy, France, and Monaco. In Pelagos Sanctuary and, in general, in the entire Mediterranean Sea, fin whales are considered vulnerable because of several anthropogenic threats, the most common of which are ship strikes, chemical pollution, and noise (*2–4*).

Postmortem investigations on well-preserved whale carcasses are necessary to gain evidence-based insight into the effect these threats have on fin whales; thus, the carcasses of all large cetaceans found stranded along the Italian coastline are systematically examined to determine the cause of death. Because morbillivirus infections have been detected during these postmortem investigations, we conducted a study to determine the effect of this natural threat on the Mediterranean fin whale population.

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DOI: http://dx.doi.org/10.3201/eid2202.150882

The Study

During 2006–2014, a total of 23 fin whales were found stranded along the coast of Italy. We systematically conducted full necropsies and microscopy- and molecular-based analyses on 9 (39%) carcasses that were in good conservation status. Of these 9 fin whales, 2 were juveniles and 2 were calves found during January 2011–February 2013 along the coasts of Tuscany, Sardinia, and Liguria, Italy. These young whales showed pathologic, immunohistochemical (IHC), biomolecular, and/or serologic evidence of dolphin morbillivirus (DMV) infection (Table 1) (*5–9*); however, not all of these cases were spatially or temporally related to known fatal DMV-associated outbreaks that occurred during 2006–2014 (online Technical Appendix Figure 1, http://wwwnc.cdc.gov/EID/article/22/2/15-0882-Techapp1.pdf). Moreover, in October 2013, a newborn male fin whale was found stranded alive on Elba Island (Tuscany); the whale died after a few hours, and postmortem investigations conducted within 24 hours of death yielded biomolecular and IHC evidence of DMV infection. Viral genome, antigen, or both were found in several tissues, along with a parasitic infection and a generalized lymphocytic depletion. Hyperimmune rabbit anti–rinderpest virus serum (provided by Pirbright Institute, Pirbright, UK) (*10*) was used to detect morbillivirus antigens; only circulating monocytes and tissue macrophages in brain and thymus stained positively (Figure 1).

DMV genome was detected in brain, lung, spleen, and thymus from the newborn whale; viral RNA was extracted from these tissues by using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Primer DMV2 (*11*) and the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) were used to synthesize cDNA; primer and viral RNA were incubated at 42°C for 60 min followed by 70°C for 5 min. Amplification was performed by using primers DMV-N1 and DMV-P2 (*11*) and Phusion Hot Start II DNA Polymerase (Thermo Scientific) with the following PCR conditions: 30 s at 98°C; 35 cycles of 10 s at 98°C, 30 s at 62°C, 1 min at 72°C; and 10 min at 72°C. Next, the DNA fragments obtained from lung and cerebral cDNA were purified, cloned into the plasmid vector pCR-Blunt II-TOPO (Invitrogen, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions, and then sequenced.

Sequences from 5 lung and 4 cerebral plasmidic colonies were analyzed. Programs in the DNASTAR

Table 1. Demographic data and examination results for 5 dolphin morbillivirus–positive fin whales stranded off the Mediterranean Sea, 2011–2013*

Whale no., sex/length, m†	Stranding date	Closest DMV outbreak	Dolphin morbillivirus–positive samples, by test		
			RT-PCR‡	IHC analysis‡	VN
1, M/17	2011 Oct 25	Dec 2010–Jun 2011	Liver, spleen, lungs	ND	ND
2, F/10	2011 Nov 3	Dec 2010–Jun 2011	Liver, spleen, lymph nodes, skeletal muscle	Negative	ND
3, M/10	2011 Nov 20	Dec 2010–Jun 2011	Negative	ND	Positive
4, F/15	2013 Mar 19	Jan–Apr 2013	Lymph nodes	ND	Negative
5, F/5	2013 Oct 11	Jan–Apr 2013	Brain, lungs, spleen, thymus	Brain, thymus	Negative

*IHC, immunohistochemical; ND, not done; RT-PCR, reverse transcription PCR; VN, virus neutralization
†Whales 1 and 4 were juveniles; whales 2 and 3 were calves.
‡Tissues routinely examined for morbillivirus: lung, liver, spleen, kidney, prescapular and pulmonary lymph nodes, brain, thymus (if available), tonsils, and skeletal muscle.

Lasergene software package (http://www.dnastar.com/t-dnastar-lasergene.aspx) were used to edit, assemble, and translate sequences. This technique enabled identification of a 1,355-bp DNA fragment from the newborn whale that encompassed partial nucleoprotein and phosphoprotein genes (i.e., the N1-P2 consensus fragment; GenBank accession no. KR337460). This fragment, from lung and cerebral samples, showed 98.89% sequence homology with the complete DMV genome (GenBank accession no. AJ608288) and 99.85% sequence homology with a DMV strain identified in long-finned pilot whales (*Globicephala melas*) that were affected by the 2006–2007 epidemic in the Mediterranean (GenBank accession no. HQ829972). Amino acid changes in KR337460, compared with AJ608288, are shown in Figure 2. The same DNA fragment was recovered from the newborn whale's spleen and thymus by using the PCR protocol mentioned above (online Technical Appendix Figure 2).

Viral hemagglutinin (H) protein mediates DMV entry into host cells by specifically binding with the whale's signaling lymphocyte activation molecule (SLAM)/CD150; thus, we also investigated variations in the H gene of the newborn whale by using the previously described cloning procedures for cerebral cDNA with 3 new overlapping primers pairs (Table 2; Figure 2). This technique enabled identification of a 1,699-bp DNA fragment encompassing a partial H gene sequence; 116 bp at the beginning of the gene were missing. The H consensus fragment, obtained from the cDNA clone for each primer pair, showed 99.41% sequence homology with the complete DMV genome (GenBank accession no. AJ608288) and 99.94% sequence homology with the DMV strain identified in long-finned pilot whales (GenBank accession no. HQ829972). We also identified 2 aa changes: the previously reported p.Val164Ala (*11*) and a novel variation, p.Ala451Thr, located within the H protein region (residues 382–582) involved in SLAM binding (Figure 2) (*12*). This variation was previously reported in other related morbilliviruses (*13*) and does not control any change in the tertiary structure of H antigen, as determined by using the SWISS-MODEL (http://swissmodel.expasy.org/) modeling program.

Conclusions

The results of our direct (IHC and biomolecular) and indirect (serologic) testing provide evidence of DMV infection or exposure in 5 (55%) of 9 fin whales that were found stranded off the Mediterranean Coast during 2011–2013.

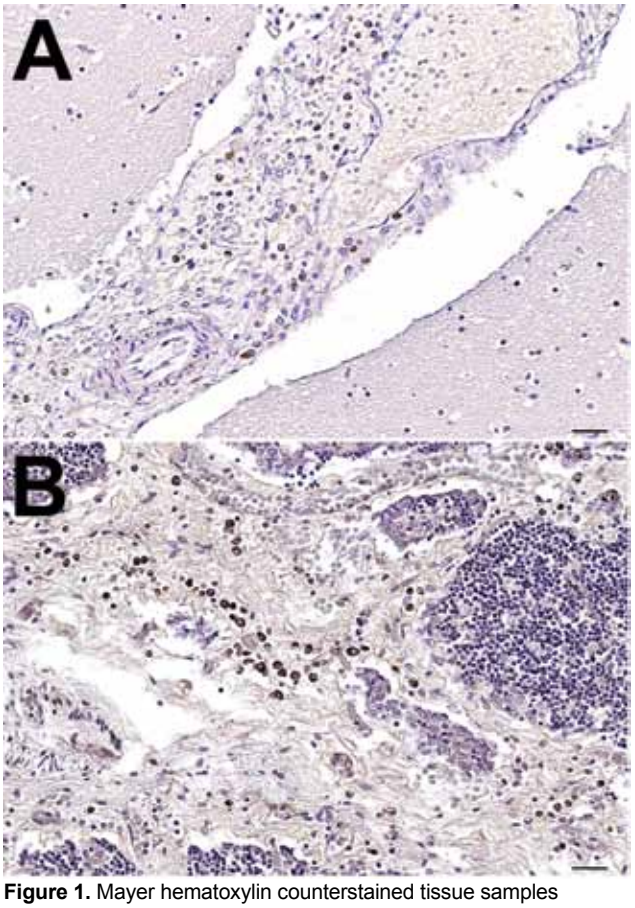

Figure 1. Mayer hematoxylin counterstained tissue samples from a newborn fin whale stranded off the Mediterranean Sea, October 2013. A) Brain tissue showing positive immunostaining for morbillivirus antigen in macrophages in the meningeal space. B) Fin whale thymus showing positive immunostaining for morbillivirus antigen in thymocytes and macrophages. For both samples, morbillivirus was detected by immunohistochemical analysis, using a rabbit hyperimmune anti–rinderpest virus serum (provided by Pirbright Institute, Pirbright, UK) (*10*). Original magnification ×40. Scale bars indicate 50 μm.

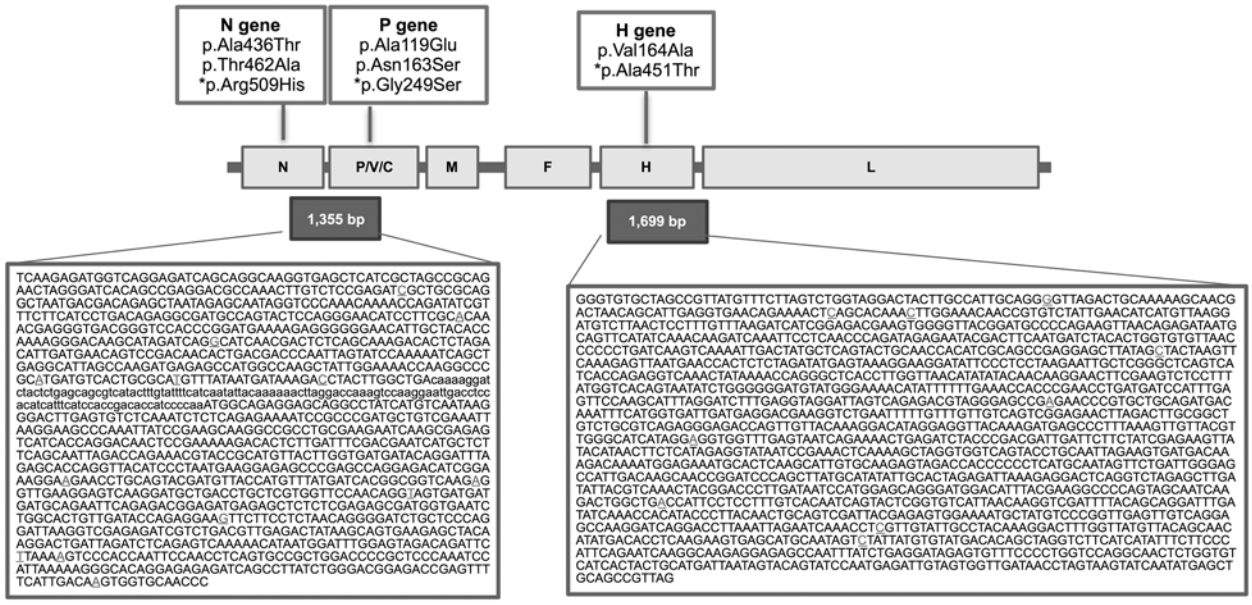

Figure 2. Genomic organization of the dolphin morbillivirus (GenBank accession no. KR337460) isolated from a newborn fin whale found stranded on Elba Island, Italy, October 2013. Boxes in first row indicate amino acid changes identified in each gene; asterisks indicate nonsynonymous amino acid substitutions. Boxes in the second row indicate the morbillivirus gene structure; horizontal lines indicate noncoding sequences. Boxes in the third row indicate the total length of the 2 analyzed virus fragments, and their sequences are shown, respectively, in boxes in the bottom row. N, nucleoprotein gene; P/V/C, phosphoprotein gene with nonstructural proteins V and C; H, hemagglutinin gene; F, fusion gene; L, large protein gene. Primers DMV-N1 and DMV-P2 (*11*) were used to amplify a 1,355-bp nt sequence (left box in bottom row) representing partial portion of N gene and P/V/C gene (shown in uppercase letters); lowercase letters indicate noncoding sequences. Three overlapping primer pairs were used to amplify a 1,699-bp nt sequence (right box in bottom row) representing the entire H gene. Underlined bases indicate nucleotide variations from the complete DMV sequence (GenBank accession no. AJ608288). The distribution of amino acid changes identified in each gene are shown in the top row.

These 5 infected whales correspond to 21.7% of the 23 whales stranded along the Italian coastline during 2006–2014. The other 4 examined whales showed no evidence of morbillivirus infection. The range of DMV-susceptible host species has progressively expanded (*5*), as highlighted by the recent report of DMV infection in a captive common seal (*Phoca vitulina*) during the 2011 outbreak (*14*). This expansion, combined with spread of DMV through the transplacental route, resulting in virus colonization of the thymus in fetuses, could represent DMV survival strategies among cetacean populations. In addition, our data argue in favor of an epidemic cluster of fatal DMV among the Mediterranean fin whale population, even though, on the basis of the amino acid sequence of the SLAM/CD150 viral receptor, this species is not included among those susceptible to DMV epidemics (*5*,*12*).

Although the single amino acid substitution, p.Ala451Thr, did not cause substantial variations in the structure of H antigen, the effect of the variation on protein functions is unclear. Recent studies showed that similar amino acid changes could affect virulence and infectivity of different *Canine distemper virus* (family *Paramyxoviridae*, genus *Morbillivirus*) strains, but such changes are often neutralized by compensatory mutations that preserve the biologic activity of H protein (*15*). Furthermore, despite the high sequence homology observed between N, P, and H genes of the DMV strain identified in the newborn fin whale in our study and in the

Table 2. Primers designed on dolphin morbillivirus isolate used for the total hemagglutinin gene sequence analysis of the virus detected in the newborn fin whale*

Primer	Nucleotide position	Sense sequence, 5′ → 3′	Fragment length, bp
DMV-10F	7206–7226	GGGTGTGCTAGCCGTTATGT	718
DMV-10R	7904–7924	TTCGTCCTCATCAATCACCA	718
DMV 11F	7799–7891	CCGAACCTGATGATCCATTT	612
DMV-11R	8391–8411	CGTAAATGTCCATCCCTGCT	612
DMV-12F	8290–8309	AACCGGATCCCAGCTTATG	800
DMV-12R	9070–9090	CCAGGTGCACTTCAGGGTAT	800

*Isolate GenBank accession no. AJ608288. Annealing temperature for all primers was 56°C. DMV, dolphin morbillivirus; F, forward; R, reverse.

isolates recovered from DMV-affected cetaceans during the 1990–1992 and the 2006–2007 epidemics (GenBank accession no. AJ608288), we cannot exclude that more prominent differences occurred in virus genes encoding for both structural and nonstructural proteins responsible for virulence and pathogenicity (e.g., P/V/C and fusion genes) (*5*); the simultaneous occurrence of primary structure differences, if any, in the SLAM/CD150 receptor should also be taken into account (*5*,*12*). In conclusion, although further studies are needed to elucidate the complex virus–host interaction dynamics and the putative influence exerted by environmental factors, DMV should be regarded as one of the major threats for the conservation of fin whales within the Mediterranean Sea.

Acknowledgments

We thank Bruno Cozzi and Maristella Giurisato for storing and delivering samples and Giuseppe Palmisano and Emanuele Zanetti for their support during necropsies in the field.

This study was partially funded by the Italian Ministry of the Environment.

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Blastomyces gilchristii as Cause of Fatal Acute Respiratory Distress Syndrome

Daniel Dalcin, Aaron Rothstein, Joanne Spinato, Nicholas Escott, Julianne V. Kus

Since the 2013 description of *Blastomyces gilchristii,* research describing the virulence or clinical outcome of *B. gilchristii* infection has been lacking. We report molecular evidence of *B. gilchristii* as an etiologic agent of fatal acute respiratory distress syndrome. *B. gilchristii* infection was confirmed by PCR and sequence analysis.

Differences in virulence have long been observed between different strains of *Blastomyces dermatitidis* (*1*,*2*). Following the 2013 description of *B. gilchristii* as a cryptic species of *Blastomyces* spp., *B. dermatitidis* and *B. gilchristii* have been recognized as etiologic agents of blastomycosis (*3*). However, research evaluating the differences in clinical outcome between *B. dermatitidis* and *B. gilchristii* infection has been lacking. Here we demonstrate that *B. gilchristii* infection can cause fatal acute respiratory distress syndrome (ARDS) in humans.

The Study

A 27-year-old woman sought care at an emergency department in a rural community in northwestern Ontario, Canada, for a nonproductive cough, right-sided chest heaviness, nausea, and abdominal pain that had developed over the previous week. She had a previous diagnosis of type 1 diabetes mellitus and a history of intravenous drug use.

On examination, deep and labored breathing was observed, and decreased air entry in the left upper lung was heard on auscultation. A chest radiograph revealed left upper lobe consolidation (Figure 1, panel A). Blood chemical analysis revealed diabetic ketoacidosis (25.4 mmol/L glucose, 3.2 mmol/L K+, 6.0 mmol/L HCO_3–, anion gap 27 mg/dL, and pH 7.0 venous blood gas). Intravenous fluoroquinolone was administered for suspected left upper lobe bacterial pneumonia, and fluid resuscitation and intravenous insulin were administered for diabetic ketoacidosis.

During the first 2 days in hospital, the patient was stable and afebrile, despite remaining tachycardic and tachypneic. From day 3 to day 4, the patient became febrile (38.3°C) and tachypnea worsened to the point that >4 L of O_2 was required to maintain O_2 saturation >90%. The patient's leukocyte count was elevated at 13.5 × 10^9 cells/L.

On day 5, the patient was observed to be using accessory muscles of respiration. A chest radiograph was performed and showed a complete whiteout of the left lung (Figure 1, panel B). Air transfer to a tertiary hospital was requested but unavailable. The patient's mean arterial blood pressure decreased from 80 to 65 mm Hg, leukocyte count increased to 18.8 × 10^9 cells/L, and hemoglobin decreased from 140 to 102 g/L. A chest radiograph revealed patchy opacification of the right lung that was not observed several hours earlier (Figure 1, panel C). After intubation and sedation, the patient was successfully transferred to the intensive care unit of a tertiary hospital.

On arrival at the intensive care unit, the patient was put on a mechanical ventilator requiring positive-end expiratory pressure to oxygenate. Antimicrobial drugs (vancomycin, piperacillin/tazobactam, and azithromycin), an antifungal drug (amphotericin B), and a vasopressor (norepinephrine) were administered, and sputum cultures were sent for bacterial and fungal culture. One day after entering the intensive care unit, the patient became bradycardic and went into cardiac arrest. Cause of death was determined to be ARDS (Figure 2).

A sputum specimen was prepared for direct microscopic examination for fungal elements by staining with Calcofluor White (Sigma-Aldrich, St. Louis, MO, USA) and for fungal culture by plating onto BHICCGE (brain heart infusion + 5% sheep blood + 50 μg/mL chloramphenicol, 0.01% cycloheximide, 20 μg/mL gentamicin, and egg white) agar and inhibitory mold agar plates and incubated at 28°C. Direct microscopic examination of the sputum specimen revealed broad-based budding yeast with refractile cell walls characteristic of *Blastomyces*. After appropriate culture and incubation, small colonies of mold were observed after 1 week. The identification of a *Blastomyces* sp. was confirmed with a molecular probe (AccuProbe *Blastomyces dermatitidis* Culture Identification Test; Gen-Probe, San Diego, CA, USA) and by conversion of the mold-form to the yeast-phase using Blasto-D medium and incubation at 37°C (*4*). The culture was preserved at –80°C as a glycerol stock with the designation 13BL347.

The isolate was later resurrected and incubated as previously described (*4*). Using a similar method described in Brown et al. (*3*), fungal DNA was extracted, the internal

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DOI: http://dx.doi.org/10.3201/eid2202.151183

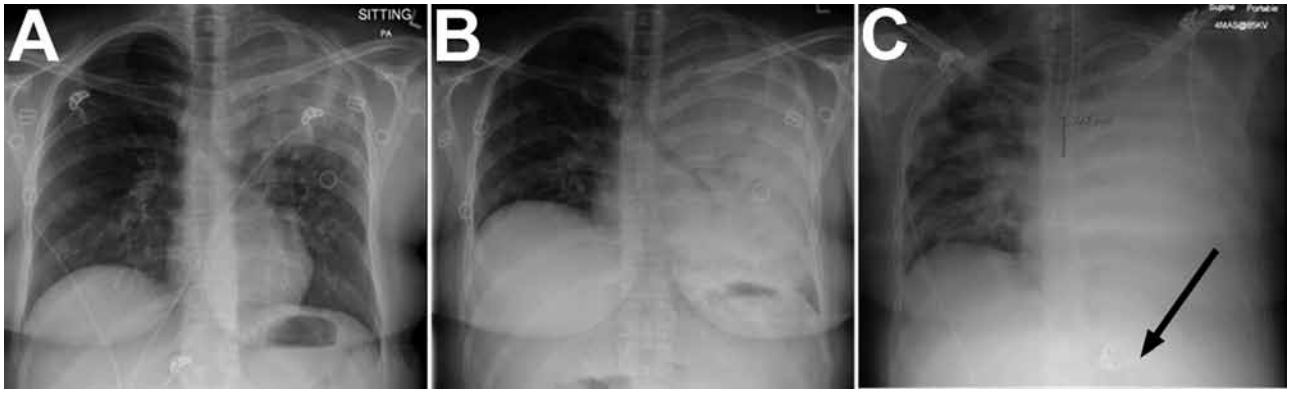

Figure 1. Chest radiograph at various stages of *Blastomyces gilchristii* infection in a 27-year-old woman, Ontario, Canada. A) Day 0: posterior–anterior (PA) chest radiograph at initial emergency department examination. Discrete confluent left upper lobe consolidation with air bronchograms are visible. B) Day 5, 15:10: PA chest radiograph demonstrating complete confluent opacification of the left hemithorax with extensive air bronchograms. C) Day 6, 23:30: PA chest radiograph postintubation with confluent left lung consolidation and new right patchy airspace opacification. Arrow indicates the correct placement of a nasogastric tube.

transcribed spacer 2 (ITS2) region was amplified by PCR using the primers described in White et al. (*5*), and the resulting product was sequenced. Sequences were analyzed, and the single nucleotide polymorphism in ITS2 at position 19 (used for differentiation of *B. dermatitidis* from *B. gilchristii*) was noted (*3*). The sequence of 13BL347 ITS2 possessed a cytosine at position 19, which is diagnostic for *B. gilchristii,* whereas thymine at that position is conserved in *B. dermatitidis*. By using a multiple sequence alignment tool, we aligned the 13BL347 ITS2 sequence to sequences of several well-characterized representative sequences of both species (*1*,*3*); it clustered with other *B. gilchristii* strains (online Technical Appendix, http://wwwnc.cdc.gov/eid/article/22/2/15-1183-Techapp1.pdf) (*6*).

Variation in clinical presentation among different genetic groups of *B. dermatitidis* has long been observed (*1*–*3*). However, since the 2013 identification of *B. gilchristii*, it is unclear as to whether the previous reports were actually reporting differences in virulence between *B. dermatitidis* and *B. gilchristii*. Therefore, we refer to the fungi that were the subject of previous blastomycosis studies as *Blastomyces* spp. rather than *B. dermatitidis*.

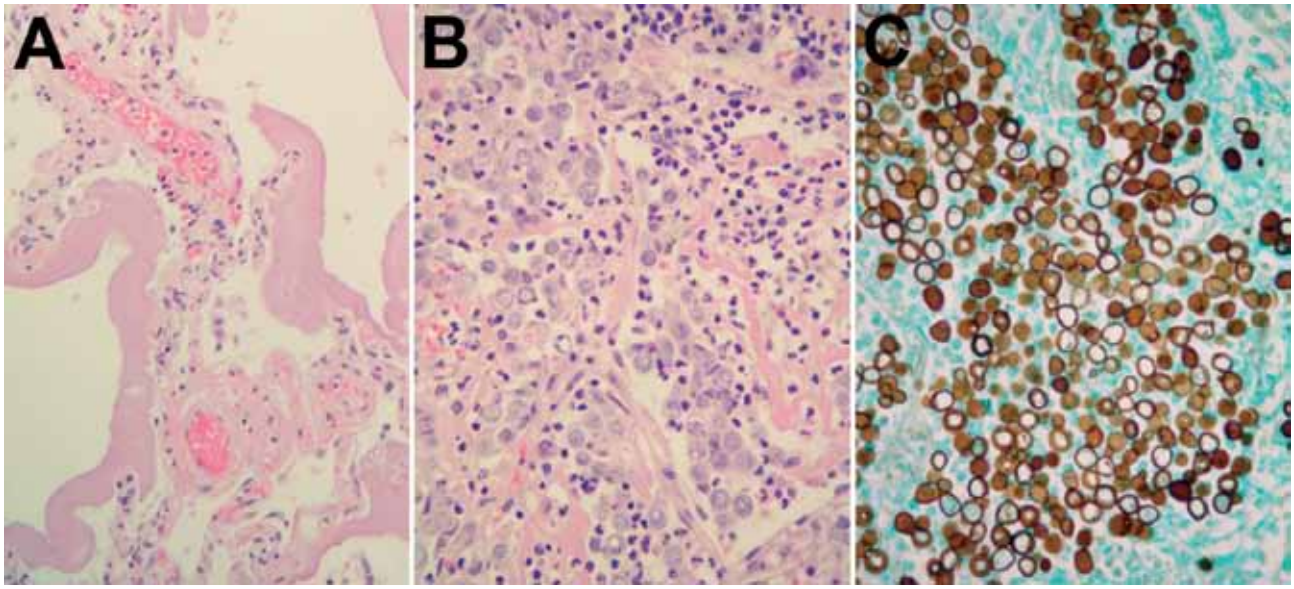

Figure 2. Histologic manifestations of *Blastomyces gilchristii* infection in a 27-year-old woman, Ontario, Canada. A) Nonconsolidated lung. Thick hyaline membranes, characteristic of diffuse alveolar damage and acute respiratory distress syndrome, line the alveoli. Hematoxylin and eosin (H&E) stain, original magnification ×400. B) Consolidated lung. Alveoli are completely filled with *B. gilchristii* yeast cells and neutrophils. *B. gilchristii* cells are pale bluish-gray with a distinct cell border. H&E stain, original magnification ×100. C) Consolidated lung containing abundant *B. gilchristii* yeast cells with characteristic broad-based buds. Grocott methenamine silver stain, original magnification ×400.

Known virulence factors of *Blastomyces* spp. include the cell surface carbohydrate polymer alpha-1,3-glucan and the *Blastomyces* adhesion 1 protein (formerly WI-1) surface adhesion molecule (*2*). To our knowledge, differences in expression of these virulence factors in *B. dermatitidis* and *B. gilchristii* have not been compared. It has been demonstrated previously that African strains of *B. dermatitidis* differ from North American strains in their growth and morphology and are thought to cause less severe disease (*2*). Genetic analysis has identified 2 distinct genetic groups of African *B. dermatitidis* that differ in expression of the *Blastomyces* adhesion 1 protein surface adhesion molecule (*2*). Whether the differences in growth, morphology, and apparent virulence in the African *Blastomyces* sp. is attributable to *B. gilchristii* and *B. dermatitidis*, intraspecies variation, or a separate undescribed genetic group remains unexplored.

There are several challenges in evaluating the virulence of *B. dermatitidis* and *B. gilchristii*. First, the clinical course of blastomycosis has been found to be correlated with the amount of inoculum (conidia) initially acquired (typically through inhalation) (*7*). In the context of human infection, it is difficult to determine if differences in disease process are attributable to differences in fungal virulence or the magnitude of inoculum. Furthermore, host factors such as immunodeficiency and variation in human leukocyte antigen profile have been shown to cause variation in immune response to *Blastomyces* spp. (*8*).

Blastomyces spp. have also been observed to lose virulence during laboratory processing, making the results of previous laboratory-based virulence studies questionable (*7*). Another challenge is that the commercially available molecular probe that is often used to confirm the identification of *Blastomyces* spp. cannot differentiate between *B. dermatitidis* and *B. gilchristii*. Currently, *B. dermatitidis* and *B. gilchristii* cannot be distinguished based on phenotype; PCR followed by sequence analysis is the only method of differentiating these species.

Conclusions

Most cases of blastomycosis-induced ARDS are preceded by a prodrome of pneumonia weeks to months before development of ARDS (*9*,*10*). However, in a minority of cases, such as the one we describe, the clinical course rapidly progresses to fatal ARDS (*9*,*10*). This patient died within 7 days of hospital admission and <24 hours after intubation. In this case, it is difficult to comment on the virulence of *B. gilchristii* because the patient's immune status is uncertain. The patient had uncontrolled hyperglycemia secondary to type 1 diabetes mellitus, history of intravenous drug use, and an unknown HIV status; these factors are known to cause immune dysfunction and might have contributed to the acuity and severity of disease. Nevertheless, our findings demonstrate that *B. gilchristii* infection can cause fatal ARDS in humans and form a basis for further virulence and epidemiologic studies of *B. dermatitidis* and *B. gilchristii*.

Acknowledgments

The authors are grateful to Marina Ulanova for providing a critical review of the manuscript and to Michael Wilson for helpful advice.

D.D. was funded by a Professional Student Research Award from the Canadian Institute of Health Research.

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Effectiveness of Meningococcal B Vaccine against Endemic Hypervirulent *Neisseria meningitidis* W Strain, England

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Serum samples from children immunized with a meningococcal serogroup B vaccine demonstrated potent serum bactericidal antibody activity against the hypervirulent *Neisseria meningitidis* serogroup W strain circulating in England. The recent introduction of this vaccine into the United Kingdom national immunization program should also help protect infants against this endemic strain.

Invasive meningococcal disease (IMD) has been declining in the United Kingdom since the early 2000s (*1*). Historically, serogroup W *Neisseria meningitides* meningococci (MenW) have been causal organisms for 1%–2% of IMD cases annually. An increase in invasive MenW disease associated with travel to the Hajj pilgrimage route during 2000–2002 was rapidly controlled after the introduction of mandatory vaccination for pilgrims (*2*). Since 2009, however, laboratory-confirmed MenW cases in England have increased each year across all age groups after rapid spread of a single endemic hypervirulent sequence type (ST) 11 clonal complex (MenW:cc11) strain (*3*). This strain has caused severe illness with unusual clinical manifestations and, for the first time in more than a decade, was associated with fatal outcomes among infants and young children.

Since the Hajj outbreak, several countries in Latin America, Africa, and the Far East have reported an increase in MenW disease and ongoing endemic transmission (*3*). In Chile, MenW has replaced serogroup B (MenB) as the most prevalent cause of IMD, identified in 58% of cases in 2012 (*4*). In Europe, an increase in MenW disease has not been observed in other countries although, in 2012, a cluster of cases related to MenW:cc11 in France was associated with travel to sub-Saharan Africa (*5*).

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DOI: http://dx.doi.org/10.3201/eid2202.150369

In England, the meningococcal quadrivalent conjugate vaccine (MenACWY, covering serogroups A, C, W, and Y) has historically been recommended for high-risk persons and travelers to disease-endemic regions and for controlling outbreaks (*6*). Beginning on September 1, 2015, a novel, protein-based, multicomponent vaccine, Bexsero (GSK Vaccines, Siena, Italy), has been offered as part of the routine immunization program in the United Kingdom; the vaccine is given to infants in 3 doses at 2, 4, and 12 months of age (https://www.gov.uk/government/publications/menb-vaccination-introduction-from-1-september-2015). Bexsero is composed of NHBA (neisserial heparin binding antigen), NadA (*Neisseria* adhesin A) and fHbp (factor H binding protein), with meningococcal outer membrane vesicles from an MenB strain from an outbreak in New Zealand (*7*). Immunization with Bexsero induces bactericidal antibodies against all vaccine antigens (*8*). Although this vaccine has been licensed for prevention of MenB disease (the most prevalent capsular group causing IMD in Europe), alleles for some or all of the vaccine antigens are also found among non-MenB meningococci, independently of the capsule. Therefore, antibodies raised by these antigens could induce complement-mediated killing of other meningococcal groups, including the endemic MenW cc11 strain.

Recently, we reported the predominance of non–cross-protective PorA (P1.5,2) and fHbp variants (variant 2 peptide 22) among endemic MenW:cc11 isolates (*3*). The other primary Bexsero antigens (NadA and NHBA), however, could potentially afford protection. We therefore assessed 1) the NadA and NHBA genotypic status of endemic MenW:cc11 isolates, and 2) the serum bactericidal antibody (SBA) activity against clinical MenW:cc11 isolates using serum samples from infants immunized with Bexsero.

The Study

A total of 73 invasive MenW:cc11 isolates from England and Wales were received by the Public Health England Meningococcal Reference Unit during July 2010–June 2013. These isolates were queried within the Meningitis Research Foundation Meningococcus Genome Library (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db = pubmlst_neisseria_mrfgenomes) for *nadA* and *nhba* and, where present, their respective allelic and peptide variants.

We used an SBA using human complement (hSBA) against 6 invasive MenW:cc11 isolates from patients 4 months–91 years of age in whom meningitis or septicemia was diagnosed in different regions of England and Wales during 2011–2012. SBA titers were expressed as the reciprocal of the serum dilution corresponding to ≥50% bacterial killing. We used pooled serum samples from phase 2 clinical trials involving infants immunized with Bexsero at 2, 3, and 4 months or 2, 4, and 6 months and after administration of a booster at 12, 18, or 24 months (*8*). Pooled prevaccination serum samples from 180 infants were used as negative controls, and pooled serum samples from 10 randomly selected adolescents who received a single MenACWY conjugate vaccine (GSK Vaccines) dose as positive controls.

Of the 6 isolates tested, 4 possessed *nadA* allele 5 (for peptide NadA-2/3.6) and *nhba* allele 17 (for NHBA peptide 29). Of the remaining 2 isolates, 1 isolate had a *nadA* allele (allele 146) that differed at a single nucleotide (C476A), causing a single amino acid change (T159K; peptide NadA-2/3.130); the other isolate had an *nhba* allele (allele 72) that differed at a single nucleotide (A376C), causing a single amino acid change (T126P; peptide 96).

hSBA titers were high and were ≥1:32 against all 6 MenW isolates, independently of the immunization schedule (Table). After the booster, higher hSBA titers were obtained than those from primary immunization, and similar responses were comparable to those among adolescents who had received a single MenACWY dose. Preimmunization serum samples showed no detectable hSBA titers against any of the 6 isolates (Table).

Conclusions

The ability of the antibodies raised by Bexsero antigens to induce SBA activity against any given meningococcal isolate depends on the presence, level of surface expression, and sequence diversity of the respective antigens. Bexsero strain coverage can be predicted by using the Meningococcal Antigen Typing System, an ELISA which measures the level of antigen expression and antigenic diversity compared with the antigen in the vaccine (9). However, the correlation between the relative potency estimated by this typing system and the ability of a meningococcal isolate to be killed by serum from immunized persons has only been defined for MenB strains.

We found that MenW:cc11 isolates causing invasive disease in England and Wales possessed alleles for NadA-2/3 peptide variants that are predicted to be highly cross-protective with the Bexsero NadA variant (*10*). The isolates also possessed alleles for NHBA peptide 29 which, although different from peptide 2 in Bexsero, has the potential to induce cross-protection even if NHBA-containing cross-protective epitopes have not been defined yet. Antibodies against Bexsero antigens can act synergistically and, therefore, the complement-mediated bactericidal killing observed could be mediated by antibodies against NadA, NHBA, or both.

In England, the ongoing MenW increase is similar to the MenC:cc11 outbreak in the mid-1990s, which was eventually controlled through mass vaccination (*11*). The increase in MenW cases has led to the rapid introduction of a national adolescent MenACWY conjugate vaccination program in August 2015 (https://www.gov.uk/government/news/new-meningococcal-vaccination-programme-expected-to-save-lives). In adolescents, a single MenACWY dose would provide direct protection and, by targeting the age group most likely to carry meningococci (*12*), could also provide indirect protection against MenW (Figure) and the other 3 capsular groups by reducing carriage and onward transmission to others.

Table. Bactericidal antibody titers in pooled serum samples from infants vaccinated with Bexsero and adolescents immunized with Menveo against 6 invasive clinical *Neisseria meningitidis* serogroup W isolates in England and Wales, UK, during 2011–2012*

	Adolescents receiving Menveo		Infants receiving Bexsero				
	Positive control†		Negative				
Isolate	Before	After	control‡	Pool 1§	Pool 2¶	Pool 3#	Pool 4**
M11–240417	<16	256	<2	64	128	>128	>128
M11–240427	<16	128	<2	32	32	64	64
M11–240802	<16	512	<2	32	>64	>64	>64
M12–240016	<16	256	<2	32	32	64	128
M11–240798	<16	512	<2	>64	>64	>64	>64
M12–240754	<16	256	<2	64	64	>64	>64

*Human serum bactericidal antibody titers against 6 meningococcal group W clinical isolates belonging to ST-11 clonal complex using pooled serum from adolescents immunized with the MenACWY conjugate vaccine, Menveo, and from infants immunized with the protein-based, multicomponent meningococcal vaccine, Bexsero.
†Pooled serum samples from adolescents 11–17 years of age before and 1 month after receiving a single dose of MenACWY (Menveo) conjugate vaccine (n = 10).
‡Pooled prevaccination serum samples from infants at 2 months of age (n = 180) (control).
§Pooled serum samples from infants who received 3 doses of vaccine at 2, 3, and 4 months of age and had a blood sample taken 1 month after the last dose of vaccine (n = 56).
¶Pooled serum samples from infants who received 3 doses of vaccine at 2, 4, and 6 months of age and had a blood sample taken 1 month after the last dose of vaccine (n = 47).
#Pooled serum samples from infants who received 3 doses of vaccine at 2, 4, and 6 months of age plus a booster at 12 months and had a blood sample taken 1 month after the booster (n = 72).
**Pooled serum samples from an additional cohort of infants who also received 3 doses of vaccine at 2, 4, and 6 months of age plus a booster at 12 months and had a blood sample taken 1 month after the booster (n = 94).

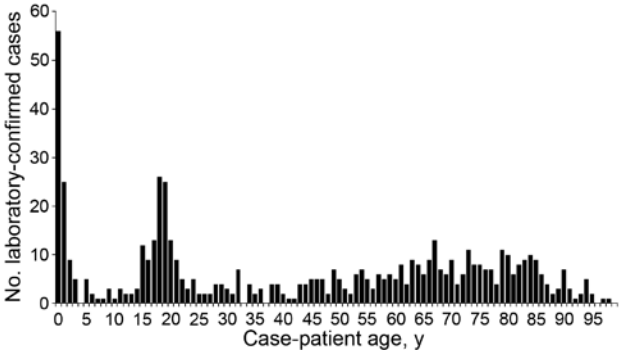

Figure. Age distribution of all laboratory-confirmed, invasive *Neisseria meningitidis* serogroup W disease cases identified in England during July 2009–December 2014.

Conversely, infants would require ≥2 MenACWY doses starting at 2 months of age, because MenW cases increase from birth and peak at 7 months of age before declining. Bexsero, which is predicted to protect against 73%–88% of invasive MenB isolates in England and Wales (*9*,*13*), could offer additional protection against MenB, which causes more cases among infants and toddlers than the other meningococcal serogroups. Among infants (<1 year of age), 101 MenB cases were reported during 2014–2015, compared with 21 MenW, 4 MenY, and 1 MenC; among toddlers, (1–4 years of age), 139 MenB cases were reported, compared with 18 MenW, 5 MenY, and 0 MenC. The difference in adolescents (15–19 years of age) is less pronounced, but 36 MenB cases were reported in this age group during 2014–2015, compared with 25 MenW, 14 MenY and 3 MenC cases (https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/476989/hpr38-3915.pdf). However, the effectiveness of Bexsero against meningococcal carriage and, therefore, its ability to provide herd protection, which is a major objective of an adolescent programme, is less certain than with conjugate vaccines (*14*). These observations and our results support the recent implementation of both the adolescent MenACWY conjugate and infant MenB immunization programmes in the UK.

J.F. and R.B. perform contract research on behalf of Public Health England for GSK, Novartis, Pfizer, Sanofi Pasteur and Sanofi Pasteur MSD, and S.N.L. performs contract research for the same companies on behalf of St. George's University of London. None of the authors have received any personal remuneration. M.M.G., A.B., and M.P. are employees of GSK Vaccines, which manufactures the MenB vaccine, Bexsero.

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Frequency and Distribution of Rickettsiae, Borreliae, and Ehrlichiae Detected in Human-Parasitizing Ticks, Texas, USA

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To describe the presence and distribution of tickborne bacteria and their vectors in Texas, USA, we screened ticks collected from humans during 2008–2014 for *Rickettsia*, *Borrelia*, and *Ehrlichia* spp. Thirteen tick species were identified, and 23% of ticks carried bacterial DNA from at least 1 of the 3 genera tested.

Ticks are vectors for a variety of microorganisms, many of which are known agents of zoonotic disease. Although much current research is focused on areas where these diseases are common, it is crucial to collect data from areas with fewer diagnoses of tickborne illness. In Texas, USA, tickborne diseases caused by *Rickettsia*, *Borrelia*, and *Ehrlichia* bacteria are diagnosed less frequently than in some areas of the United States (*1*); however, those agents have been documented to occur (*2*), and many medically relevant tick species, capable of carrying and transmitting these pathogens, are established in various geographic areas of Texas (*1*). Long-term surveillance data encompassing consecutive seasons and a wide geographic range are necessary to ascertain disease transmission risks associated temporally or geographically with established or emerging tickborne pathogens and their vectors. The University of North Texas Health Science Center Tick-Borne Disease Research Laboratory (UNTHSC-TBDL), the primary tick-testing facility for Texas Department of State Health Services Zoonosis Control (TX DSHS), receives ticks continually throughout the year. The data collected from this testing provide an assessment of the prevalence of tick species and associated tickborne bacterial agents collected in Texas.

The Study

From October 1, 2008, through September 30, 2014, ticks removed from humans were sent by TX DSHS to UNTHSC-TBDL, where they were tested by using PCR-based methods, then underwent by DNA sequence analysis to determine the presence of *Rickettsia*, *Borrelia*, and *Ehrlichia* spp. Morphologic identification of tick species was implemented by entomologists at TX DSHS. Ticks that could not be classified morphologically were identified at UNTHSC-TBDL by sequencing mitochondrial 16S rDNA (data not shown).

Each tick was sent to UNTHSC-TBDL in an individual collection tube. Upon arrival, ticks were processed according to the laboratory's standard protocol, as described by Williamson et al. (*2*). After bead pulverization, we extracted DNA using the E.Z.N.A. Mollusc DNA Isolation Kit (Omega Bio-Tek, Norcross, GA, USA) following the manufacturer's protocol.

DNA from each specimen was screened in duplicate by PCR for *Rickettsia*, *Borrelia,* and *Ehrlichia* spp. as previously described (*2*) by using primers listed in Table 1. PCR products were evaluated, and presumptive-positive amplicons were purified for sequencing (*2*). Cycle sequencing reactions were performed in both directions by using BigDye Terminator version 3.1 chemistry (Life Technologies, Carlsbad, CA, USA). Dideoxy chain termination products were detected electrophoretically on an ABI 310 or 3130xL Genetic Analyzer (Life Technologies). Sequence analysis was performed by using Sequencher version 4.8/5.0 (GeneCodes, Ann Arbor, MI, USA). Analyzed sequences were compared with reference data in GenBank (http://blast.ncbi.nlm.nih.gov/). Sequences were submitted to GenBank under accession nos. KP861333–KP861347.

The TX DSHS submitted 1,112 ticks to UNTHSC-TBDL during October 1, 2008–September 30, 2014, of which 1,062 originated in Texas. Thirteen tick species were identified; most were *Amblyomma americanum* (55.7%), followed by *Dermacentor variabilis* (15.0%), *Rhipicephalus sanguineus* (13.0%), *Ixodes scapularis* (5.6%), *A. maculatum* (5.4%), and *A. cajennense* (2.9%). Approximately 23.3% of ticks originating in Texas tested positive for DNA from *Rickettsia*, *Borrelia*, or *Ehrlichia* bacteria (Table 2; online Technical Appendix Table, http://wwwnc.cdc.gov/EID/article/22/2/15-0469-Techapp1.pdf). Of these bacteria, most belonged to spotted fever group rickettsiae (SFGR); *A. americanum* was the most common tick species found to carry an SFGR agent. The most frequent SFGR sequences detected demonstrated

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DOI: http://dx.doi.org/10.3201/eid2202.150469

Table 1. Primers used for screening of human-parasitizing tick specimens, Texas, USA, October 1, 2008–September 30, 2014

Primer name	Gene	Primer sequence, 5′ → 3′	Specificity	Amplicon, bp	Reference
Borrelia spp.					
FlaLL	*flaB*	ACATATTCAGATGCAGACAGAGGT	Genus	664	(*3*)
FlaRL	*flaB*	GCAATCATAGCCATTGCAGATTGT	Genus		(*3*)
FlaLS	*flaB*	AACAGCTGAAGAGCTTGGAATG	Genus	330	(*3*)
FlaRS	*flaB*	CTTTGATCACTTATCATTCTAATAGC	Genus		(*3*)
BL-Fla 522F	*flaB*	GGTACATATTCAGATGCAGACAGAGGG	*B. lonestari*	660	(*2*)
BL-Fla 1182R	*flaB*	GCACTTGATTTGCTTGTGCAATCATAGCC	*B. lonestari*		(*2*)
BL-Fla 662F	*flaB*	CTGAAGAGCTTGGAATGCAACCTGC	*B. lonestari*	198	(*2*)
BL-Fla 860R	*flaB*	GAGCTAATCCCACCTTGAGCTGG	*B. lonestari*		(*2*)
BL-16S 227F	16S	TCACACTGGAACTGAGATACGGTCC	Genus	693	(*2*)
BL-16S 920R	16S	GAATTAAACCACATGCTCCACCGC	Genus		(*2*)
Rickettsia spp.					
Rr.190 70P	*rompA*	ATGGCGAATATTTCTCCAAAA	Genus	532	(*4*)
Rr.190 602N	*rompA*	AGTGCAGCATTCGCTCCCCCT	Genus		(*4*)
BG1–21	*rompB*	GGCAATTAATATCGCTGACGG	Genus	650	(*5*)
BG2–20	*rompB*	GCATCTGCACTAGCACTTTC	Genus		(*5*)
Ehrlichia spp.					
Ehr DSB 330F	*dsb*	GATGATGTCTGAAGATATGAAACAAAT	Genus	398	(*6*)
Ehr DSB 728R	*dsb*	CTGCTCGTCTATTTTACTTCTTAAAGT	Genus		(*6*)
Ehr map1F	*map*1	ATTTTTACCTGGTGTGTCCTTTTCTGA	Genus	873	(*7*)
Ehr map1R	*map*1	CCTTCCTCCAATTTCTATACC	Genus		(*7*)
Ehr Pmap2F	*map*1	GACACCAAGGCAGTATACGG	Genus		(*7*)
Ehr Pmap2R	*map*1	CTAAGTCAGTACCAATACCTGCAC	Genus		(*7*)
Tick DNA					
16S-1	mt16S	CCGGTCTGAACTCAGATCAAG	Unknown	300	(*8*)
16S+2	mt16S	TTGGGCAAGAAGACCCTATGAA	Unknown		(*8*)

100% identity to *Candidatus* Rickettsia amblyommii *rompA* (GenBank accession no. EF194096). *Candidatus* R. amblyommii was detected in both *A. americanum* and *A. cajennense* ticks and showed prevalence rates of 30.3% and 32.3%, respectively. The second most common SFGR *rompA* sequences were 100% homologous to the previously termed rickettsial *I. scapularis* endosymbiont, which has been officially named *R. buchneri* (accession no. KP172259) (*9*). Five *A. maculatum* specimens contained DNA sequences identical to *R. parkeri rompA* (accession no. KC003476). Sequences that shared 100% similarity to 1 specific *R. rhipicephali* isolate (accession no. U43803) and 99% similarity to other *R. rhipicephali rompA* isolates (accession nos. EU109175–EU109178) were obtained from 4 *D. variabilis* ticks. Sequences isolated from 2 *D. andersoni* ticks were identical to *R. peacockii rompA* and *rompB* (accession nos. FM883671 and CP001227, respectively). Tick species was confirmed by sequencing mitochondrial 16S rDNA. Sequences from both specimens aligned 99% with *D. andersoni* (accession no. EU711343) and 94% with *D. variabilis* (accession no. L34300). *D. andersoni* is not known to inhabit Texas (*1*,*10*), so this finding could suggest a novel geographic association.

The total prevalence of borreliae detected was 1.1%. DNA sequences sharing 100% identity to *B. lonestari* were found in 8 *A. americanum* ticks (1.4%). As seen by Stromdahl et al., the *B. lonestari* isolates matching sequences in this study depended on the insertion or deletion of a nucleotide triplet, AAG (*11*). Sequences from 7 tick samples matched 100% with *B. lonestari flaB* isolates containing the additional triplet (accession no. AY850063), and 1 sequence was identical to *B. lonestari flaB* isolates lacking the triplet (accession no. AY850064). Of the 8 *A. americanum*

Table 2. Number of positive bacterial DNA sequences identified for each human-parasitizing tick species, Texas, USA, October 1, 2008–September 30, 2014*

	No. positive								
	Borrelia			*Ehrlichia*	*Rickettsia*				
Tick	UNID	*burgdorferi*	*lonestari*	*chaffeensis*	amblyommii†	*parkeri*	*peacockii*	*rhipicephali*	*buchneri*
Amblyomma americanum	0	0	8	2	179	0	0	0	0
A. cajennense	0	0	0	0	10	0	0	0	0
A. maculatum	2	0	0	0	0	5	0	0	0
Dermacentor variabilis	1	0	0	0	0	0	0	4	0
D. andersoni	0	0	0	0	0	0	2	0	0
Ixodes scapularis	0	1	0	0	0	0	0	0	44
Rhipicephalus sanguineus	0	0	0	0	0	0	0	0	0
Total	3	1	8	2	189	5	2	4	44

*Only tick species originating in Texas that tested positive for *Borrelia*, *Ehrlichia*, or *Rickettsia* spp. by DNA sequence analysis are shown. Additionally, 2 *A. maculatum* ticks from Texas were positive for Panola Mountain *Ehrlichia*. UNID, unidentified species.
†*Candidatus* species.

ticks from which the *B. lonestari* sequences were obtained, 6 were co-infected with *Candidatus* R. amblyommii. DNA extracts from 1 *I. scapularis* tick contained a sequence consistent with *B. burgdorferi* sensu stricto (s.s.) and was co-infected with *R. buchneri*. The *flaB* sequence matched 100% to (accession no. CP002228), and 99% to (accession no. CP009656) *B. burgdorferi* s.s. reference sequences. The *Borrelia* 16S rDNA sequence showed 100% identity to (accession no. CP009656) and differed by 1 single nucleotide polymorphism from (accession no. CP002228) *B. burgdorferi* s.s. reference sequences. A *flaB* gene sequence from 1 *D. variabilis* tick shared 100% identity with *Candidatus* B. texasensis (accession no. AF264901). Samples from 2 *A. maculatum* ticks showed *flaB* sequences matching 90% identity values to *B. turcica* (accession no. AB109243), a reptilian *Borrelia* sp. Those *flaB* sequences were identical to a novel *Borrelia* sp. (accession no. KF395230) previously found in *A. maculatum* ticks in Mississippi and known to share a phylogenetic clade with *B. turcica* (*12*). *Borrelia* 16S rDNA primers produced nonspecific amplification with these 2 samples.

Phylogenetic analysis was performed by using MEGA version 5.1 (http://www.megasoftware.net) using GenBank reference sequences to examine relationships between the *Borrelia* sp. from this study, *B. turcica*, and both Lyme disease–associated and relapsing fever borreliae (Figure). The results supported findings by Lee et al. that the novel *Borrelia* sp. *flaB* sequences were more closely related to the reptilian *Borrelia* than the other 2 *Borrelia* groups (*12*).

Two *A. americanum* ticks contained DNA sharing 100% identity with *Ehrlichia chaffeensis dsb* (accession no. CP000236). One of these ticks was co-infected with *Candidatus* R. amblyommii. Prevalence of *E. chaffeensis* in the *A. americanum* specimens tested was 0.34%. In addition, 2 of 42 *A. maculatum* ticks tested for the emerging pathogen Panola Mountain *Ehrlichia* sp. (PME) (*7*) each produced a *map*1 sequence that was 100% homologous to 2 separate PME reference sequences (accession nos. EU272356, EU272358). These sequences differed from each other by 1 single nucleotide polymorphism. This finding represents a novel association, as *A. americanum* is the known vector for PME (*7*). A subset of 141 *A. americanum* ticks was also tested for PME, with negative results.

Conclusions

Frequency of tickborne zoonoses in Texas remains low compared with some regions of the United States. We report the detection of known pathogens along with bacteria of unknown pathogenicity in human-parasitizing ticks commonly found in Texas. Our findings underscore the importance of better characterization and continued surveillance of the frequency and distribution of tick species and the bacterial agents they carry. Continued monitoring in low-risk areas provides data regarding the presence of potential emerging pathogens and vectors not yet commonly identified, which could pose unidentified threats to public health.

Acknowledgments

We thank Rhonda Roby for advice regarding this work. We also thank Bonny Mayes, Jim Schuermann, Dave Florin, and staff at Texas DSHS Zoonosis Control for collection and taxonomic identification of tick samples.

This project was financially supported by the State of Texas.

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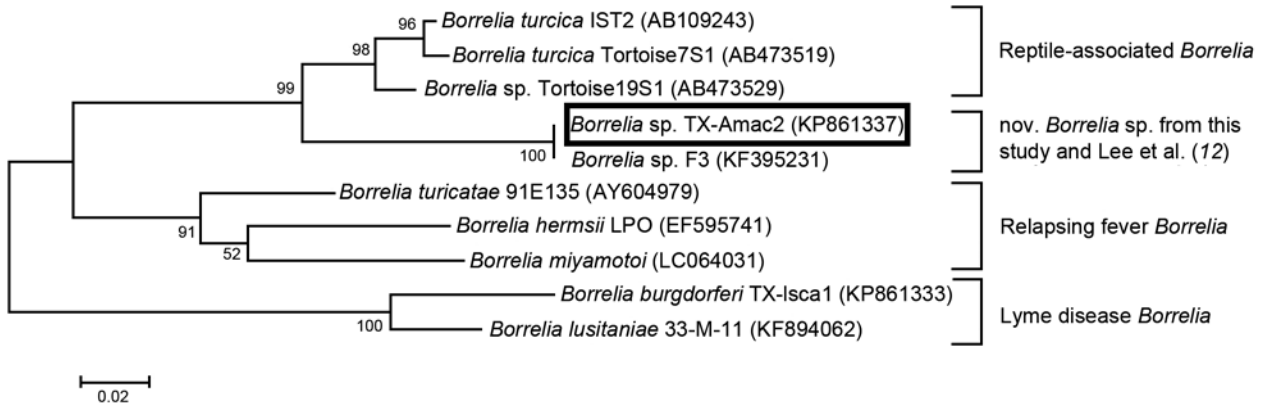

Figure. Maximum-likelihood tree showing that the novel *Borrelia* sp. identified in *Amblyomma maculatum* ticks from Texas in this study (box) and from Mississippi (*12*) shares a closer phylogenetic relationship to *B. turcica* than to to other *Borreliae* groups. Analysis is based on *flaB* sequences (267 bp). GenBank accession numbers are shown in parentheses. Tree was constructed using the Tamura 3-parameter model with a bootstrap value of 1,000 replicates. Scale bar indicates substitutions per nucleotide position.

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High Prevalence of *Borrelia miyamotoi* among Adult Blacklegged Ticks from White-Tailed Deer

Seungeun Han, Graham J. Hickling, Jean I. Tsao

We compared the prevalence of *Borrelia miyamotoi* infection in questing and deer-associated adult *Ixodes scapularis* ticks in Wisconsin, USA. Prevalence among deer-associated ticks (4.5% overall, 7.1% in females) was significantly higher than among questing ticks (1.0% overall, 0.6% in females). Deer may be a sylvatic reservoir for this newly recognized zoonotic pathogen.

Borrelia miyamotoi, a relapsing fever group spirochete detected throughout the range of *Ixodes ricinus* complex ticks, has been implicated recently in human disease in the Northern Hemisphere (*1*–*3*). Enzootic maintenance of *B. miyamotoi*, however, has remained enigmatic since its discovery in Japan in 1995 (*4*). *B. miyamotoi* has been detected in several rodent species and their attached ticks, but the importance of rodents to the enzootic cycle remains uncertain (*5*,*6*). *B. miyamotoi* also has been detected in ticks feeding on European red deer (*Cervus elaphus*) (*7*); domestic ruminants (*8*); and recently by our group, in blacklegged ticks removed from white-tailed deer (*Odocoileus viginianus*) in the eastern United States (*9*). White-tailed deer are key hosts for adult *I. scapularis* ticks and a source of food for juvenile ticks. Thus, if deer are reservoirs for *B. miyamotoi*, in addition to maintaining tick populations, they may play a critical role in the epizootiology of this transovarially transmitted pathogen.

As an initial test of the hypothesis that white-tailed deer may be reservoir hosts, we collected questing and deer-associated adult *I. scapularis* ticks and assayed them for *B. miyamotoi*. We predicted that infection prevalence would be higher among deer-associated adult *I. scapularis* ticks than among questing adults. Furthermore, because females take larger blood meals than do males, we hypothesized that infection prevalence would be highest among deer-associated female ticks.

The Study

All ticks were collected at Fort McCoy in central Wisconsin, USA, where *I. scapularis* ticks are well-established and where several *I. scapularis*–borne pathogens have been detected, including *Borrelia burgdorferi*, *Anaplasma phagocytophilum*, *Babesia microti*, and *B. miyamotoi* (*10*). Questing ticks were collected from vegetation at monthly intervals during April–November (2010–2012) by dragging or flagging as described in Rulison et al. (*11*). Adult ticks were collected from hunter-harvested deer in November 2010.

Total DNA was extracted from individual ticks by using the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA, USA) as described (*12*). We assayed for *B. miyamotoi* infection using a quantitative PCR that targets the 16S rDNA gene and can detect 1 spirochete per tick (*6*,*12*). To confirm the identity of positive samples, we sequenced a fragment of the 16S-23S intergenic spacer region (*12*). We then compared *B. miyamotoi* prevalence between groups, using a Fisher exact test; $\alpha = 0.05$.

We tested 730 questing adult ticks and 355 adult ticks collected from 44 deer (33 males, 7 females, and 4 of undetermined sex) from 49 deer that were checked. Because prevalence of *B. miyamotoi* infection among questing adult ticks did not vary significantly by year (Table; $p>0.05$ for each sex), we pooled data across the years to increase statistical power.

B. miyamotoi–infected adult ticks were collected from 9 of the 44 tick-infested deer (20.5%, all males). Infestation with infected ticks was not significantly correlated with deer age or sex (both $p>0.1$). The infection prevalence among deer-associated adult *I. scapularis* ticks (4.5%), however, was significantly higher than that among questing adults (1.0%; $p = 0.0004$), and the infection prevalence among attached male ticks was significantly lower than that among attached female ticks (2.5% vs. 7.1%; $p = 0.035$, 1-tailed test). The infection prevalence among attached female ticks (7.1%) was 11.8× greater than that among questing female ticks (0.6%; $p<0.0001$, 1-tailed test).

We successfully sequenced a fragment of the intergenic spacer region from 34 of the 39 *B. miyamotoi*–positive samples. All sequences showed 99% similarity with published sequences for *B. miyamotoi* in GenBank (for example, accession no. AY363706). Four representative sequences have been deposited in GenBank (accession nos. KT321365–KT321368).

Conclusions

Lyme borreliosis is the most common vectorborne disease in the United States; ≈30,000 new cases are reported to the Centers for Disease Control and Prevention each year (*13*).

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DOI: http://dx.doi.org/10.3201/eid2202.151218

Table. Prevalence of *Borrelia miyamotoi* among *Ixodes scapularis* ticks collected from white-tailed deer, Fort McCoy, Wisconsin, USA

Origin of ticks	Year	No. *B. miyamotoi*–positive ticks/no. tested (% positive)		
		M	F	Total
Questing on vegetation	2010	1/65 (1.5)	0/49 (0)	1/114 (0.9)
	2011	4/169 (2.4)	1/140 (0.7)	5/309 (1.6)
	2012	0/177 (0.0)	1/130 (0.8)	1/307 (0.3)
	2010–2012	5/411 (1.2)	2/319 (0.6)	7/730 (1.0)
Removed from deer	2010	5/199 (2.5)	11/156 (7.1)	16/355 (4.5)

Given that *B. miyamotoi* uses the same vector ticks as *B. burgdorferi* and that the range of *I. scapularis* ticks continues to expand, it seems inevitable that the human population will be increasingly exposed to *B. miyamotoi* (*13*). *B. miyamotoi* has been found in rodents, but prevalence rates are so low that whether rodents play a key role as reservoirs is questionable (*6*).

If white-tailed deer are reservoir hosts for *B. miyamotoi*, adult ticks removed from deer would be expected to have a higher infection prevalence than sympatric host-seeking ticks collected from vegetation. Furthermore, because female *I. scapularis* ticks take larger blood meals than do males, the difference in prevalence among questing ticks should be most pronounced in female ticks. Both of these predictions are supported by our data: 1) the infection prevalence of adult ticks removed from deer was >4.5× that of questing adults, and 2) the infection prevalence of engorging females was >11× that of questing females.

Our data indicate that white-tailed deer at least permit *B. miyamotoi* to remain viable in the feeding ticks, in marked contrast to the situation with the Lyme disease agent, which is rapidly lysed by deer blood complement (*14*). Further research is needed to clarify how the ecoepidemiology of *B. miyamotoi* differs from that of *B. burgdorferi* and thus help inform public health management regarding diagnosis, treatment, and prevention of disease.

We note 2 caveats that arise from possible alternative explanations for the observed increase in *B. miyamotoi* infection prevalence among deer-associated adult ticks. First, spirochete numbers in infected questing adults could have been below the detection threshold of our quantitative PCR so that many questing ticks tested negative when they were positive (type II error). Then, as ticks were engorging, *B. miyamotoi* spirochetes may have reproduced sufficiently to rise above the threshold of detection. However, the mean PCR cycle threshold values at which *B. miyamotoi* was detected in questing ticks versus deer-fed ticks did not differ significantly (27.9 vs. 32.1, respectively; Wilcoxon rank sum test, $p = 0.20$). Thus we see no sign that false-negative rates would differ. Until more is known about the growth kinetics of *B. miyamotoi* in ticks that are engorging on a competent host, we cannot rule out this explanation. Nonetheless, if blood meal amplification occurs, it would strengthen the possibility that deer could be reservoir hosts.

The second caveat is that infection prevalence of ticks attached to deer could be elevated if venereal transmission of *B. miyamotoi* occurred during copulation. Preprandial mating has been documented in *I. scapularis* ticks, and mate guarding is common (*15*); however, that venereal transmission alone could produce the 4.5–11× increase in prevalence reported here seems unlikely.

If deer prove to be notable reservoirs for this pathogen, deer management practices to reduce tick populations and Lyme disease risk may provide additional health benefits by weakening *B. miyamotoi* transmission dynamics. Further supportive evidence for deer as a key reservoir could come in part from surveys of deer for infection with *B. miyamotoi* and by comparing infection prevalence of ticks removed from deer versus infection prevalence among ticks removed from other host species. A definitive answer, however, will require logistically challenging controlled transmission studies that quantify reservoir competence and characterize the course of infection in deer. The findings reported here point to the need for such studies.

Acknowledgments

We thank Fort McCoy Military Installation for permission to conduct the research and D. Beckmann, many students, and numerous hunters for facilitating sample collection. We are also grateful to I. Arsnoe for sample and database management, M. Wiseman and J. Landgraf for technical assistance, L. Beati and her students for assistance with DNA extractions, and H. Ginsberg, N. Ogden, and 2 anonymous reviewers for helpful comments.

Funding was provided by the National Science Foundation (Emerging Infectious Disease Award EF-0914476) (to J.I.T. and G.J.H.) and Michigan State University's graduate program in Comparative Medicine Integrative Biology (to S.H.).

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Bordetella pertussis Strain Lacking Pertactin and Pertussis Toxin

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A *Bordetella pertussis* strain lacking 2 acellular vaccine immunogens, pertussis toxin and pertactin, was isolated from an unvaccinated infant in New York State in 2013. Comparison with a French strain that was pertussis toxin–deficient, pertactin wild-type showed that the strains carry the same 28-kb deletion in similar genomes.

Pertussis has resurged in the United States in recent decades; >48,000 cases were reported in 2012 (http://www.cdc.gov/pertussis/surv-reporting/cases-by-year.html). Suggested causes include improved surveillance and diagnostics, waning immune response to acellular vaccines introduced in the United States in the 1990s (DTaP [diphtheria, tetanus, and pertussis]; Tdap, [tetanus, diphtheria, and pertussis]), and changes to circulating *B. pertussis* strains, which led to a mismatch with vaccine strains (*1*). Components of acellular pertussis vaccines in the United States are pertactin (Prn), pertussis-toxin (Pt), filamentous hemagglutinin, and sometimes fimbrial proteins 2/3. Since 2010, multiple mutations have been documented in the Prn-encoding gene (*prn*), which have spread rapidly across the United States and other countries (*2*,*3*). Pt-deficient *Bordetella pertussis* isolates are rare, with 1 report from France (*4*). To our knowledge, *B. pertussis* that lacks Pt and an additional acellular vaccine immunogen has not been documented.

The Case

Prodromal pertussis symptoms developed on March 4, 2013, in an 11-month-old white, non-Hispanic infant from New York State while the family was traveling outside the state. Cough reportedly began on March 14, 2013, and 12 days later (March 26) he was brought to his healthcare provider (HCP) with symptoms consistent with pertussis. Since the child's birth, the diagnosing HCP had seen the child only once; no visits to other HCPs were known. Per parental report, the case-patient was experiencing paroxysmal cough, apnea, and posttussive vomiting. No thoracic radiograph was obtained. A 5-day course of oral azithromycin was prescribed; the parent reported that the infant received treatment for 3 consecutive days, beginning March 26, 2013. The infant was not reported to have any pertussis-associated complications (seizures, pneumonia, or encephalopathy) and had only light coughing as of April 11, 2013.

The infant was unvaccinated because the parents refused administration of all vaccines. Three siblings, ages 12, 10, and 8 years, lived with the infant and were undervaccinated; they had received 2, 1, and 3 doses, respectively, of pertussis-containing vaccines. No coughing illness was reported among the siblings. The mother reported that she received Tdap vaccine during her pregnancy with the case-patient, but receipt of vaccine could not be verified.

A nasopharyngeal swab specimen was collected from the infant on March 26, 2013, for testing at a commercial laboratory. The isolate was also forwarded to New York State's public health laboratory, the Wadsworth Center, where it was found to be positive for *B. pertussis* by PCR targeting IS*481* and BP283 (*5*). Both laboratories yielded positive culture results for *B. pertussis*. No other testing was performed.

The Wadsworth Center forwarded the isolate, designated I979, to the Centers for Disease Control and Prevention (CDC; Atlanta, Georgia, USA) for confirmatory identification and molecular typing as part of the Enhanced Pertussis Surveillance program (*6*). PCR amplification of the gene encoding the first subunit of Pt (*ptxA*) was unsuccessful while the CDC multitarget real-time PCR diagnostic assay was performed (*7*). Amplification of the promoter region (*ptxP*) and *ptxA* was also unsuccessful during multilocus sequence typing targeting acellular vaccine component genes *ptxA*, *ptxP*, *prn*, and *fim3* (*8*).

Further characterization of I979 and French strain FR3749 (*4*) was undertaken by multilocus sequence typing, multilocus variable-number tandem-repeat analysis, pulsed-field gel electrophoresis (*9*), and whole-genome sequencing. Long sequencing reads were obtained with the Pacific Biosciences RS II (Menlo Park, CA, USA) at >120× coverage and assembled de novo into a single contig by using HGAP v3 and Quiver v1 (Pacific Biosciences). Assembly structure was confirmed with a genome

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DOI: http://dx.doi.org/10.3201/eid2202.151332

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Table 1. Characterization of *Bordetella pertussis* strains I979 and FR3749 in comparison to strain Tohama I*

Strain	GenBank accession no.	Length, bp	No. IS*481*	*prn* type	Prn	Reference
FR3749	CP010965	4,079,396	249	2	+	This study
I979	CP010966	4,082,551	252	2	–	This study
Tohama I	NC_002929.2	4,086,189	238	1	+	(*10*)

*Prn, pertactin; + positive; –, negative.

optical map after restriction digestion with *Kpn*I (OpGen, Gaithersburg, MD, USA). The final sequence was polished with short reads obtained with Illumina MiSeq and CLC Genomics Workbench v7.5.1 (QIAGEN, Valencia, CA, USA) with >90× coverage. Completed genomes were submitted to the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) with GenBank accession nos. CP010965 (FR3749) and CP010966 (I979). Basic genome metrics are listed in Table 1.

Prn production was determined by ELISA (*2*,*11*). Pt production was examined through Western blot analysis of cultures grown in cyclodextrin-modified Stainer-Scholte liquid medium to optical density (OD) 600 nm = 0.1 (*12*). Proteins precipitated with trichloroacetic acid were washed, reduced, and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Pt detection is described in Figure 1.

I979 and FR3749 share the same multilocus variable-number tandem-repeat analysis type 27 and *prn-2* genotype, the most common recent types (*3*,*13*). I979 and FR3749 are *fim3–1* and *fim3–2*, respectively. The *fim3* locus has fluctuated between these 2 alleles recently (*3*,*8*). Pulsed-field gel electrophoresis indicated that I979 displays profile CDC306, and FR3749 displays CDC046. I979 and FR3749 both lacked Pt production, as shown by Western blot (Figure 1). I979 also failed to produce Prn, whereas FR3749 was positive for Prn production by ELISA, within the range of negative (OD 0.3–0.6) and positive (OD 1.2–1.6) controls.

Comparison of assembled I979 and FR3749 genomes with that of Tohama I (GenBank accession no. NC_002929.2) (*10*) indicated that the entire *ptx/ptl* operon is missing as the result of a putative deletion spanning 28,040 bp (Figure 2). Both genomes contain a conserved, truncated IS*481* immediately upstream of the deletion and a single IS*481* (FR3749) or 2 tandem IS*481* sequences (I979) immediately downstream (Figure 2). Within Tohama I, the region absent from I979 and FR3749 encodes 30 predicted genes bound by 2 NCATGN motifs, the target sequence for IS*481* insertion (Table 2). The insertion element IS*1002* is located within the 3′ end of this region, and shares a GCATGG motif with IS*481* immediately downstream. The 3′ deletion boundary is between IS*1002* and IS*481* (Figure 2). Whole-genome alignment, using progressiveMauve (*14*), of I979 and FR3749 with Tohama I revealed structural variation through genomic rearrangements and inversions. In particular, I979 and FR3749 genomes differ by a single, large inversion, the coordinates of which correspond to 2 conserved insertions of IS*481* in opposing orientations (online Technical Appendix Figure). I979 and FR3749 differ by 31 single nucleotide polymorphisms, each differing from Tohama I by 204 and 173 single nucleotide polymorphisms, respectively. FR3749 contains wild-type *prn* at position 1613, whereas I979 *prn* contains an IS*481* insertion, the most common cause of Prn-deficiency, at position 1613 (*2*,*11*).

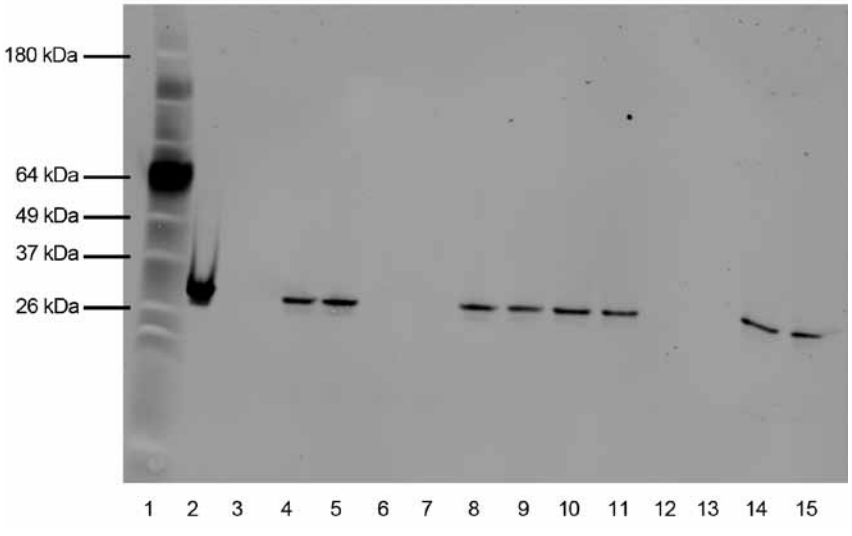

Figure 1. Western blot of pertussis toxin (Pt) expression in *Bordetella pertussis* Tohama I, I979, FR3749, and 3 additional recent isolates. All isolate lanes were loaded with 10-μg of protein, extracted after growth for 48 hours. Protein was transferred with the iBlot Dry Blotting system (Invitrogen, Carlsbad, CA, USA). The primary antibody consisted of 1b7 anti-PTX S1 monoclonal antibody at a concentration of 20 μg/mL diluted in 0.01 M PBS/Tween with 5% milk. The secondary antibody was a FITC-conjugated goat anti-mouse pAb from AbCam, diluted 1:1,000 with 0.01 M PBS/Tween with 5% milk. Lane 1, benchmark protein ladder, 6–180 kDa (Life Technologies, Grand Island, NY, USA); lane 2, Pt positive control, 2 μg; lane 3, empty; lanes 4 and 5, J024 (pertactin (Prn)+/Pt+); lanes 6 and 7, I979 (Prn-/Pt-); lanes 8 and 9, Tohama I (Prn+/Pt+); lanes 10 and 11, I978 (Prn-/Pt+); lanes 12 and 13, FR3749 (Prn+/Pt-); lanes 14 and 15, I974 (Prn-/Pt+).

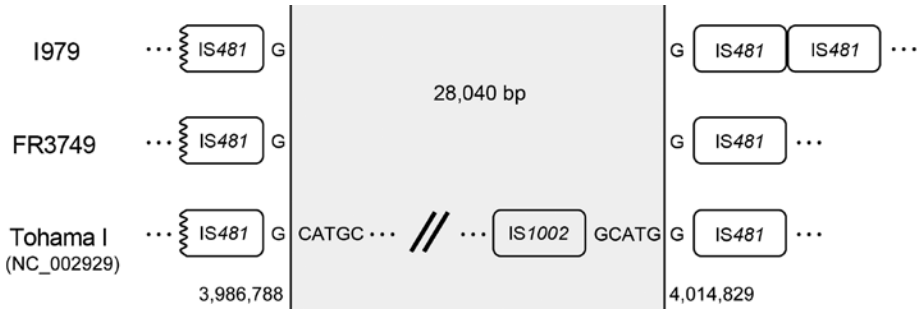

Figure 2. Map of the 28-kb region deleted in *Bordetella pertussis* strains I979 and FR3749, compared with vaccine strain Tohama I. Vertical lines indicate the deletion boundaries, at a nucleotide G of the IS*481* 6-base insertion site motif. The deleted region is flanked by a single IS*481* on each end in Tohama I and FR3749; I979 contains a second tandem IS*481* downstream of the deletion. In Tohama I, IS*1002* is located in the deletion region immediately upstream of IS*481,* and they share the 6-base motif.

Conclusions

B. pertussis strain I979, identified in our study, is both Prn- and Pt-deficient. Loss of Pt in *B. pertussis* is a rare occurrence; only 2 isolates have been documented in 8 years. Both I979 and FR3749 were isolated from unvaccinated infants (11 months and 3 months old, respectively), who exhibited typical pertussis symptoms, although FR3749 had difficulty colonizing and multiplying in respiratory tracts of adult mice (*4*). *B. pertussis* isolates with deletions at other sites across the genome, including part or all of *prn*, were reported previously (*4*,*15*). During the past 5 years, US *B. pertussis* isolates have become nearly 100% Prn-deficient (*2*,*3*) (unpub. data), and Prn-deficient isolates have been obtained from vaccinated persons (*11*). The loss of Pt may represent a higher fitness cost to *B. pertussis* than the loss of Prn. In addition, the possibility that only the Pt-deficient isolates were recovered from patients who were co-infected with wild-type and mutant *B. pertussis* cannot be discarded. Further testing in models to understand the clinical relevance of Prn- and Pt-deficient strains in vaccinated and unvaccinated persons is warranted.

Although incidence of combined Pt- and Prn-deficiency in *B. pertussis* is rare, any increased mutation in these

Table 2. Genes in *Bordetella pertussis* vaccine strain Tohama I deleted from *B. pertussis* I979 and FR3749 genomes, including the entire *ptx/ptl* operon*

Protein ID, GenBank accession no.	Gene	Product
NP_882281.1		Hypothetical protein
NP_882282.1	*ptxA*	Pertussis toxin subunit 1
NP_882283.1	*ptxB*	Pertussis toxin subunit 2
NP_882284.1	*ptxD*	Pertussis toxin subunit 4
NP_882285.1	*ptxE*	Pertussis toxin subunit 5
NP_882286.1	*ptxC*	Pertussis toxin subunit 3
NP_882287.1	*ptlA*	Type IV secretion system protein PtlA
NP_882288.1	*ptlB*	Type IV secretion system protein PtlB
NP_882289.1	*ptlC*	Type IV secretion system protein PtlC
NP_882290.1	*ptlD*	Type IV secretion system protein PtlD
NP_882291.1	*ptlI*	Type IV secretion system protein PtlI
NP_882292.1	*ptlE*	Type IV secretion system protein PtlE
NP_882293.1	*ptlF*	Type IV secretion system protein PtlF
NP_882294.1	*ptlG*	Type IV secretion system protein PtlG
NP_882295.1	*ptlH*	Type IV secretion system protein PtlH
		tRNA-Asn
NP_882296.1		Membrane protein
NP_882297.1		AraC family transcriptional regulator
NP_882298.1		Hypothetical protein
NP_882299.1		Membrane protein
NP_882300.1		Hypothetical protein
NP_882301.1		Peptide ABC transporter substrate binding protein
NP_882302.1		Transport system permease
NP_882303.1		Transport system permease
		Pseudogene
NP_882304.1	IS*481*	Transposase
NP_882305.1	*argJ*	Bifunctional ornithine acetyltransferase/N-acetylglutamate synthase
NP_882306.1		Hypothetical protein
NP_882307.1		Hypothetical protein
NP_882308.1	IS*1002*	Transposase

*ID, identification; *ptx*, pertussis toxin gene; *ptl*, pertussis toxin liberation gene.

or other acellular vaccine immunogens may have serious implications for the efficacy of current vaccines. Global epidemiologic, culture-based, and molecular-based monitoring of *B. pertussis* is critical for understanding current trends of the disease it causes.

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Acknowledgments

B. pertussis isolate I979 was contributed by the state of New York through participation in the Enhanced Pertussis Surveillance of the Emerging Infections Program Network. In particular, we thank Kathy Root, Kimberlee Musser, and Tammy Quinlan for providing clinical data and isolate I979 and for performing pertussis PCR testing. We also thank Nicole Guiso, Institut Pasteur, Paris, France, for sharing isolate FR3749.

Funding for genome sequencing was provided internally by CDC as part of its Advanced Molecular Detection Initiative.

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The Merits of Malaria Diagnostics during an Ebola Virus Disease Outbreak

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Malaria is a major public health concern in the countries affected by the Ebola virus disease epidemic in West Africa. We determined the feasibility of using molecular malaria diagnostics during an Ebola virus disease outbreak and report the incidence of *Plasmodium* spp. parasitemia in persons with suspected Ebola virus infection.

The Ebola virus disease (EVD) epidemic occurring in West Africa is unprecedented in its duration and scale; as of October 28, 2015, a total of 28,575 suspected, probable, and confirmed cases, including 11,313 deaths, have been reported (*1*). Healthcare workers have been severely affected, and the epidemic has resulted in an almost complete breakdown of the public health infrastructure that undoubtedly resulted in many deaths from otherwise treatable conditions and diseases. Particularly concerning are the effects of lapses in childhood vaccination, antenatal and emergency obstetric care, HIV treatment, and malaria control (*2*–*7*).

Malaria is a major public health concern in Guinea, Liberia, and Sierra Leone, the 3 countries most affected by the EVD epidemic. In 2012, these countries accounted for ≈9 million malaria cases and 30,566 associated deaths (*8*). Symptoms of Ebola virus (EBOV) infection and malaria overlap to a great extent, especially early during the course of disease; fever, headache, chills, and vomiting are observed frequently in both diseases. Malaria transmission occurs year-round in Liberia. Therefore, it is recommended that every patient with suspected malaria receive treatment for presumptive malaria when they first seek medical care at an Ebola treatment unit (ETU) or triage point (*9*,*10*). A diagnostic test to detect *Plasmodium* spp. parasitemia was implemented in the joint Centers for Disease Control and Prevention–National Institutes of Health (CDC–NIH) diagnostic laboratory located at the Eternal Love Winning Africa (ELWA) campus in Monrovia, Liberia.

Plasmodium spp. parasitemia can be detected by using rapid diagnostic tests (RDTs), light microscopy, or PCR. Of these methods, PCR is the most sensitive (0.004 parasites/μL) (*11*), and light microscopy is the reference diagnosis standard (5–10 parasites/μL). Both methods require highly qualified personnel and, in the case of PCR, specialized equipment to perform the test and analyze the results. An RDT produces results quickly and is simple to use, but it is the least sensitive method (>100 parasites/μL) (*12*), and most RDTs detect only *P. falciparum*. Light microscopy analysis poses difficulties in an EVD outbreak because of the required handling of infectious material and the need for proper personal protective equipment, so this diagnostic service usually is discontinued during EVD outbreaks. Therefore, because all blood samples submitted to

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DOI: http://dx.doi.org/10.3201/eid2202.151656

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the ELWA laboratory were analyzed by real-time quantitative reverse transcription PCR (qRT-PCR) for the presence of EBOV, we chose to screen for *Plasmodium* spp. parasitemia also by using qRT-PCR. Here we determine the feasibility of using molecular *Plasmodium* spp. diagnostics during an EVD outbreak and report the incidence of *Plasmodium* spp. parasitemia in persons with suspected EBOV infection.

The Study

During October 12, 2014–March 28, 2015, samples collected from 1,058 persons in Liberia with suspected EBOV infection were submitted to the CDC–NIH ELWA laboratory. The samples used for this research were collected for public health surveillance and not human subjects research, so institutional review board review and approval were not required. Early during the study period (October–November), most of the patients from whom samples were collected received a diagnosis of EBOV infection (Figure 1, panel A); toward the end, however, few cases of EBOV infection were diagnosed in Liberia. The number of patients who received a diagnosis of *Plasmodium* spp. parasitemia remained stable over time; thus, despite the lack of positive EBOV test results in the final months of the study period, overall, 40%–60% of patients each week received a diagnosis of EBOV infection, *Plasmodium* spp. parasitemia, or both (Figure 1, panel A). Of 1,058 samples tested, 259 (24.5%) were positive for EBOV alone, 243 (23%) were positive for *Plasmodium* spp. alone, and 47 (4.4%) were positive for both (Figure 1, panel B). Of 311 *Plasmodium*-positive samples that were further analyzed, 296 (95%) were positive for *P. falciparum* (Figure 1, panel C), confirming that *P. falciparum* was the main *Plasmodium* species causing parasitemia in our cohort.

The cycle threshold (C_t) values observed in the *Plasmodium* spp. qRT-PCR results are a proxy for parasite load (Figure 2). A high C_t value corresponds to a low-level parasitemia; the lower the C_t value, the higher the number of *Plasmodium* parasites detected. All patients in the cohort were triaged as having suspected EBOV infection, and thus all had clinical symptoms that might have been caused by EBOV, *Plasmodium* spp., or a different pathogen. Of note, significantly fewer *Plasmodium* parasites were detected in patients with EBOV infection than in patients who were

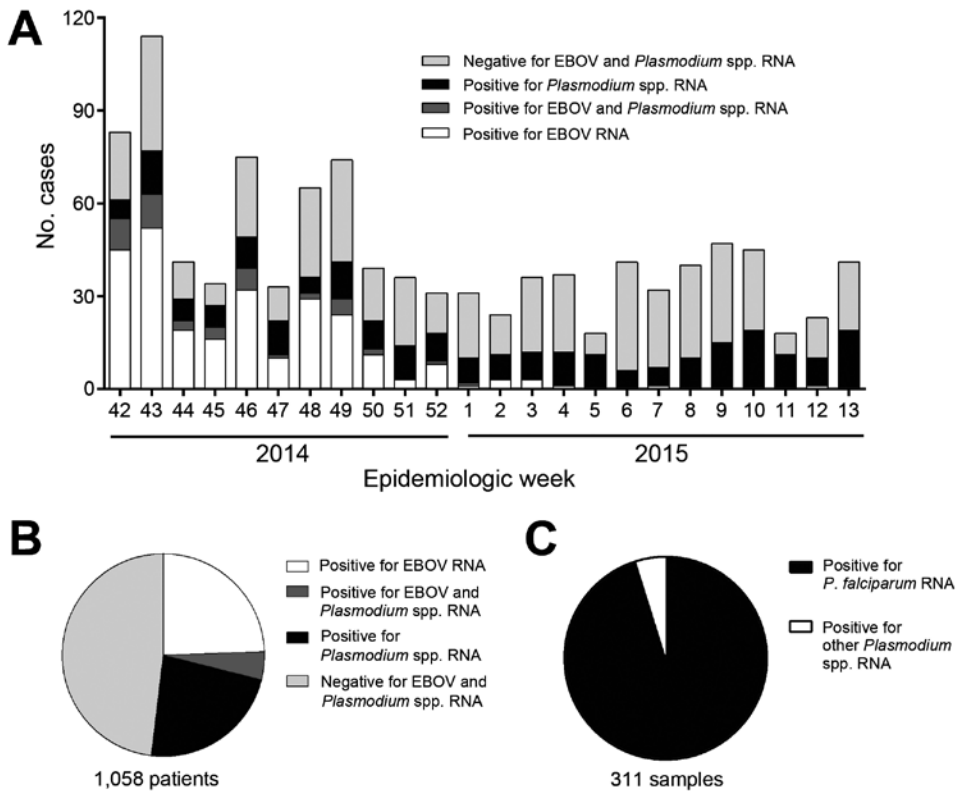

Figure 1. Prevalence of Ebola virus (EBOV) and *Plasmodium* spp. RNA in patient samples submitted to the Centers for Disease Control and Prevention–National Institutes of Health diagnostic laboratory at the Eternal Love Winning Africa campus in Monrovia, Liberia, from October 12, 2014 (epidemiologic week 42), through March 28, 2015 (week 13). Whole blood samples were inactivated, and RNA was extracted by using the QIAAmp Viral RNA Mini Kit (QIAGEN, Hilden, Germany). These samples were then tested for the presence of EBOV RNA and *Plasmodium* spp. RNA by real-time quantitative reverse transcription PCR (qRT-PCR) (*13*). A) Number of patients, as determined by qRT-PCR, positive for EBOV, *Plasmodium* spp., both, or neither (i.e., no EBOV and no *Plasmodium* spp.), by epidemiologic week. B) Total number of patients receiving a laboratory diagnosis of Ebola viremia, *Plasmodium* spp. parasitemia, both, or neither. C) A subset of 311 *Plasmodium* spp. qRT-PCR–positive samples that were retested with a qRT-PCR specific for *P. falciparum* (*14*).

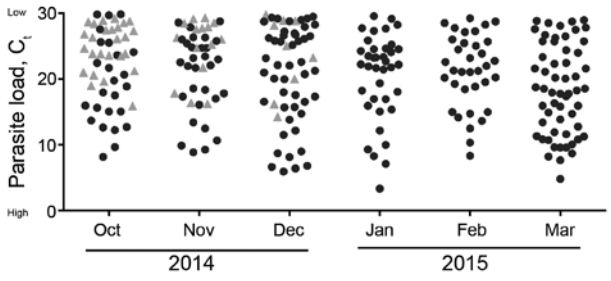

Figure 2. Inverse parasite load in patients with *Plasmodium* spp. parasitemia over time by month of sample submission, for samples submitted to the Centers for Disease Control and Prevention–National Institutes of Health diagnostic laboratory at the Eternal Love Winning Africa campus in Monrovia, Liberia, from October 12, 2014, through March 28, 2015. Cycle threshold (C_t) values were detected by using real-time quantitative reverse transcription PCR. Triangles represent parasite loads in parasitemic patients co-infected with Ebola virus; circles represent patients with *Plasmodium* spp. parasitemia only. In West Africa, similar to other malaria-endemic regions, a large proportion of the population is infected with *Plasmodium* parasites without developing clinical disease. To compensate for this and the higher sensitivity of the PCR assay compared with light microscopy, we used a cutoff of $C_t \leq 30$ rather than $C_t \leq 40$ under the assumption that a 10-C_t difference would compensate for the ≈1,000-fold higher sensitivity of PCR over microscopy. This principle could be carried further to assume that a $C_t \leq 25$ would be in the range detectable by the rapid diagnostic test. Of note, high C_t values correspond to low parasitemia levels and vice versa.

not infected with EBOV (average *Plasmodium* C_t 24.7 vs. 20.37; $p<0.01$ by unpaired Student *t*-test), likely because clinical symptoms in these patients were caused by the EBOV infection rather than malaria. Some of the patients in our cohort with *Plasmodium* spp. parasitemia might have been asymptomatic carriers, especially those with a low-level parasitemia.

Conclusions

One could argue that because all patients with febrile illness who were seen at many of the ETUs were given antimalarial treatment when they first sought medical care, providing laboratory testing for *Plasmodium* spp. parasitemia is not useful. Presumptive artemisinin-based combination treatment is recommended for all patients seen at ETUs, followed by prompt malaria diagnostic testing so that appropriate measures can be taken if oral treatment cannot be sustained because of clinical symptoms (*9*,*10*). Moreover, mathematical models predict a large increase in malaria in the epidemic region because of lapses in malaria control (*7*). Therefore, *Plasmodium* spp. parasitemia testing of all febrile patients seen at healthcare facilities could be used to determine whether this increase is indeed occurring so that countermeasures can be scaled up accordingly.

Differential diagnostic testing should be expanded to identify the cause of disease in the patients in our cohort whose samples tested negative for EBOV and *Plasmodium* spp., as was attempted in 1 laboratory in Sierra Leone (*15*). However, this kind of testing was difficult for several reasons. First, during the height of the EVD epidemic in Liberia, most laboratories were already working close to capacity while testing for EBOV alone. Testing for additional pathogens would have required the allocation of additional resources, including equipment and personnel. Second, it might not be possible to perform diagnostics using a whole blood sample for all the pathogens of differential diagnostic importance that can cause signs and symptoms similar to those of EVD (e.g., typhoid, bacterial sepsis, shigellosis, cholera, leptospirosis, dengue fever, rickettsioses, relapsing fever, meningitis, viral hepatitis, influenza, Lassa fever). Third, each of these pathogens likely is the cause of disease in only a small subset of febrile patients. Last, the prevalence of these pathogens likely differs in each outbreak region. Because broad-spectrum antimicrobial drugs and antimalarial drugs are routinely administered to all patients seen at an ETU, consideration for additional testing should focus on endemic pathogens that would not respond to these treatments.

By using PCR-based detection for *Plasmodium* spp. parasitemia, the need for additional handling of clinical specimens possibly infected with EBOV was eliminated. Thus, the addition of PCR-based diagnostic tests to detect *Plasmodium* spp. does not pose an additional safety risk to laboratory staff. Also, it is less time-consuming to add additional PCR reactions to a PCR run than to separately perform microscopy or an RDT on each sample, and these additions would add only ≈15 minutes to the overall time needed from sample submission to reporting of results. Moreover, if a multiplex approach is used, the additions would not require extra time or reagents. Taken together, our findings suggest that PCR-based testing for *Plasmodium* spp. parasitemia can be implemented easily and safely in laboratories performing EBOV diagnostics to assist with case-patient management during EVD outbreaks in malaria-endemic areas.

Acknowledgments

The authors would like to thank Mulbah Jallah, Monrovia, Liberia, for his excellent support and assistance in operating the laboratory at ELWA, as well as Kay Menk and Dawn Clifton for logistical support. We further acknowledge the World Health Organization headquarters in Geneva, Switzerland; the World Health Organization Regional Office for Africa; the Liberia Ministry of Health and Social Welfare; CDC; NIH; and ELWA. Last, we would like to thank the people of Liberia for their hospitality and cooperation.

This work was funded in part by the Intramural Research Program of the National Institute of Allergy and Infectious Diseases, NIH.

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Microevolution of Outbreak-Associated Middle East Respiratory Syndrome Coronavirus, South Korea, 2015

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During the 2015 Middle East respiratory syndrome coronavirus outbreak in South Korea, we sequenced full viral genomes of strains isolated from 4 patients early and late during infection. Patients represented at least 4 generations of transmission. We found no evidence of changes in the evolutionary rate and no reason to suspect adaptive changes in viral proteins.

Middle East respiratory syndrome coronavirus (MERS-CoV), first detected in Saudi Arabia in 2012, is a novel human pathogen that causes severe respiratory illness (*1*). Phylogenetic analyses and transmission studies suggest a zoonotic origin in dromedaries (*2*,*3*). Human-to-human transmission among close contacts of patients (e.g., family members and persons in healthcare settings) has been described (*4*). As of October 2015, the World Health Organization had received reports of 1,593 cases (including at least 568 deaths), most of which were reported from the Arabian Peninsula.

In South Korea, the first imported MERS-CoV case was identified on May 20, 2015, in a 68-year-old man who had traveled to the Middle East 2 weeks earlier (*5*). Another 185 persons in South Korea were subsequently infected during a 4-week period, mainly through in-hospital transmission. The unusually large number of cases, which occurred during at least 4 sequential generations of human-to-human transmission, raised questions regarding potential adaptations of MERS-CoV to the human host. To determine the possibility of virus adaptation, we repeatedly sequenced complete genomes for MERS-CoV from 4 patients representing different generations of transmission during the South Korea outbreak.

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DOI: http://dx.doi.org/10.3201/eid2202.151700

The Study

Institutional review boards of the Seoul National University Hospital and the National Medical Center approved this study. We tested samples from 4 patients, designated as patients 14, 35, 163, and 168 (Table 1; online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/2/17-1700-Techapp1.pdf). Patients 14, 35, and 168 were second-, third-, and fourth-generation case-patients, respectively; each had recorded exposure histories (*5*) (Figure 1). Patient 163 had a recorded transmission history that traced back to patient 119. However, for 1 interim transmission, the place and approximate time of exposure could be reconstructed, but individual contacts could not be determined. Thus, patient 163 might belong to at least the fourth, but potentially the fifth, generation of transmission (*5*).To identify virus changes, we obtained 2 samples from each of the 4 patients, 1 at the early and 1 at the late stage of infection. Clinical samples were tested for MERS-CoV RNA by reverse transcription PCR (RT-PCR) targeting the upE (upstream of E) and ORF1 (open reading frame 1) genes (*6*). Using virus quantity estimates as a basis, we determined full genomes by amplifying overlapping PCR products and sequencing as previously described (*7*).

Genome sequences with a minimum length of 29,831 bp (99.04% of the genome) were obtained from each specimen and submitted to GenBank (accession nos. KT374050– KT374057). All sequences clustered phylogenetically with MERS-CoV strains identified during the outbreak and with a sequence from a linked case in China (*8*,*9*). Our findings confirmed previously described evidence for recombination between MERS-CoV clades (*10*). MERS-CoV strain Hu/Riyadh KSA_2959_2015, the closest related strain outside the South Korea outbreak, was used as an outgroup to reconstruct the phylogeny of the 8 viral genomes (online Technical Appendix Figure); the strain was isolated in Riyadh, Saudi Arabia, during February 2015.

[1]These authors contributed equally to this article.

Table 1. Characteristics of cases and samples in a study of the microevolution of 8 isolates obtained from 4 patients during a MERS-CoV outbreak, South Korea, 2015*

Patient no., date of symptom onset	Sample type	Date of sample collection	Cycle threshold for upE/ORF1a	Transmission generation (source of transmission)
14, May 25				Second (patient 1 to 14)
Sample 1	ETA	May 31	24.0/25.2	
Sample 2	Sputum	Jun 13	29.0/31.5	
35, May 29				Third (patient 1 to 14 to 35)
Sample 1	Sputum	Jun 3	24.7/25.3	
Sample 2		Jun 18	27.5/28.2	
168, June 17	ETA			Fourth (patient 1 to 14 to 76 to 168)
Sample 1	Sputum	Jun 21	31.6/32.3	
Sample 2	Sputum	Jun 24	28.9/28.3	
163, June 13				Fourth or fifth (patient 1 to ? to 52 to 119 to 163)
Sample 1	Sputum	Jun 19	20.2/20.9	
Sample 2	Sputum	Jun 29	28.4/28.8	

*ETA, endotracheal aspirate; MERS-CoV, Middle East respiratory syndrome coronavirus; ORF1a, open reading frame 1a gene; upE, upstream of E gene.

The 8 strains from South Korea shared 99.8%–99.9% nt identity with Hu/Riyadh KSA_2959_2015, deviating by 24–27 positions across the genome. Sequences for the viruses showed 13 variant nucleotide positions: 6 in the ORF1ab gene, 5 in the spike gene, and 1 each in accessory genes ORF4a and ORF5 (Figure 2). Of the 13 variants, 11 caused nonsynonymous substitutions (online Technical Appendix Table 2). To analyze substitutions along the transmission tree, we reconstructed the unknown sequence of the index case-patient's virus based on an isolate from his wife (GenBank accession no. KT029139), a virus from a patient who traveled to China (accession no. KT006149), the co-ancestral strain from Saudi Arabia (accession no. KT026453), and all sequences determined in this study. Considering that sequencing errors were possible in strains KT029139 and KT006149 due to cell culture adaptation and differences in sequencing technique (*8*,*9*), and assuming no mutation reversion within short human-to-human passage, an unequivocal ancestral sequence reconstruction was possible (*11*). The index case-patient showed overt symptoms by May 11, indicating virus exposure occurred around May 1 (*5*). This date coincides with days (May 1 and 2) that the index case-patient spent in Saudi Arabia, where, at the time, viruses most closely related to the South Korea strains were circulating (*10*). Thus, that starting sequence, as determined by ancestral sequence reconstruction, was assumed to have existed on May 1.

Substitutions observed along the transmission tree corresponded, on average, to 3.78×10^{-6} substitutions/site/day (Table 2). The average substitution rate within individual patients (comparing first and second samples for each patient) was not significantly different: 3.44×10^{-6} substitutions/site/day. The between-patient evolutionary rate was projected to be 1.3×10^{-3} substitutions/site/year, which is within the CI of an earlier estimate of the evolutionary rate for MERS-CoV: 1.12×10^{-3} substitutions/site/year (95% CI 8.76×10^{-4} to 1.37×10^{-3}) (*12*). This finding suggests that the exclusively human-to-human transmission observed in this outbreak has not led to a change in the apparent evolutionary rate of the virus. No similar mutations occurred

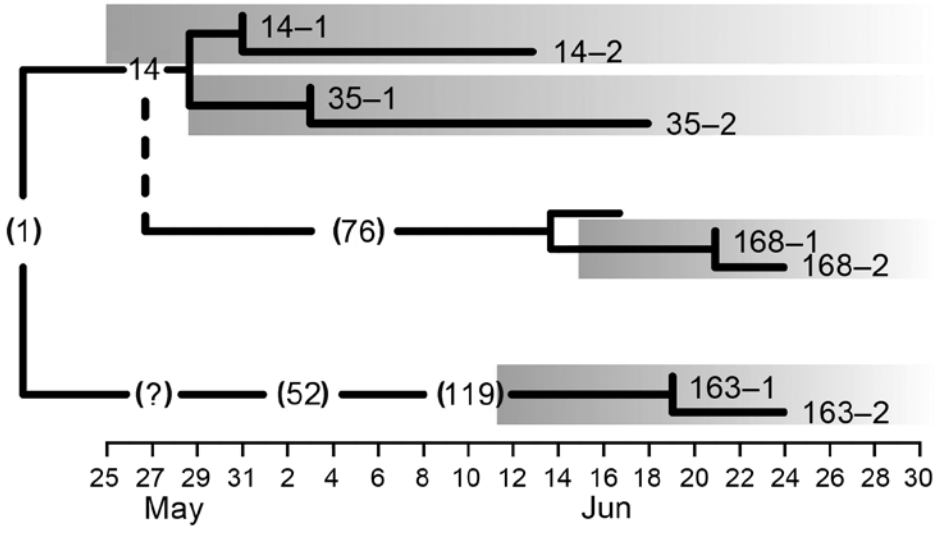

Figure 1. Transmission tree timeline for 8 Middle East respiratory syndrome coronavirus strains isolated during an outbreak in South Korea, 2015. Numbers without parentheses indicate patients in this study; numbers inside parentheses indicate patients not included in this study. The index case-patient is represented by (1). Numbers 1 and 2 following patient identification numbers indicate separate samples that were sequenced. The left edge of each shaded box indicates date of symptom onset for that patient; solid black vertical lines indicate sampling dates. Dashed vertical line indicates transmission from patient 14 to patient 76; (?) indicates unknown source of infection.

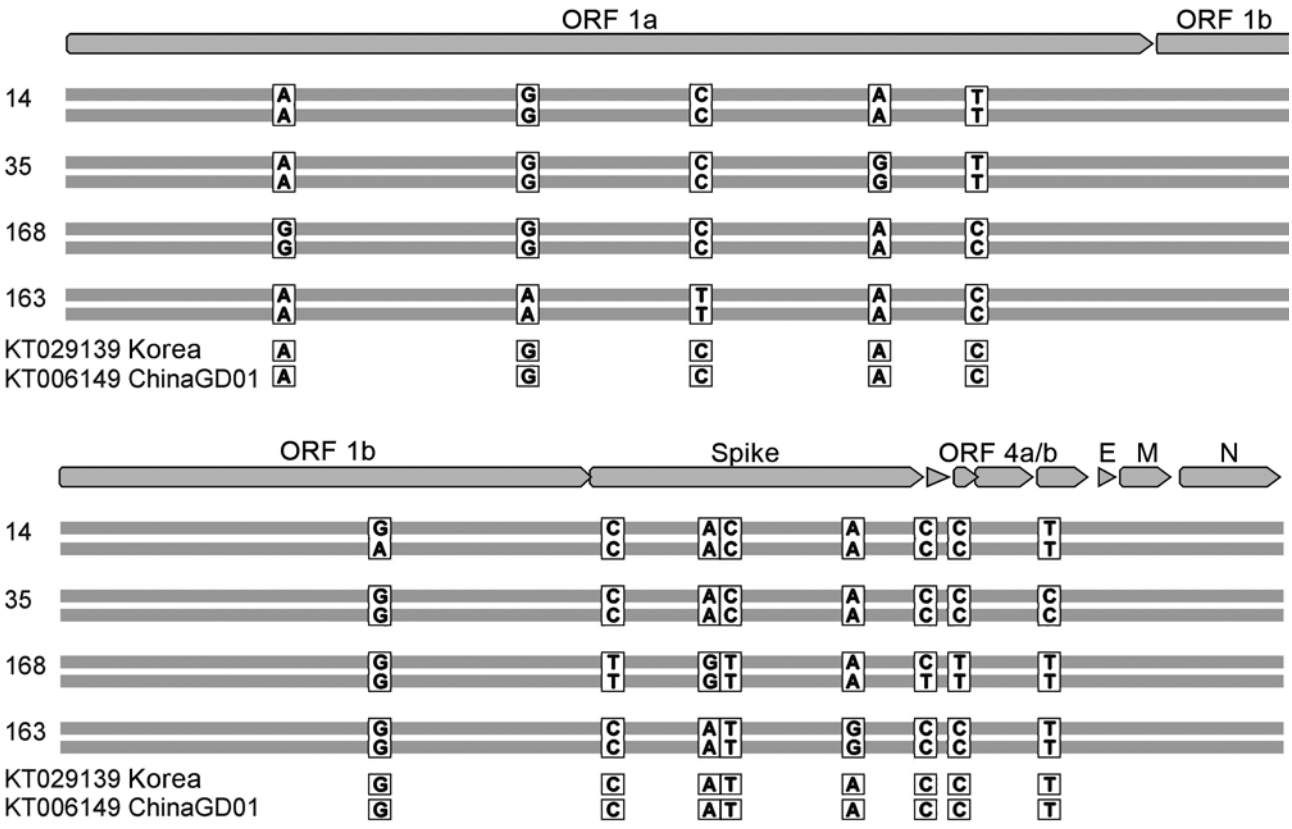

Figure 2. Location of the 13 variant nucleotide positions identified in genomes of 8 Middle East respiratory syndrome coronavirus strains isolated during an outbreak in South Korea, 2015. Case-patient numbers are indicated on the left, as are GenBank accession numbers for 2 related strains. For each case, the top and bottom horizontal bars represent the genome sequence generated from the first and second samples, respectively. Letters indicate matching nucleotide positions between samples. Sample collection dates are shown in Table 1. E, small envelope gene; M, matrix gene; N, nucleocapsid gene; ORF, open reading frame.

in parallel transmission chains leading up to patients 35, 168, and 163, suggesting that quasispecies sampling during transmission events has been stochastic rather than selective. Selective sampling would have been expected in the hypothetical case of emergence of a mutated virus with increased transmissibility or replication level.

The MERS-CoV spike glycoprotein targets the cellular receptor DPP4 (dipeptidyl peptidase 4). The receptor-binding domain (RBD) consists of residues E382 to C585 (*13*,*14*). In this study, we identified 2 RBD variants: D510G and I529T. Recent mutagenesis studies indicate that alterations of key residues within this region (DS10A and ES13A) could substantially reduce the efficiency of binding and virus entry (*13*,*15*). Because aspartate and glycine have similar physicochemical properties, the D510G variant found in this study might resemble D510A in its potential to reduce receptor binding.

Table 2. Substitution rates in strains within and between cases in a study of the microevolution of 8 isolates obtained during a MERS-CoV outbreak, South Korea, 2015*

	Within cases			Between cases		
Variable	Days between samples	Substitutions/ observation period	Substitutions/ site/d	Days after start of transmission chain†	Substitutions/ observation period†	Substitutions/ site/d
Patient no.						
14	13	1	2.58×10^{-6}	30	3	3.35×10^{-6}
35	15	0	0	33	5	5.08×10^{-6}
168	3	1	1.12×10^{-5}	51	6	3.94×10^{-6}
163	10	0	0	49	4	2.74×10^{-6}
Mean (SD)	NA	NA	3.44×10^{-6} (4.59×10^{-6})	NA	NA	3.78×10^{-6} (8.64×10^{-7})

*MERS-CoV, Middle East respiratory syndrome coronavirus; NA, not applicable.
†Determined on the basis of the reconstructed ancestral sequence existing on May 1, 2015.

Patient 168, a 36-year-old man who worked as a radiology technologist at a university hospital in Seoul, South Korea, carried the D510G variant virus. He was exposed to the virus via patient 76 on June 6. Symptoms developed on June 17, and he was immediately admitted to an isolation room in the hospital. RT-PCR was conducted at admission, but MERS-CoV RNA was not detected until 2 days later, suggesting an initially low level of virus replication. After MERS-CoV infection was confirmed by RT-PCR, combined interferon and ribavirin treatment was administered. The patient's vital signs were stable, and no lung lesions were seen on chest radiographs during hospitalization. He was discharged on June 30 after 2 consecutively negative RT-PCR results (June 28 and 30). The patient's mild clinical course and indicators of a low level of virus replication warrant further virologic study of a potentially attenuating effect of the D510G variant.

Another RBD variant, I529T, was carried by study patients 14 and 35. Although further studies are needed, physicochemical considerations suggest that this variant is unlikely to affect virus binding. Of note, patient 14 transmitted the virus to >80 tertiary case-patients and was thus considered a superspreader; however, persons infected by this patient, including patient 35, were not superspreaders.

Conclusions

Our study is limited by the absence of full-genome information for the index case-patient's virus and by the effect of outbreak interventions that may have concealed phenotypic virus changes. However, we found no evidence for an increase in the evolutionary rate or for adaptive changes over at least 4 generations of transmission. Changes that were observed were likely caused by transmission bottleneck effects.

C.D. has been supported by the European Commission research grant PREPARE and by the Deutsche Forschungsgemeinschaft (grant DR772/10-1).

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Nanopore Sequencing as a Rapidly Deployable Ebola Outbreak Tool

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Rapid sequencing of RNA/DNA from pathogen samples obtained during disease outbreaks provides critical scientific and public health information. However, challenges exist for exporting samples to laboratories or establishing conventional sequencers in remote outbreak regions. We successfully used a novel, pocket-sized nanopore sequencer at a field diagnostic laboratory in Liberia during the current Ebola virus outbreak.

Disease outbreaks in resource-limited or remote areas pose unique challenges to outbreak responses. These challenges are exemplified by the ongoing Ebola virus (EBOV) outbreak in West Africa that began in 2014 (*1*) and is unprecedented in its size and duration. Correspondingly, the magnitude of the international response, encompassing ≈50 Ebola treatment units (ETUs) and >2 dozen diagnostic laboratories, has been equally unprecedented. These laboratories often are operated under improvised field conditions to keep them close to active, sometimes remote transmission sites (*2*,*3*).

Rapidly obtaining genome sequences during disease outbreaks is crucial for clarifying patterns of virus evolution, monitoring the validity of diagnostic assays, and investigating transmission chains (*4*,*5*). Further, rapid results may help determine the efficacy of sequence-dependent countermeasures, such as siRNAs or antibody treatments. In the past, obtaining timely genome sequences has been difficult because of political and logistical obstacles that limited the export of samples to laboratories capable of performing these analyses. As an example, during the first year of the outbreak in West Africa, only 2 reports of genome sequences from patients were published (*1*,*6*). Similarly, establishing conventional Sanger or next-generation sequencing technologies in affected countries is logistically challenging because of the size and weight (≈40 to ≈100 kg) of the necessary equipment, the high potential for transport damage related to the sensitive optics many of these machines incorporate, limitations on supportive infrastructure, and complex sample processing procedures. An additional challenge is the required installation or calibration of sequencing machines, which often has to be done by field engineers employed by the manufacturers, who may be reluctant to send their employees into outbreak areas. However, Kugelman et al. recently reported the successful deployment of an Illumina MiSeq, a well-established, conventional next-generation sequencing platform (Illumina, San Diego, CA, USA), to West Africa; the platform became operational in February 2015 (*5*).

Seeking a platform that would be more rapidly deployable and reliable under field conditions, we established protocols and evaluated the feasibility of nanopore sequencing technology under outbreak conditions using a pocket-sized (≈10 × 4 × 2 cm, 75 g) MinION sequencing device (Oxford Nanopore Technologies, [https://www.nanoporetech.com/]). Because of its small size, this device can easily be transported into remote locations; furthermore, it requires no special setup or calibration procedures and can be operational immediately after arrival in an outbreak area. Further, data turnaround is very rapid, and consequently, nanopore sequencing is being developed as a rapid diagnostic tool for management of outbreaks of various diseases (*7*,*8*). The MinION device senses individual DNA molecules based on modulation of ion currents across nanopores as the molecules are passing through. These modulations are

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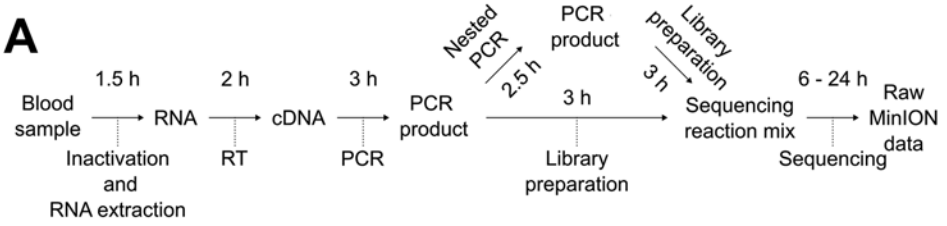
DOI: http://dx.doi.org/10.3201/eid2202.151796

dependent on the physical properties of the nucleotides and allow determination of the nucleotide sequence (*9*).

The Study

To facilitate sequencing of the RNA genome of EBOV, we developed and tested an approach based on reverse transcription PCR, in which whole virus genomes were amplified in overlapping fragments (Figure 1, panels A, B; online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/2/15-1796-Techapp1.pdf). This approach was first validated in a regular laboratory setting in the Rocky Mountain Laboratories of the National Institutes of Health (NIH) by using blood samples from nonhuman primates experimentally inoculated with EBOV strain Makona-Gueckedou-C07 (*10*,*11*). This validation showed that sequencing information was obtainable for the complete genome with an average of 7,038 reads at every nucleotide position (read depth; online Technical Appendix Figure 1, panel A). We observed no sequence differences when comparing the consensus sequence derived from these data to those obtained by using Sanger sequencing (online Technical Appendix Figure 1, panel B). Furthermore, by analyzing linearized plasmid DNA of known sequence, we established the accuracy of the MinION device as ≈84% for a single read (online Technical Appendix Figure 1, panels C, D). On the basis of this information, and the fact that read depth can compensate for miscalled nucleotides in individual reads by piling up reads covering the same region, we determined the theoretical probability for ≥1 miscalled base (TPMB) in a complete MinION-sequenced EBOV genome to be <5% when the read depth is ≥33 at all positions (online Technical Appendix Figure 1, panels E and F).

After having validated this approach, MinION devices were taken to the Centers for Disease Control and Prevention (CDC)/NIH field laboratory that provided diagnostic support for ETUs in Monrovia, Liberia, during August 2014–May 2015. All equipment and reagents necessary for sequencing could be easily transported as checked luggage by a single person on a commercial carrier. In Liberia, temperatures in the laboratory area used for sequencing ranged from 28 to 32°C, necessitating the use of an improvised heat sink for the devices, which consisted of a metal plate of ≈30 × 30 cm (online Technical Appendix Figure 2, panels A, B). Under field conditions, we initially failed to produce complete genomes with high confidence because of problems with



C

5% C_t <21 18% C_t <24 57% C_t <31

Sample	C_t	Genome at read depth ≥1×	Genome at read depth ≥33×	Mean read depth	TPMB, %	GenBank ID
11	17.0	15.7	14.1	270	NA	NA
14	17.8	100.0	100.0	5,250	8.77×10^{-56}	KR075003.1
13	18.5	100.0	99.2	471	0.49	KR075002.1
3	19.9	100.0	100.0	11,916	5.70×10^{-75}	KR074997.1
6	20.0	100.0	100.0	3,208	3.32×10^{-50}	KR074998.1
12	20.2	100.0	97.2	516	6.17	KR075001.1
5	20.7	100.0	100.0	7,950	1.43×10^{-31}	KR074999.1
4	20.8	100.0	100.0	1,734	2.43×10^{-11}	KR074998.1
1	21.1	100.0	100.0	2,841	3.96×10^{-27}	KR074996.1
2	22.9	94.3	94.1	3,651	NA	KU042955.1
15	23.5	97.5	94.3	1,216	NA	KU042954.1
8	24.1	53.5	46.7	1,347	NA	NA
10	28.6	91.5	81.1	640	NA	NA
7	29.3	36.1	24.6	156	NA	NA
9	30.5	68.5	63.7	350	NA	NA

Figure 1. MinION sequencing. A) Experimental and B) bioinformatics workflows. Times indicated are the approximate duration for each procedure. RT, reverse transcription. C) Sequencing results showing Ebola virus load (expressed as C_t value), percentage of the genome with a minimum read depth of ≥1 or ≥33, mean read depth, theoretical probability for a miscalled base (TPMB), and GenBank accession numbers of complete and nearly complete genomes. Brackets at left indicate percentage of Ebola virus–positive patient samples below each of the 3 cutoff cycle threshold (C_t) values used in this study (C_t <21, <24, <31). Sample 8 was from an oral swab; all others were from blood. NA, not available.

PCR yields (online Technical Appendix Figure 3, panels A, B). However, by implementing a second PCR step, we circumvented this problem and obtained high quality complete genome sequences for 8 of 9 high-virus load samples (cycle threshold ≤21) (Figure 1, panel C; online Technical Appendix Figure 4, panel A). In lower virus load samples, we could obtain only incomplete genome sequences; however, even in those samples regions for which sequencing information was available generally showed high read depths (online Technical Appendix Figure 4, panel B), suggesting that further optimization of PCRs might also allow complete coverage for these samples. Furthermore, even incomplete genome sequences can provide valuable information during an outbreak, allowing analysis of individual genes and the tracing of transmission chains (*12*).

Using this updated protocol, we achieve a sustained capacity of 4 full-length genomes per day for a single person conducting the laboratory work using 2 MinION devices (Figure 1, panels A, B). However, with the exception of the first 2 sequencing runs, bioinformatics analysis during this mission was mainly completed after returning to the NIH, to maximize the time for raw data acquisition (online Technical Appendix).

Phylogenetic analysis of the complete genomes generated in Monrovia, Liberia, showed them being clearly distinct from Sierra Leone or early Guinea sequences of EBOV-Makona (online Technical Appendix Figure 5) but clustering well with all other sequences found in samples from Liberia. These results suggest that EBOV in Liberia resulted from a single introduction or a limited number of introductions with genetically similar viruses. When analyzing the obtained full-length sequences and comparing them to a consensus sequence from the outbreak (*13*), we observed few mutations, most in noncoding regions or synonymous mutations (Figure 2); none affected siRNA target sequences or the diagnostic targets used in the CDC/NIH laboratory.

Using Bayesian analysis including these sequences, we estimated the nucleotide substitution rate during the outbreak at 1.36×10^{-3}, consistent with recently published values (*5*,*13*–*15*). In a root-to-tip-analysis, the sequences we obtained showed substitution rates comparable to other sequences from the outbreak (online Technical Appendix Figure 6). Overall, these data suggest that EBOV has remained relatively stable genetically during the outbreak.

Conclusions

We found that, because of the device's small size and comparatively modest resource requirements, nanopore sequencing has tremendous potential for use in remote and resource-limited areas, and its implementation could revolutionize the capacity of public health professionals to perform sequencing during future disease outbreaks. Although we used a directed approach to sequencing, approaches not dependent on prior pathogen identification (i.e. for diagnostic use of the MinION device) are currently being developed (*7*) and will even further increase this technology's usefulness in future outbreaks.

Acknowledgments

We thank Mulbah Jallah, James Brayer, Dawn Clifton, Kay Menk, Joyce Walczynski, W. Lesley Shupert, Kristi Schmidt, Rob Stevens, Rohn Wood, and Mark Pineda for support of this project. We further acknowledge the World Health Organization Headquarters, Geneva; the World Health Organization Regional Office for Africa; Médecins Sans Frontières; and the Ministry of Health and Social Welfare, Liberia.

This work was supported in part by the Intramural Research Program of NIH, National Institute of Allergy and Infectious Diseases, and used the high-performance computational capabilities of the Biowulf Linux cluster at NIH (http://biowulf.nih.gov). The complete genome sequences reported in this manuscript were deposited in GenBank under the accession numbers KR074996.1–KR075003.1 and the nearly complete sequences under accession numbers KU042954 and KU042955.

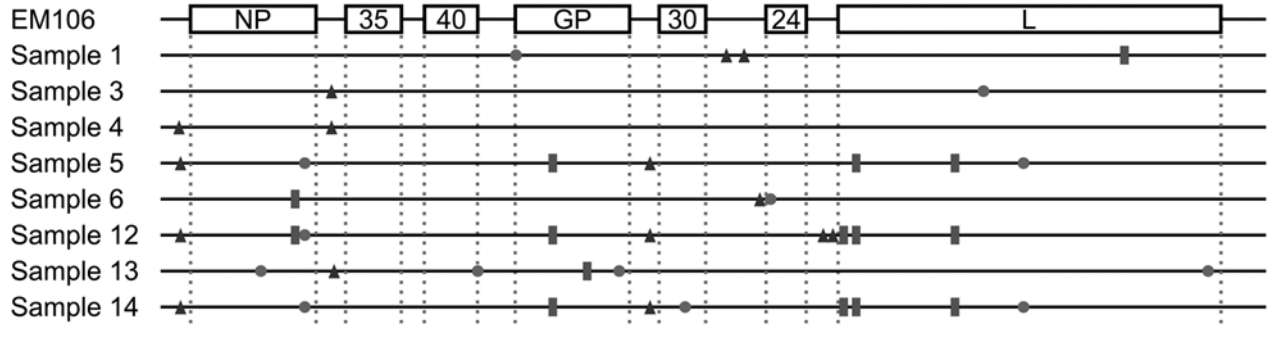


Figure 2. Observed mutations in the 8 fully nanopore-sequenced Ebola-positive blood samples compared to a reference sequence from June 2014 (SLI/Makona-EM106, GenBank accession number KM233036.1). Squares indicate nonsynonymous mutations, circles indicate synonymous changes, and triangles indicate changes in noncoding regions. NP, nucleoprotein; GP, glycoprotein.

T.H., A.G., K.R., and J.P. are participants in the ONT MinION Access Programme, and received some of the MinION devices and Flow Cells used for this study free of charge or at reduced cost. T.H. was invited by Oxford Nanopore Technologies to present part of this work at the London Calling meeting in London, UK. The deidentified patient specimens used in this manuscript were collected as part of public health surveillance and not as human subjects research. The NIH Office of Human Subjects Research has determined that federal regulations for the protection of human subjects do not apply.

Dr. Hoenen was a visiting postdoctoral fellow at the Laboratory of Virology of the National Institutes of Health in Hamilton, Montana, USA, for major phases of this study, and has recently joined the Friedrich-Loeffler-Institut on the island of Riems, near Greifswald, Germany, where he is head of the Laboratory for Molecular Biology of Filoviruses. His research focuses on the molecular biology of filoviruses and their interactions with host cells, with the ultimate goal of finding new antivirals against filoviruses and other hemorrhagic fever viruses.

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Acute Colitis Caused by *Helicobacter trogontum* in Immunocompetent Patient

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DOI: http://dx.doi.org/10.3201/eid2202.150287

To the Editor: In industrialized countries, diarrhea and vomiting associated with acute gastrointestinal illness is estimated to occur at a rate of ≈1 episode per person per year; ≈0.3% of patients are hospitalized because of severe symptoms associated with colitis or fever. The most commonly identified infectious agents of non-nosocomial diarrhea are calicivirus and *Salmonella*, *Campylobacter*, *Giardia*, and *Cryptosporidium* spp. However, for numerous cases, the causative agent is not identified. *Helicobacter* species other than *H. pylori*, but not *H. trogontum*, have emerged as causes of gastrointestinal and systemic disease, mainly in immunocompromised patients (*1*). We report a case of community-acquired colitis with bacteremia caused by *H. trogontum* in an immunocompetent patient and emphasize the diagnostic difficulties.

The patient was a 31-year-old woman with a history of recurrent epigastralgia, vomiting, diarrhea, and weight loss of 10 kg over an 8-year period. In April 2014, she was admitted to the Hôpital Européen Georges Pompidou emergency ward (Paris, France) because of abdominal pain, nonbloody diarrhea, fever, and chills, which had persisted for 3 days. Examination revealed a mildly tender abdomen without hepatosplenomegaly, signs of slight dehydration, and tachycardia. Leukocyte count was 13.2×10^9 cells/L (neutrophils, 11.4×10^9), and C-reactive protein level was 191 mg/L. Abdominal computed tomography images showed nonspecific right and transverse colitis (Figure, panel A).

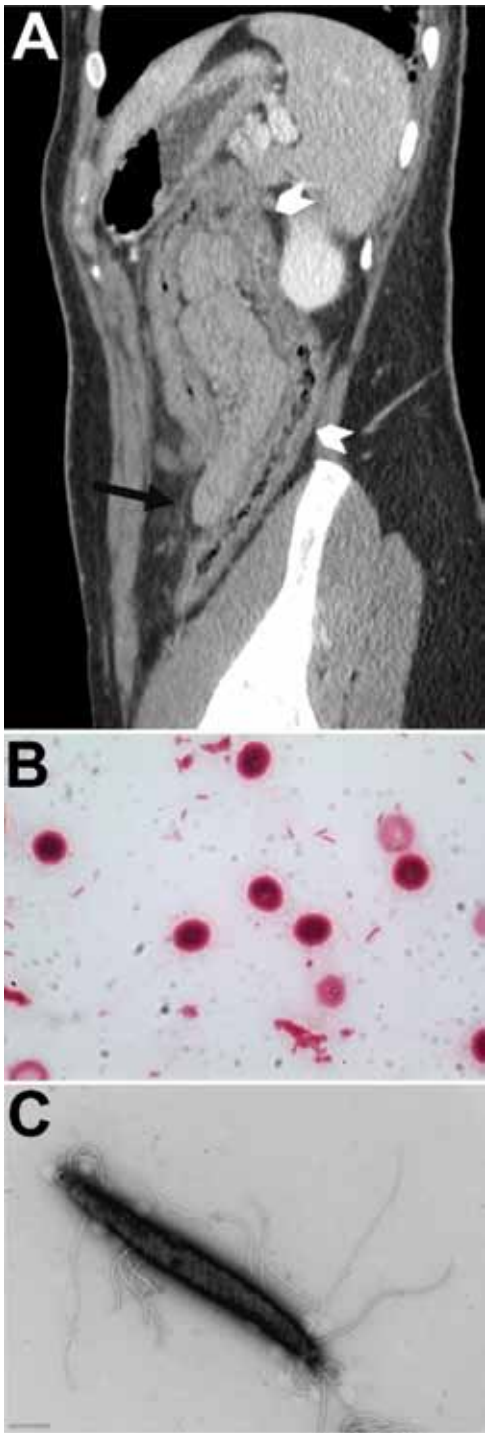

Figure. Computed tomographic image of patient with *Helicobacter trogontum* infection and micrographs of *H. trogontum*. A) Paramedian sagittal section of an abdominopelvic scan after injection of contrast medium in the portal phase, showing thickening of the transverse and right colon (white arrowheads) with tubular appearance and discrete thickening of the fat stranding (gray arrow). B) Gram-stained blood culture smear. Original magnification ×1,000. C) Transmission electron micrograph of negatively stained *H. trogontum* showing bipolar flagella. Scale bar indicates 0.5 μm.

[1]These authors contributed equally to this article.

One day after admission, the patient was discharged with empirically prescribed ciprofloxacin and metronidazole for 7 days. After 4 days, aerobic blood culture was positive for motile, fusiform, gram-negative bacilli, suggestive of strictly aerobic bacteria that could not be identified directly (Figure, panels B, C). After 7 days of incubation under a microaerophilic atmosphere only, a blood subculture isolate was obtained; 23S and 16S rDNA sequencing (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/2/15-0287-Techapp1.pdf) identified this isolate as *H. trogontum*. Of note, use of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Bruker Daltonik GmbH, Bremen, Germany) did not enable identification of the bacterium.

No common pathogens were detected in fecal samples. Upper and lower gastrointestinal endoscopic examinations conducted 1 month after discharge revealed no notable abnormalities. No immunocompromised condition was found. At most recent follow-up examination, the patient was free of symptoms.

The genus *Helicobacter* currently comprises 48 formally named species belonging to the gastric or enterohepatic group according to their ecologic niche. *H. trogontum* (enterohepatic group) has been isolated from apparently health animals (rat and piglet intestinal mucosa and swine feces), but its characteristics are typical of pathogenic bacteria ([*2,3*], online Technical Appendix). The apparent in vitro susceptibility of the isolate to metronidazole and the favorable patient outcome reported here are in agreement with the finding that metronidazole is an effective treatment for *H. trogontum* infection in rats ([*4*]; online Technical Appendix), but there are no antimicrobial drug susceptibility data for *H. trogontum* isolated from animals. We assume that the immunocompetent patient reported here had chronic colitis caused by *H. trogontum*, followed by an episode of acute colitis with bacteremia after several years of intermittent symptoms.

The rarity of reported *H. trogontum* infections might be linked to the difficulty associated with culturing and identifying the bacterium or to a low level of exposure to this pathogen. The mode of transmission, probably from animals to humans, remains unclear. Methods for isolation and rapid identification of *H. trogontum*, including the updating of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry databases, are needed for further elucidation of its pathogenic properties and the mode of contamination.

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Accuracy of Dengue Reporting by National Surveillance System, Brazil

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DOI: http://dx.doi.org/10.3201/eid2202.150495

To the Editor: Dengue is an underreported disease globally. In 2010, the World Health Organization recorded 2.2 million dengue cases (*1*), but models projected that the number of symptomatic dengue cases might have been as high as 96 million (*2*). Brazil reports more cases of dengue than any other country (*1*); however, the degree of dengue underreporting in Brazil is unknown. We conducted a study to evaluate dengue underreporting by Brazil's Notifiable Diseases Information System (Sistema de Informação de Agravos de Notificação [SINAN]).

From January 1, 2009, through December 31, 2011, we performed enhanced surveillance for acute febrile illness

Table. Accuracy of a national surveillance system for recording cases of suspected dengue among patients with acute febrile illness who visited an emergency health unit of Salvador, Brazil, January 1, 2009–December 31, 2011*

Notification status of AFI patients†	Laboratory status of AFI patients, no.‡		Dengue prevalence, %§	% (95% CI)				MF¶
	Dengue	Nondengue AFI		SENS	SPEC	PPV	NPV	
Overall								
Reported	57	26	25.8	5.7 (4.4–7.3)	99.1 (98.7–99.4)	68.7 (58.1–77.6)	75.1 (73.7–76.5)	12.0
Not reported	940	2,841						
Study year								
2009								
Reported	4	4	10.3	3.3 (1.3–8.3)	99.6 (99.0–99.9)	50.0 (21.5–78.5)	90.0 (88.1- 91.6)	15.0
Not reported	116	1,039						
2010								
Reported	27	8	33.8	6.0 (4.1–8.6)	99.1 (98.2–99.5)	77.1 (61.0–87.9)	67.4 (64.8- 69.9)	12.9
Not reported	423	873						
2011								
Reported	26	14	31.2	6.1 (4.2–8.8)	98.5 (97.5–99.1)	65.0 (49.5–77.9)	69.9 (67.3- 72.3)	10.7
Not reported	401	929						
Age group, y								
5–14								
Reported	23	15	25.5	5.8 (3.9–8.6)	98.7 (97.9–99.2)	60.5 (44.7–74.4)	75.4 (73.2–77.5)	10.4
Not reported	372	1,139						
≥15								
Reported	34	11	26.0	5.6 (4.0–7.8)	99.4 (98.9–99.6)	75.6 (61.3–85.8)	75.0 (73.2–76.7)	13.8
Not reported	568	1,702						
Monthly dengue prevalence§								
≥20%								
Reported	52	18	38.1	6.7 (5.2–8.7)	98.6 (97.7–99.1)	74.3 (63.0–83.1)	63.1 (61.0–65.2)	11.1
Not reported	722	1,235						
<20%								
Reported	5	8	12.1	2.2 (1.0–5.2)	99.5 (99.0–99.8)	38.5 (17.7–64.5)	88.0 (86.5–89.5)	17.2
Not reported	218	1,606						

*AFI, acute febrile illness; SINAN, Sistema de Informação de Agravos de Notificação (Notifiable Diseases Information System, Brazil); SENS, sensitivity; SPEC, specificity; PPV, positive predictive value; NPV, negative predictive value; MF, multiplication factor.
†Notification status of the AFI patients was ascertained from the SINAN database on reported dengue cases for the city of Salvador. SINAN database was obtained from the Salvador Secretary of Health in January 2013.
‡Acute-phase serum samples from AFI patients were systematically tested for dengue by nonstructural protein 1 ELISA and IgM ELISA; convalescent-phase serum samples were also tested by IgM ELISA.
§Among AFI patients assisted at the sentinel surveillance emergency unit.
¶Laboratory-confirmed dengue/AFI patients reported as having a suspected case of dengue.

(AFI) in a public emergency unit in Salvador, Brazil. The surveillance team enrolled outpatients ≥5 years of age with measured (≥37.8°C) or reported fever. Patients or their legal guardians provided written consent. The study was approved by the Oswaldo Cruz Foundation Ethics Committee, Brazil's National Council for Ethics in Research, and the Yale Institutional Review Board.

We collected participants' blood samples at study enrollment and ≥15 days later. Acute-phase serum samples were tested by dengue nonstructural protein 1 ELISA and IgM ELISA (Panbio Diagnostics, East Brisbane, Queensland, Australia). Convalescent-phase serum samples were tested by IgM ELISA. In concordance with case-reporting guidelines in Brazil (*3*), we defined dengue cases by a positive nonstructural protein 1 ELISA result or a positive acute-phase or convalescent-phase IgM ELISA result. All others were classified as nondengue AFI.

We then identified which study patients were officially reported to SINAN as having a suspected case of dengue. In Brazil, notification of suspected dengue cases is mandatory. A suspected case is defined as illness in a person from an area of dengue transmission or *Aedes aegypti* mosquito infestation who has symptoms of dengue (fever of ≤7 days' duration, plus ≥2 of the following symptoms: nausea/vomiting, exanthema, myalgia, arthralgia, headache, retro-orbital pain, petechiae/positive tourniquet test, or leukopenia). We used Link Plus software (CDC-Link Plus Production 2.0; Centers for Disease Control and Prevention, Atlanta, GA, USA) to perform probabilistic record linkage from our database with official reports in the SINAN data-

base. The records were matched based on the patients' first names, last names, and dates of birth. We then manually reviewed the matches to confirm the pairs.

On the basis of the results, we calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value of the national surveillance system. We calculated accuracy measurements with 95% CIs for the overall study period and for each study year, age group (5–14 vs. ≥15 years), and seasonal prevalence of dengue (months of low vs. high dengue transmission, defined by dengue detection in <20% vs. ≥20% of the AFI patients, respectively). We estimated multiplication factors by dividing the number of dengue cases in our study by the number of study patients who were reported to SINAN as having dengue.

Of the 3,864 AFI patients identified during the 3-year study period, 997 (25.8%) had laboratory evidence of dengue infection, and 2,867 (74.2%) were classified as having nondengue AFI. Of the 997 dengue cases, 57 were reported to SINAN (sensitivity 5.7%) (Table). Of the 2,867 nondengue AFI cases, 26 were reported to SINAN as dengue cases (false-positive ratio 0.9%, specificity 99.1%). None of these 26 cases had laboratory confirmation in the SINAN database. The PPV for reporting to SINAN was 68.7%, and the negative predictive value was 75.1% (Table). PPV was higher among patients ≥15 years of age, which might be attributable to atypical presentations of dengue in children (*4*,*5*).

We found that 1 in 4 patients with AFI had laboratory evidence of dengue infection. However, for every 20 dengue patients that we identified, only about 1 had been reported to SINAN as having dengue. During periods of low dengue transmission, only about 1 in 40 dengue cases identified was reported. Conversely, among the patients who were reported as having dengue, 31.2% did not have the disease; this percentage reached 61.5% in low-transmission periods.

We estimated that overall, there were 12 dengue cases per reported case in the community, but in months of low dengue transmission, this ratio was >17:1 (Table). Comparable results have been observed in Nicaragua, Thailand, and Cambodia (*6*–*8*). By applying the estimated multiplication factor to the study period's mean annual incidence of 303.8 reported dengue cases/100,000 Salvador residents (*9*), we estimated that the actual mean annual dengue incidence for Salvador was 3,645.7 cases/100,000 residents.

We showed that dengue surveillance substantially underestimated disease burden in Brazil, especially in what are considered low-transmission periods. Dengue underreporting has been attributed to passive case detection, which fails to identify persons with dengue who do not seek health care (*1*). We also showed that surveillance failed to detect dengue cases among symptomatic patients seeking health care.

Novel surveillance tools, such as active syndromic surveillance and point-of-care testing, should be applied to improve estimates of dengue incidence. Furthermore, given the recent emergence of chikungunya and Zika viruses in Brazil (*10*), improved surveillance and laboratory diagnostics are needed to avert misclassification and mismanagement of cases.

Acknowledgments

We thank those who participated in study data collection and sample processing, especially Helena Lima, Juan Calcagno, and André Henrique Gonçalves; Nivison Nery Jr, Renan Rosa, and Delsuc Evangelista Filho for their assistance with data management; Monique Silva for her assistance with administrative matters; and Federico Costa and Jose Hagan for their advice while the study was being conducted. We also want to thank the São Marcos Emergency Center staff; the Pau da Lima Health District, Salvador Secretariat of Health; and Pau da Lima community leaders and resident associations.

Financial support was provided by the National Council for Scientific and Technological Development (grant 550160/2010-8 and scholarships to M.M.O.S., M.S.R., I.A.D.P., M.K.,A.I.K., M.G.R., and G.S.R.); the Bahia Foundation for Research Support (grant PNX0010/2011); the Federal University of Bahia (grants PROPI 2013 and PRODOC 2013); the National Institutes of Health (grants R01 AI052473, U01 AI088752, R25 TW009338, and D43 TW00919); the Oswaldo Cruz Foundation (scholarships to A.M.K., M.M.O.S., A.S.T., and J.S.C.); and the Coordination for the Improvement of Higher Education Personnel, Brazil Ministry of Education (scholarships to M.K. and T.L.Q).

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Aberrant *Ascaris suum* Nematode Infection in Cattle, Missouri, USA

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DOI: http://dx.doi.org/10.3201/eid2202.150686

To the Editor: *Ascarididae* is a family of parasitic nematodes, commonly known as intestinal roundworms, that affects humans and various animals, including pigs, dogs, cats, horses, raccoons, and marine mammals. The *Ascaris suum* nematode is a common parasite of swine. There has been a sizeable decrease in the number of cases of infection with this nematode because swine husbandry has become more modernized and industrialized (*1*).

The *A. lumbricoides* nematode is the primary roundworm of humans. However, sporadic aberrant *A. suum* nematode infections have been reported in humans worldwide. Although uncommon in industrialized countries, several human cases of infection with this nematode have been reported (*2,3*), including an outbreak in Maine, USA, in 2010–2013 (*4*). Similar to human cases, cases of aberrant infection of *A. suum* nematodes in cattle are rare; no infections have been reported in North America since the 1960s (*5*).

In September 2010, a 1.5-year-old heifer from a farm was brought to Veterinary Medical Diagnostic Laboratory (VMDL), University of Missouri (Columbia, MO, USA), for postmortem examination. The heifer died with clinical signs of respiratory distress, coughing, tachypnea, and general weakness. The farm contained 15 heifers, all of which had shown similar clinical signs for 2–3 weeks. Two heifers had died 2 days before being brought to the VMDL but no necropsy was performed. New cattle had not recently been introduced to the herd, and animals were current for all vaccinations.

Necropsy showed bilaterally inflated lungs with moderate emphysema, diffusely firm red parenchyma, and abundant blood-tinged froth in the bronchi and trachea. Interstitial pneumonia was diagnosed on the basis of gross appearance, and viral pneumonia or acute bovine pulmonary emphysema and edema, especially that caused by toxic Perilla mint (*Peri indicutescens*), was suspected. However, microscopic examination showed diffuse, severe, fibrinous, eosinophilic and histiocytic interstitial pneumonia and multiple nematode larvae in bronchi and alveolar sacs (Figure). Nematode larvae were ≈100 μm in diameter and had a cuticle, pseudocoelom, coelomyarian musculature, large lateral cords, lateral alae, and a digestive tract. Bacteriological and molecular diagnostic tests did not indicate a viral or bacterial etiology. Fecal examination showed few coccidian and strongylid eggs.

Dictyocaulus viviparous, a trichostrongyle, is the pathogenic bovine lungworm. However, morphologic characteristics of nematodes in this study were more consistent with ascarids. There is a bovine roundworm (*Toxocara viturolum*), but it is uncommon in the United States.

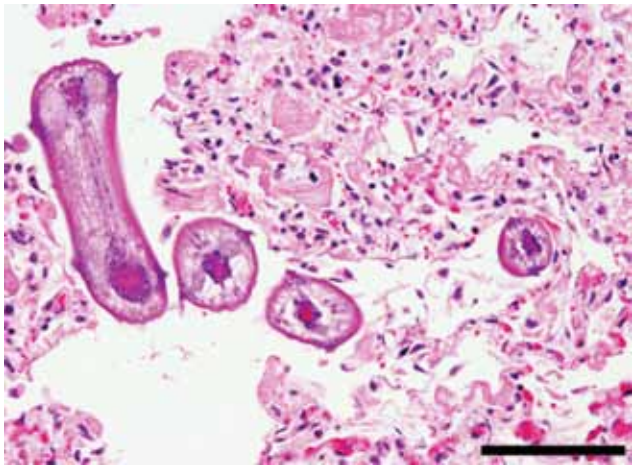

Figure. Multiple cross-section of *Ascaris suum* nematode larvae in the lung of cattle. Larvae have prominent lateral alae and lateral cords. Several scattered eosinophils and macrophages and abundant fibrin are also shown. Scale bar indicates 200 μm.

In tropical and subtropical regions, especially in Africa and Southeast Asia, *T. viturolum* roundworms infect suckling bovine calves (*6*). *T. viturolum* roundworms have been identified in Florida, USA, in 2010 (*7*) and Canada in 2012 (*8*). Although *T. viturolum* roundworms cause diarrhea and weight loss in calves ≤3 months of age, infection with these roundworms is largely asymptomatic in adults.

Further investigation indicated that the source of infection was a pair of young-adult, free-range pigs living alongside the affected cattle. The cattle had been fed a round bale of hay to which the pigs had access. Parasitologic examination of the hay bale showed contamination with *A. suum* nematode eggs, indicating that the hay bale was probably exposed to pig feces. The hay bale was removed from the remaining cattle, and clinical signs gradually resolved without additional loss.

To unambiguously identify the nematodes, genomic DNA was extracted from formalin-fixed, paraffin-embedded tissue of infected lung. On the basis of the mitochondrial DNA sequence of *A. suum*, primers were designed to amplify a partial sequence encoding NADH dehydrogenase subunit 5: VMDL-F2 (5′-TGCTAAAGGTTGGGTTTATGGA-3′) and M3-R (5′-CCTACTGCGTAGAGCCAGA-3′). PCR was performed by using the GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA) under the following conditions: 95°C for 4 min; 45 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 1 min; and final extension at 72°C for 5 min.

The resulting amplicon was purified by using spin chromatography (QIAGEN, Valencia, CA, USA) and sequenced with amplification primers at the University of Missouri DNA Core Facility. The resulting 354-bp sequence (GenBank accession no. KT808321) was compared with sequences in GenBank by using blastn (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch) and found to be 100% identical with that of *A. suum* nematode, thus confirming the identity. The sequence was only 98.3% identical with that of *A. lumbricoides* nematode, the species that had the next closest match (6 polymorphisms).

Increasing interest in and demand for organic meat results in proliferation of small, suburban farms that raise free-range animals. Reports have warned the community about major increases in zoonotic parasitic infections in organically raised pigs compared with animals raised under modern husbandry practices (*9,10*). The recent zoonotic case in Maine also involved a farm that grew and sold organic vegetables and organic livestock, including pigs (*4*). Until new proven preventive protocols are established, health personnel and veterinarians should be well informed about the risk for aberrant parasitic infections in pigs and possible transmission to humans and other domestic animals.

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Vectorborne Infections, Mali

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DOI: http://dx.doi.org/10.3201/eid2202.150688

To the Editor: As in many West Africa nations, vectorborne diseases represent a substantial health burden in Mali; however, beyond malaria, the incidence and etiology of many of these diseases is poorly understood. Of the estimated 14.1 million persons living in sub-Saharan Mali, ≈70% live in remote rural settings with an ecologic landscape that puts inhabitants at an increased risk for contact with rodent and arthropodborne diseases. We retrospectively analyzed serum samples for evidence of recent (IgM+) and previous (IgG+) infection with chikungunya (CHIKV), dengue (DENV), West Nile (WNV), Lassa (LASV), Crimean-Congo hemorrhagic fever (CCHFV), and Ebola (EBOV) virus, as well as Old World hantaviruses (OW-HANV) and *Leptospira* spp., which is regularly misdiagnosed as an acute viral infection.

We tested 376 deidentified serum samples collected from acutely ill patients who had a history of fever and hemorrhagic, diarrheal, or icteric syndromes (online Technical Appendix Figure, http://wwwnc.cdc.gov/EID/article/22/2/15-0688-Techapp1.pdf). Research on samples from humans was conducted in accordance with the policies and regulations of the National Institutes of Health and adhered to the principles of the Belmont Report (1979) (http://www.hhs.gov/ohrp/humansubjects/guidance/belmont.html). This research was conducted under an institutional review board–approved document.

Samples had previously tested negative for acute *Plasmodium falciparum* malaria and yellow fever virus infections. Commercially available IgM capture and conventional IgG ELISAs were used for serologic testing for CHIKV (GenWay Biotech, San Diego, CA, USA); DENV (all four serotypes) and WNV (both from Focus Diagnostics, Cypress, CA, USA); OW-HANVs (Euroimmun, Luebeck, Germany); and *Leptospira* spp. (Abnova, Taipei City, Taiwan). Conventional IgM/IgG ELISAs were used for LASV (Corgenix, Broomfield, CO, USA) and CCHFV (Vector-Best, Novosibirsk, Russia), and reagents for the EBOV IgM/IgG ELISA (infected/uninfected cell lysates) were prepared at the Rocky Mountain Laboratories (Hamilton, MT, USA) and validated with serum from experimentally infected monkeys. With the exception of the CHIKV, *Leptospira* spp., and in-house EBOV assays, the tests conducted in this study are under preclinical development for human diagnostic assays.

Samples were tested at a 1:100 dilution according to manufacturer specifications (CHIKV, CCHFV, WNV, DENV, OW-HANVs, LASV, and *Leptospira* spp.) or in-house quality-control assessments (EBOV), in a

Table. IgM and IgG seroprevalence rates of selected vectorborne pathogens in samples submitted from suspected yellow fever cases in Mali, 2009–2013*

Pathogen	IgM/IgG positivity	No. (%) samples					
		2009, n = 77	2010, n = 107	2011, n = 71	2012, n = 34	2013, n = 87	Total, N = 376
Chikungunya virus	IgM+	4 (5.2)	4 (3.7)	5 (7.0)	2 (5.9)	5 (5.7)	20 (5.3)
	IgG+	5 (6.5)	9 (8.4)	2 (2.8)	2 (5.9)	7 (8.0)	25 (6.6)
	IgG+/IgM+	0	1	0	0	0	1
West Nile virus	IgM+	0	0	0	0	1 (1.1)	1 (0.27)
	IgG+	33 (42.9)	46 (43.0)	28 (39.4)	13 (38.2)	27 (31.0)	147 (39.1)
	IgG+/IgM+	0	0	0	0	0	0
Dengue virus	IgM+	6 (7.8)	11 (10.3)	4 (5.6)	4 (11.8)	4 (4.6)	29 (7.7)
	IgG+	32 (41.6)	46 (43.0)	31 (43.7)	13 (38.2)	28 (32.2)	150 (40.0)
	IgG+/IgM+	3	6	1	1	2	13
Leptospira spp.	IgM+	7 (9.1)	23 (21.5)	1 (1.4)	3 (8.8)	20 (23.0)	54 (14.4)
	IgG+	12 (15.6)	19 (17.8)	15 (21.1)	8 (23.5)	20 (23.0)	74 (19.7)
	IgG/IgM+	1	2	1	0	3	7
OW-HANV	IgM+	5 (6.5)	6 (6.5)	5 (7.0)	2 (6.7)	7 (8.0)	27 (7.2)
	IgG+	2 (2.6)	8 (7.5)	6 (8.5)	2 (6.7)	3 (3.4)	21 (5.6)
	IgG+/IgM+	0	0	0	0	0	0
Lassa virus	IgM+	0	0	0	0	1 (1.1)	1 (0.27)
	IgG+	0	0	0	0	0	0
	IgG+/IgM+	0	0	0	0	0	0
CCHFV	IgM+	5 (6.5)	5 (4.7)	2 (2.8)	0	6 (6.9)	18 (4.8)
	IgG+	2 (2.6)	3 (2.8)	2 (2.8)	1 (2.9)	3 (3.4)	11 (2.9)
	IgG+/IgM+	0	0	0	0	0	0
Ebola virus	IgM+	0	0	0	0	0	0
	IgG+	0	0	0	0	0	0
	IgG+/IgM+	0	0	0	0	0	0

*OW-HANV, Old World hantavirus; CCHFV, Crimean-Congo hemorrhagic fever virus.

blinded fashion. Serologic reactivity was assessed according to manufacturer recommendations. For the EBOV ELISA, samples were deemed positive if optical density at 405 nm was >3 SD above that of the average of known negative samples.

Serologic evidence suggestive of acute infection (IgM+) with 1 of the pathogens tested for was observed for 39.9% of samples (Table). At 14.4%, *Leptospira* spp. was the most prevalent probable etiologic agent of acute disease identified. Of mosquitoborne viruses tested, DENV had the highest prevalence at 7.7%, followed by CHIKV (5.3%) and WNV (0.27%). Of rodentborne pathogens, OW-HANVs had a seroprevalence of 7.2%, whereas LASV was considerably lower (0.27%). CCHFV IgM was documented in 4.8% of samples. Overall, little annual variation in the IgM seroprevalence was noted, except for *Leptospira* spp., for which 2 obvious peaks in seroprevalence were observed (Table).

Most IgM+ samples demonstrated serologic reactivity in only 1 assay. The exception was 2 samples that were IgM+ for hantaviruses and *Leptospira* spp., an acute dual infection that might be underrecognized (*1*). With the exception of DENV, few samples were both IgM+ and IgG+, suggesting the results were not attributable to IgM persistence. The DENV IgM+/IgG+ results might represent IgM persistence. However, because the ELISA detected all 4 serotypes, it is plausible that some results represent recent infection with DENV in the presence of IgG reactive with a different serotype.

The relatively high IgG seroprevalence for most of the pathogens tested supports the findings of the IgM assays and further suggest the circulation of and potential for human exposure to these agents in Mali (Table). Geographically, serologic evidence of infections with *Leptospira* spp., DENV, WNV, OW-HANVs, and CHIKV was observed throughout Mali (online Technical Appendix). No samples were reactive with EBOV, and the low incidence of LASV infection is not surprising because the samples analyzed here were collected outside of the 1 documented LASV-endemic region in Mali (*2*).

We used commercially available diagnostic platforms, primarily IgM capture and conventional IgG ELISAs, many of which are validated for human diagnostics. Ideally, diagnostics for zoonotic diseases would not rely on IgM/IgG serologic analysis because of caveats including IgM persistence and cross-reactivity between closely related pathogens (*3*,*4*). In the industrialized world, as well as in several countries throughout Africa, molecular approaches are often used to genetically identify pathogens, or follow-up convalescent-phase serum samples are collected to determine seroconversion or increased antibody titers or to conduct plaque reduction neutralization assays. Unfortunately, because of the nature of the samples available, including time of collection, storage history, and remaining volume, many of these tests were not feasible for our study.

Despite these limitations, these serologic findings indicate that flaviviruses, bunyaviruses, and togaviruses, as well as *Leptospira* spp., are contributing to human illness in Mali. These results add to those recently documented in studies conducted in Sierra Leone, implying that several of these zoonotic pathogens are widely distributed yet underreported throughout West Africa (*5*,*6*).

Acknowledgments

We thank Joseph Shott, Richard Sakai, and Salif Camara for logistical support, and Randal Schoepp for helpful comments and suggestions.

Tragically, a coauthor, Darin Oottamasathien, who assisted in the development of the LASV diagnostics, lost her life all too soon. We wish to honor her memory.

This work was funded by the International Centers for Excellence in Research program, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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Transdermal Diagnosis of Malaria Using Vapor Nanobubbles

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DOI: http://dx.doi.org/10.3201/eid2202.151203

To the Editor: Establishing reliable noninvasive methods for diagnosis of malaria has been a challenge. Lukianova-Helb et al. should be applauded for developing such a method on the basis of hemozoin (Hz) detection (*1*). The authors reported a proof of principle and are preparing for "large-scale studies in humans" (*2*). Such large endeavors should be based on firm evidence, so it is surprising that the results presented were from a single patient, remarkable for the unusual quadruple drug treatment (*2*). In such a scenario, to compensate for the limited data, the results should be of convincing scientific quality.

However, the case described raises several doubts that could have been addressed, such as the reliability of the diagnosis if only a thin film and a rapid test were used (co-infection excluded) and why parasitemia was not determined at the time of the device test (instead of 4 hours before and 9 hours after). What developmental stages were the parasites in at the time of the evaluation (for example, already early trophozoites containing Hz or Hz-rich gametocytes)? Why was the patient not re-evaluated to find out if repeated measurements would become appropriately negative (test-of-cure)?

The methods and results used in the study contrast with the extraordinary numbers for the limit of detection (LOD): 0.0001% in human blood and 0.00034% in a rodent model (*1*,*2*). However, the LOD is a virtual, inferred parasitemia rate based on the detection of free Hz added to uninfected blood (*1*). An LOD can be obtained from serially diluted cultures or samples (*3*). In rodent models, detection of Hz tends to be much easier (*4*). Moreover, in *Plasmodium falciparum* infections, only immature forms have been observed, with little or no detectable Hz (*5*).

The prospects of a noninvasive test for malaria are exciting. However, in times of cost restraints, any diagnostic test or intervention should provide sufficiently convincing results before consideration of resource-intensive large-scale trials.

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etymologia

Hemozoin [he"mo-zo'in]

From the Greek *haima* ("blood") + *zoon* ("animal"), hemozoin is a pigment produced by malaria parasites from hemoglobin in the host's red blood cells. This pigment was first observed by Johann Heinrich Meckel in 1847 in the blood and spleen of a mentally impaired person. In 1849, Rudolf Virchow made the connection to malaria, but it was initially believed that it was produced in the patient's spleen as a part of the immune response to malaria. In 1880, Charles Louis Alphonse Laveran observed pigmented parasites in the blood of an Algerian soldier and realized that the parasites, not the patient, produce "malaria pigment." The term "hemozoin" was coined by Louis Westenra Sambon.

Isolated *Plasmodium falciparum* hemozoin, Ernst Hempelmann, via Wikipedia

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In Response: The letter by Rebelo et al. (*1*) that questions our previously described noninvasive malaria diagnostics (*2*,*3*) misinterprets both articles. The main objection comes to our alleged call for "large-scale studies in humans"; no such statement appeared in our 2014 article (*2*), and in the 2015 article (*3*), we clearly stated that large-scale studies will be considered after the optimization of a new prototype and improving its sensitivity. The authors' final questioning of our eligibility for resources is a nonscientific opinion.

Concerning the quality of the standard clinical diagnosis, both thin blood film analysis and rapid diagnostic test results were obtained in a certified US clinical laboratory and returned consistent data. The lack of re-evaluation of the patient and the diagnostic timing are indeed limitations but were caused by the clinical restrictions. Our goal in the 2015 article (*3*) was to demonstrate the first noninvasive diagnosis of malaria in a human, which was achieved. The additional parameters discussed in the letter were not the subject of this study. Their letter further misinterprets our 2014 study, stating that parasitemia was virtual in that article; in fact, we studied actual infections among mice (*2*).

The criticism of Rebelo et al. might have been fueled by their own limited detection of hemozoin with flow cytometry and microscopy (*4*), in which they used parasite cultures and an unspecified number of malaria patients. That the methods they used might not have performed well does not mean that the novel technology we described, based upon a different mechanism, would have the same limitations in detecting hemozoin.

In conclusion, we agree with the need for optimization of the technology and additional testing. We are currently developing and testing our technology in a malaria-endemic country. Nevertheless, the letter by Rebelo et al. does not alter the fact that our novel noninvasive malaria diagnostic technology worked in a human.

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Malaria in French Guiana Linked to Illegal Gold Mining

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DOI: http://dx.doi.org/10.3201/eid2202.151292

To the Editor: French Guiana, an overseas territory of France and part of the European Union, is located on the northeast coast of South America (Figure). During 2008–2014, the number of malaria cases reported in French Guiana drastically decreased (*1*). The littoral area (≈30 km–wide Atlantic Ocean coastal band between the cities of Awala-Yalimapo and Ouanary) and the lower part of the Maroni River bordering Suriname (between the cities of Maripasoula and Saint-Laurent du Maroni) are considered malaria free, but this status may not reflect malaria transmission in the inland rainforest (*2–4*). Since 2008, French Armed Forces have been involved in military operations to control and reduce illegal gold mining activities in forested areas. Soldiers and military policemen usually spend 1–3 weeks in illegal gold mining sites in remote rainforest areas before returning to the littoral area or to bases on rivers bordering Suriname and Brazil. Despite malaria prevention strategies (*5*), these deployments have resulted in several outbreaks and increased malaria incidence among French forces (*6*). Most malaria episodes occurred during or just after deployments, so presumed locations of exposure can be easily identified.

Information about malaria cases was collected during 2008–2014 by the French Armed Forces' epidemiologic surveillance system by using a mandatory, specific form that captured putative place of malaria exposure and biologic data for case-patients (*6*). Geographic coordinates of presumed places of contamination were uploaded into a geographic information system (ArcGIS; http://www.esri.com/software/arcgis/) to produce a malaria distribution map.

During 2008–2014, a total of 1,070 malaria cases were reported to the French Armed Forces' epidemiologic surveillance system. *Plasmodium vivax* accounted for 78.8% (843/1,070), *P. falciparum* for 18.0% (193/1,070), and mixed infection (*P. vivax* and *P. falciparum*) for 3.2% (34/1,070). Places where malaria exposure occurred were identified for 742 cases of single malaria (586 *P. vivax* and 156 *P. falciparum*) infections (Figure). Cases occurring along the Maroni and Oyapock Rivers delimiting the frontiers with Suriname and Brazil, respectively, accounted for 25.3% (188/742). The other cases (74.7%, 554/742) were associated with exposures during military operations in illegal gold mining sites.

Entomologic investigations were conducted in 2 malaria epidemic locations where French forces were deployed: Eau-Claire and Dagobert. Collected *Anopheles* spp. mosquito specimens were identified by using morphologic keys specific to the Guyana Shield, a geomorphologic formation underlying French Guiana and other areas (*7*). Nonidentifiable *Anopheles* mosquito specimens were further identified molecularly (*8*). PCR products from the internal transcribed spacer 2 gene were sequenced, and *Anopheles* species were identified by comparing sequences to those in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) by searching with BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Testing for *P. falciparum* and *P. vivax* infections was conducted for all *Anopheles* spp. specimens by using nested PCR, as described (*9*).

In May 2013, a malaria outbreak occurred 1 month after military deployment of 100 soldiers at Eau Claire (3.56075°N, –53.21268°E; Figure), where 1 Mosquito Magnet trap (Woodstream Corporation, Lititz, PA, USA) baited with octenol was used to sample *Anopheles* mosquitoes during April 22–May 12, 2013 (*10*). The attack rate among the soldiers was 5.0% (5/100): 4 *P. vivax* and 1 *P. falciparum* malaria cases. Fifty-three *Anopheles* mosquito specimens were caught during the 20 days before the outbreak and identified as comprising 4 species (online Technical

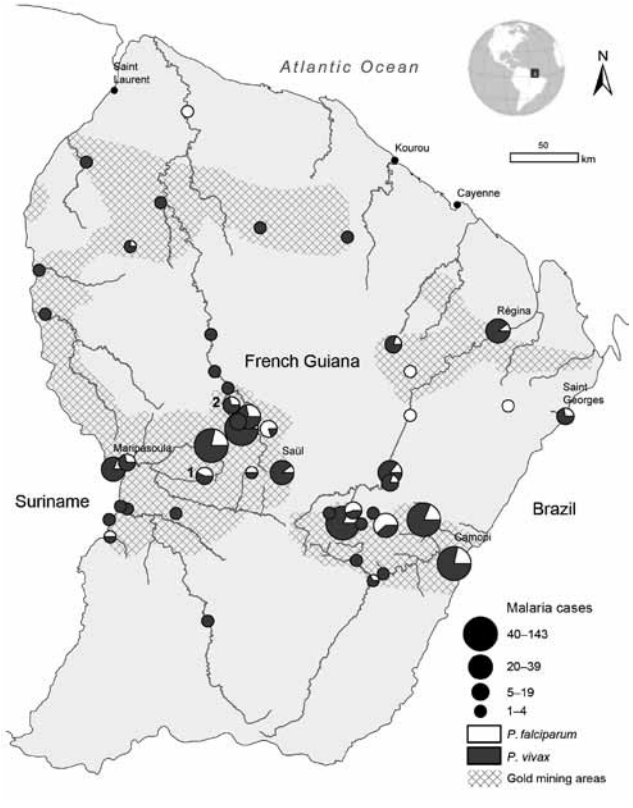

Figure. Geographic distribution of presumed places of exposure for 742 single-infection *Plasmodium vivax* (586) and *P. falciparum* (156) malaria cases reported among French Armed Forces in French Guiana, 2008–2014. Numbers on map show illegal gold mining sites where entomologic investigations were conducted; 1 indicates Eau Claire; 2 indicates Dagobert.

Appendix Table, http://wwwnc.cdc.gov/EID/article/22/2/15-1292-Techapp1.pdf).). *P. falciparum* infection was detected in 2 *Anopheles* species: 1 (12.5%) of 8 *An. ininii* and 1 (5.0%) of 19 *An. nuneztovari s.l.* mosquitoes collected; *P. vivax* infection was found in 1 (5.5%) of 19 *An. nuneztovari s.l.* mosquitoes.

In September 2013, another malaria outbreak occurred 3 weeks after the deployment of 15 soldiers in Dagobert (4.06028°N, -53.70667°E; Figure). The attack rate among these soldiers was 53.3% (8/15): 7 *P. vivax* infections and 1 co-infection with *P. vivax* and *P. falciparum*. Mosquitoes were collected 3 months later by using human landing catches during 5 consecutive days. The area had been free of illegal gold mining activities since the 15 soldiers were deployed. A total of 321 *Anopheles* mosquitoes were collected in this location; 95.6% were identified as the same 4 species as in the Eau Claire mosquito collection (online Technical Appendix Table). Only 1 specimen (0.4%, 1/282), *An. darlingi* mosquito, was infected with *P. vivax*.

These results suggest a high level of malaria transmission involving *An. darlingi* and other *Anopheles* species as primary vectors of malaria in the rainforest. The findings probably highlight malaria hyperendemicity in communities of undocumented gold miners, who are often mobile and pose a challenge for controlling malaria and other infectious diseases in the region. Indeed, these gold miners could reintroduce malaria in areas where competent vectors exist in the coastal part of French Guiana and in Surinam and Brazil, which border French Guiana. This potential for transmission could seriously threaten the success of malaria elimination programs in the Guiana Shield. Further studies are needed to better evaluate malaria epidemiology in these undocumented populations to determine how best to adapt strategies to control malaria transmission in this subregion of South America.

Acknowledgments

We thank military physicians who participated in malaria epidemiologic surveillance in French Guiana and France during 2008–2014, especially E. de Parseval, N. Barthes, J.-P. Boudsocq, C. Ilcinkas, P.-A. Poutou, G. Samy, E. Martinez, F.-X. Le Flem, and C. Marchand. We also thank P. Gaborit, R. Carinci, and J. Issaly for their support in the entomologic studies.

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Importation of Fosfomycin Resistance *fosA3* Gene to Europe

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DOI: http://dx.doi.org/10.3202/eid2202.151301

To the Editor: The wide spread of *Enterobacteriaceae* resistant to last-resource therapeutic options, including

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extended-spectrum β-lactams, fluoroquinolones, and aminoglycosides, has re-ignited interest in old antimicrobial drugs, such as fosfomycin (*1*). Fosfomycin resistance rates are generally low (<10%) but substantially higher when carbapenemase producers are considered (15%–34%) (*1*–*3*). Resistance phenotypes have been more thoroughly investigated in *Escherichia coli* and linked to chromosomal mutations in the target (*murA*) or transporter (*glpT* and *uhpT*) genes or less frequently to plasmid-mediated fosfomycin resistance genes (*fosA*, *fosB*, *fosC*) encoding glutathione S-transferases that inactivate the drug (*4*). *fosA3* is the most prevalent gene variant, disseminated mainly in *E. coli* isolates from clinical and nonclinical origins (healthy persons, companion and food animals) in countries in Asia (China, South Korea, and Japan) (*2*–*6*) and only recently in a migratory bird in Europe (*7*). We investigated the occurrence and molecular features of 43 fosfomycin-resistant *Enterobacteriaceae* isolates (21 *E. coli*, 21 *Klebsiella pneumoniae*, and 1 *Morganella morganii*). These isolates were identified among 461 third-generation cephalosporin-resistant *Enterobacteriaceae* isolates from a community laboratory in northern Portugal during a 13-month period (August 2012–August 2013).

We screened for carriage of plasmidborne fosfomycin resistance genes (*fosA*, *fosA3*, *fosB*, *fosC2*) by PCR and sequencing (*2*,*5*). Chromosomal mutations in *murA*, *glpT*, and *uhpT* were investigated for 9 representative *E. coli* isolates (*8*) and 7 representative *K. pneumoniae* isolates with variable MICs to fosfomycin (≥64 mg/L) by PCR and comparison of sequences with reference wild-type strains (*E. coli* ATCC25922 and *K. pneumoniae* type strain JCM1662) (*8*; this study). Fosfomycin-resistant isolates represented 9.3% (43/461) of the collection surveyed during the study period, which is in line with rates reported for clinical isolates from other countries (*2*,*3*). Bacterial identification and antimicrobial drug susceptibility testing to β-lactams and non–β-lactams were performed by automated methods and further confirmed by disk diffusion and agar dilution (for fosfomycin, MIC cutoff 32 mg/L) according to European Committee on Antimicrobial Susceptibility Testing guidelines (http://www.eucast.org). We screened bla_{ESBL} genes ($bla_{\text{CTX-M}}$, bla_{TEM}, bla_{SHV}) by PCR and sequencing (*9*).

One (2.3%) of 43 *E. coli* isolates carried *fosA3*, $bla_{\text{CTX-M-15}}$, and $bla_{\text{TEM-1}}$ and contained mutations in GlpT (L297F, T348N, Q443E, E444Q) and UhpT (E350Q) (GenBank accession nos. KT832798 and KT832797, respectively), most of which were previously associated with fosfomycin resistance (*8*). This isolate was detected in a urine sample from a 61-year-old man who had a clinical history of chronic prostatitis and was associated with a urinary tract infection (UTI) acquired after travel to Asia (China, Philippines). *aac-6'-lb-cr*, $bla_{\text{OXA-I}}$, and *rmtB* genes were negative by PCR. This isolate exhibited fosfomycin MIC ≥256 mg/L and was concomitantly resistant to cefotaxime, cefepime, aztreonam, ciprofloxacin, gentamicin, kanamycin, netilmycin, streptomycin, sulphonamide, tetracycline, tobramycin, and trimethoprim but not to carbapenems, amoxicillin/clavulanic acid, or cefoxitin. In other *E. coli* isolates, fosfomycin resistance phenotypes were linked to mutations in transporter proteins UhpT (8 isolates, E350Q) and GlpT (3 isolates, premature stop codons resulting in truncated proteins of 45, 134, or 442 aminoacids [GenBank accession nos. KT832799, KT832800, and KT832801, respectively]); however, no amino acid changes were detected in *K. pneumoniae* isolates. The detection of *fosA3* in a clinical *E. coli* isolate in Europe is alarming because of its association with $bla_{\text{CTX-M-15}}$, which is highly disseminated in Portugal and other European Union countries (*9*), whereas fosfomycin is increasingly being used to treat UTIs caused by extended-spectrum β-lactams–producing *E. coli* (*1*).

Strain typing (identification of *E. coli* phylogroups and multilocus sequence typing; http://mlst.warwick.ac.uk/mlst/) revealed that this isolate belonged to phylogenetic group D_1 and the sequence type 393 clone (*9*). This clone was not previously detected among *fosA3*-carrying isolates (*3*,*4*), but it is distributed worldwide (including Asia) linked to community-acquired UTI and multidrug resistance patterns (*9*).

Conjugative assays (solid/broth mating at 24°C/37°C using *E. coli* Hb101 azide and kanamycin resistant as recipient) and plasmid typing assessed by PCR-based replicon typing, IncFII typing formula (FAB), I-*Ceu*I pulsed-field gel electrophoresis, and hybridization (*5*) showed that both *fosA3* and $bla_{\text{CTX-M-15}}$ were co-located in a conjugative F2:A-:B- plasmid (transconjugant MIC to fosfomycin ≥256 mg/L). Moreover, the genetic environment of *fosA3* was assessed by PCR mapping and sequencing (*2*,*6*), showing a composite transposon containing an insertion sequence *26* 323 bp upstream *fosA3*; the *orf1*, *orf2*, and *orf3* genes (homologous to regulatory ones in *K. pneumoniae* 342); and an insertion sequence (IS) *26* downstream (GenBank accession no. KT734860). The genetic platform (IS*26* composite transposon) and the IncFII plasmid variant (F2:A-:B-) are main vehicles for disseminating *fosA3* among clinical isolates, companion and food animals in Asian countries (*3*,*5*,*6*), or $bla_{\text{CTX-M-15}}$ worldwide (*10*). Thus, epidemiologic and molecular data suggest that the detection of *fosA3* in a clinical isolate in Europe is associated with a travel-related infection acquired after international travel to Asia. The acquisition of *fosA3* by a successful clone and an efficient resistance plasmid, which might entail subsequent dissemination and alerts to the need of close monitoring of fosfomycin resistant isolates, is of particular concern.

This work received financial support from European Union FEDER (Fundo Europeu de Desenvolvimento Regional) funds through COMPETE (Programa Operacional Fatores de Competitividade), and National Funds (Fundação para a Ciência e Tecnologia) through project UID/Multi/04378/2013. The work also received financial support from the European Union (FEDER funds) under the framework of QREN (Quadro de Referência Estratégica Nacional through project NORTE-07-0124-FEDER-000066. C.R. and Â.N. were supported by fellowships from Fundação para a Ciência e Tecnologia (SFRH/BD/84341/2012 and SFRH/BPD/104927/2014, respectively).

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Mycoplasma pneumoniae Monoclonal P1 Type 2c Outbreak, Russia, 2013

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DOI: http://dx.doi.org/10.3201/eid2202.151349

To the Editor: *Mycoplasma pneumoniae* is a major cause of respiratory infections among children and young adults and is responsible for up to 40% of all community-acquired pneumonia. In 2011, an epidemic of *M. pneumoniae* infection was reported in several countries in Europe and Asia and in Israel that primarily involved adhesin P1 type 1 strains and only a few P1 type 2 strains (*1*,*2*). The spread of *M. pneumoniae* was polyclonal (*1*–*3*), except in a few semiclosed settings, such as schools (*4*). Recently, a new adhesin P1 type 2 variant, named 2c, was reported (*5*,*6*) and accounted for 8.3% of 96 *M. pneumoniae*–positive samples in Germany (*7*).

In 2013, an increase in the number of community-acquired pneumonia cases was reported in children and their adult contacts from 2 towns in Russia separated by 45 km, Ozerniy and Duchovshina, during January–March and October–November, respectively. To characterize the outbreak in Ozerniy, we collected 13 throat swabs from 9 symptomatic children and 4 asymptomatic adults who were the parents or grandparents of the affected children. All children attended the same school and were treated in the same district hospital as inpatients or outpatients. In Duchovshina, throat swab samples were collected from 17 children and 2 adults. The children attended the same school, and the preschool-aged children visited the same daycare center 1 km away. One adult patient was the first aid driver who

transported the children to the hospital. The other adult patient was a community center worker who spent time with the children. In both cities, the symptomatic patients received β-lactams as initial therapy before testing.

All specimens were processed in the laboratory of molecular diagnostics of the Smolensk State Medical Academy (Smolensk Russia). Nucleic acids were extracted by using the DNA-sorb-AM nucleic-acid extraction kit (InterLabService, Moscow, Russia), and *M. pneumoniae* was subsequently detected by using the AmpliSens *Mycoplasma pneumoniae/Chlamydophila pneumoniae*-FRT PCR kit (InterLabService). Two *M. pneumoniae* molecular typing methods, adhesin P1 typing and multilocus variable-number tandem-repeat analysis (MLVA), were performed as previously described (*1*,*5*,*7*). Macrolide resistance-associated mutations were searched using real-time PCR and melting curve analysis (*1*).

The *M. pneumoniae* isolates from the specimens collected in Ozerniy were all adhesin P1 type 2c and belonged to 4 distinct MLVA types, 1 of which, MLVA type 73563, has not been previously reported (Table). Without including the instable MPN1 marker (*8*), we observed only 2 MLVA types. The 19 *M. pneumoniae* isolates from the specimens collected in Duchovshina also were all P1 type 2c, and all belonged to the same MLVA type, 43562 (type M). No macrolide resistance–associated mutation was observed in any city.

For comparison purposes, because no previous data regarding *M. pneumoniae* molecular epidemiology in Russia were available, we retrospectively characterized 29 specimens, not from an outbreak, that were previously randomly collected for community-acquired pneumonia etiologic studies during October 2006–October 2007 and February–October 2010 by the laboratory of Smolensk State Medical

Table. Characteristics of 61 *Mycobacterium pneumoniae*–positive respiratory tract specimens collected in Ozerniy and Duchovshina, Russia*

City or region and specimen designation	Sample source	Patient age, y	Hospitalization status	Respiratory clinical syndrome	Date of collection	MLVA type†	MLVA type without MPN1‡	PCR-RFLP type	Macrolide resistance genotype
Ozerniy									
38795	Throat swab	12	Inpatient	Pneumonia	2013 Feb 15	53562 (S)	3562	2c	Wild type
38796	Throat swab	10	Inpatient	Pneumonia	2013 Feb 15	73562 (Y)	3562	2c	Wild type
38799	Throat swab	10	Inpatient	Pneumonia	2013 Feb 15	73563	3563	2c	Wild type
38812	Throat swab	9	Inpatient	Pneumonia	2013 Feb 15	73562 (Y)	3562	2c	Wild type
38814	Throat swab	11	Inpatient	Pneumonia	2013 Feb 15	73562 (Y)	3562	2c	Wild type
38941	Throat swab	33	Outpatient	Asymptomatic	2013 Feb 20	73563	3563	2c	No amp
38945	Throat swab	10	Inpatient	Pneumonia	2013 Feb 20	73563	3563	2c	No amp
38946	Throat swab	10	Inpatient	Pneumonia	2013 Feb 20	73563	3563	2c	Wild type
38960	Throat swab	33	Outpatient	Asymptomatic	2013 Feb 20	73563	3563	2c	Wild type
38962	Throat swab	51	Outpatient	Asymptomatic	2013 Feb 20	73562 (Y)	3562	2c	Wild type
39042	Throat swab	9	Inpatient	Pneumonia	2013 Feb 25	73562 (Y)	3562	2c	Wild type
39048	Throat swab	64	Outpatient	Asymptomatic	2013 Feb 25	63562 (V)	3562	2c	Wild type
39293	Throat swab	8	Inpatient	Pneumonia	2013 Mar 7	73562 (Y)	3562	2c	Wild type
Duchovshina									
43593	Throat swab	13	Inpatient	Pneumonia	2013 Oct 18	43562 (M)	3562	2c	Wild type
43596	Throat swab	15	Outpatient	Pneumonia	2013 Oct 18	43562 (M)	3562	2c	Wild type
43597	Throat swab	12	Inpatient	Pneumonia	2013 Oct 18	43562 (M)	3562	2c	Wild type
43692	Throat swab	5	Inpatient	Pneumonia	2013 Oct 22	43562 (M)	3562	2c	Wild type
43693	Throat swab	10	Inpatient	Pneumonia	2013 Oct 23	43562 (M)	3562	2c	Wild type
43694	Throat swab	9	Inpatient	Pneumonia	2013 Oct 22	43562 (M)	3562	2c	Wild type
43695	Throat swab	13	Inpatient	Pneumonia	2013 Oct 23	43562 (M)	3562	2c	Wild type
43804	Throat swab	9	Inpatient	Pneumonia	2013 Oct 27	43562 (M)	3562	2c	Wild type
43805	Throat swab	6	Inpatient	Pneumonia	2013 Oct 27	43562 (M)	3562	2c	Wild type
43806	Throat swab	14	Inpatient	Pneumonia	2013 Oct 27	43562 (M)	3562	2c	Wild type
43843	Throat swab	6	Inpatient	Pneumonia	2013 Oct 30	43562 (M)	3562	2c	Wild type
43888	Throat swab	58	Outpatient	Pneumonia	2013 Oct 31	43562 (M)	3562	2c	Wild type
43890	Throat swab	4	Inpatient	Pneumonia	2013 Oct 31	43562 (M)	3562	2c	Wild type
43919	Throat swab	41	Outpatient	Pneumonia	2013 Nov 1	43562 (M)	3562	2c	Wild type
43989	Throat swab	10	Inpatient	Pneumonia	2013 Nov 6	43562 (M)	3562	2c	Wild type
43990	Throat swab	13	Inpatient	Pneumonia	2013 Nov 6	43562 (M)	3562	2c	Wild type
43991	Throat swab	8	Inpatient	Pneumonia	2013 Nov 6	43562 (M)	3562	2c	Wild type
44174	Throat swab	10	Inpatient	Pneumonia	2013 Nov 14	43562 (M)	3562	2c	Wild type
44176	Throat swab	8	Inpatient	Pneumonia	2013 Nov 14	43562 (M)	3562	2c	No amp

*MLVA, multilocus variable-number tandem-repeat; no amp, no amplification with the real-time PCR used to detect 23S rRNA mutations associated with macrolide resistance (*1*); RFLP, restriction fragment length polymorphism. An expanded version of this table is available online at http://wwwnc.cdc.gov/EID/article/22/2/15-1349-T1.htm.

†The profiles are named according to a string of allele numbers in order of MPN1, MPN13, MPN14, MPN15, and MPN16 markers showing the number of repeats at each locus. When available, the naming according to Degrange et al. is shown in parentheses (*1*).

‡The profiles are named according to a string of allele numbers in order of MPN13, MPN14, MPN15 and MPN16 markers showing the number of repeats at each locus. The instable MPN1 marker (*8*) was removed.

Academy (Table). Of these specimens, 12 (41%) were P1 type 1, 15 (52%) were P1 type 2a, and only 2 (7%) were P1 type 2c. A polyclonal distribution with 8 distinct MLVA types was observed, with the MLVA type M representing 11 (38%) of the identified MLVA types. Without the MPN1 marker, 3 MLVA types were observed. No macrolide resistance–associated mutation was detected, similar to what was observed in the 32 specimens collected in 2013. This finding is consistent with the low prevalence of macrolide resistance reported in northern Europe (*6*,*7*).

We report 2 outbreaks of *M. pneumoniae* infections that occurred in the first and last quarter of 2013 in western Russia (Smolensk region). Despite the high predominance of P1 type 1 strains reported in the recent literature (*1*,*2*,*7*), these 2 outbreaks, reported in semiclosed settings involved only the newly described P1 type 2c variant; 1 outbreak represented a monoclonal phenomenon. In the Smolensk region, the circulation of both type 1 and 2 strains was observed a few years before the outbreak; most of these strains were P1 type 2a variants, and only a minority were type 2c variants, suggesting that the new type 2c variant had spread throughout this region of Russia since at least 2006. In other parts of the world, a switch between type 1 and type 2 strains might be occurring. Indeed, in the United States, P1 type 1 isolates predominated before 2010 but dropped to 50% of isolates in 2013, and type 2 and type 2 variant strains increased (*9*). This cyclic pattern of type 1 or type 2 predominance in the population has previously been reported (*10*).

In conclusion, we detected no macrolide resistance in western Russia. The P1 type 2c variant spread throughout this region and can be responsible for monoclonal outbreaks. The epidemiologic monitoring of *M. pneumoniae* P1 types will assess the potential switch to P1 type 2 in the United States and other parts of the world and detect the possible emergence of the P1 type 2c variant.

This study was supported by internal funding.

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Initial Costs of Ebola Treatment Centers in the United States

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DOI: http://dx.doi.org/10.3201/eid2202.151431

To the Editor: The 2014–2015 outbreak of Ebola virus disease (EVD) in West Africa was unprecedented in scale and scope. During the outbreak, 11 patients with

EVD were cared for in the United States (*1*). Safely caring for patients with suspected EVD requires specialized protocols and training for hospital staff in the use of personal protective equipment (PPE) and isolation precautions (*2*,*3*). The care of a hospitalized patient with confirmed EVD in high-level isolation units requires large specialized teams of nurses, physicians, laboratory technologists, environmental service workers, and waste management specialists, and inpatient care may continue for weeks (*3*,*4*). The staff-to-patient ratio necessary to care for a patient with EVD in high-level isolation is much higher than that in a typical intensive care unit because of the extensive PPE used and the need for partners to assist with PPE donning and doffing.

In response to preparedness challenges in the United States, the Centers for Disease Control and Prevention recommended a multitiered framework of hospitals with advanced capabilities for Ebola care: frontline facilities, Ebola assessment hospitals, and Ebola treatment centers (ETCs) (*2*). Within this federal framework, 55 hospitals in the United States have been designated by their states as ETCs, which have the advanced capabilities required to provide medical care to patients with confirmed EVD throughout their illness (*5*). Although the cost of preparing these healthcare facilities to care for EVD patients was believed to be substantial (*5*–*7*), we aimed to directly survey the ETCs to determine the costs incurred to prepare their facilities to manage and treat EVD patients.

In April 2015, we sent a 19-question electronic survey to all 55 ETCs, including the 3 preexisting biocontainment patient care units (online Technical Appendix, http://wwwnc.cdc.gov/EID/article/22/2/15-1431-Techapp1.pdf). Participation was voluntary, and individual responses were confidential. The survey assessed the ETCs' general organization and the costs incurred to establish the ETC. Of the ETCs, 45 indicated interest in participating in the establishment of the United States Highly Infectious Diseases Network to establish infection control metrics and competencies for high-level patient isolation centers. The Institutional Review Board of the University of Nebraska Medical Center declared this study exempt.

Of the 55 ETCs, 47 (85.5%) responded to the survey; 45/47 reported the total costs incurred to establish their ETC, and 43/47 provided a detailed assessment of costs. The 45 ETCs reporting total costs incurred a cumulative total of $53,909,701 (mean $1,197,993/ETC) to establish the ETCs (Table). The most costly activity was facility construction and modifications. Costs incurred to provide initial training for staff averaged $267,075 (range $10,000–$1,624,639). Each ETC spent $172,581 (mean per facility; range $3,000–$560,000) on other expenses not included in the 5 specified categories (Table). Examples of additional costs included computer hardware and software, nonmedical equipment, office supplies, and employee apparel. Costs and expenses allocated to specific purchases varied by region (online Technical Appendix Figures 1, 2).

With the exception of 3 hospitals that had preexisting biocontainment units, 52 hospitals had to undertake novel activities to prepare to care for patients with EVD, including development of plans, recruitment of facility leadership, recruitment and training of a multidisciplinary team of volunteers, and purchase of specialized supplies and equipment. The nearly $54 million in previously unbudgeted expenses was a substantial financial burden on the ETCs. Wide variations for overall expenditures and for specific types of expenditures were noted.

Because 10 ETCs did not report financial data, the overall costs reported here do not fully estimate the expenses incurred by ETCs. Furthermore, these overall costs represent only the initial start-up costs of establishing ETCs and do not include the costs of ongoing maintenance such as resupplying validation reagents for the laboratory, purchasing supplies and equipment, continual training of staff, or testing the units and programs.

This study had limitations. We could not validate self-reported data from the ETCs with information from expense reports. We also acknowledge that many additional hospitals undertook similar efforts to those of the designated ETCs but were not included in this survey (*8*). The costs incurred by public and private public health organizations also were not included.

In conclusion, we have described the initial preparation costs incurred by designated ETCs in the United States. The substantial start-up costs as well as ongoing maintenance costs of EVD programs underscore the need for specialized

Table. Initial costs in US$ incurred by 45 Ebola treatment centers in the United States*

Cost scale	Total costs	Construction/ facility modifications	PPE supplies	Staff training	Unit planning	Laboratory equipment	Non-PPE and nonlaboratory supplies and equipment
Average	1,197,993	420,502	213,347	267,075	176,713	99,106	172,581
Median	1,000,000	202,980	110,000	150,000	82,000	84,000	100,000
High	6,556,457	3,839,000	1,067,573	1,624,639	1,200,000	317,406	560,000
Low	51,500	8,500	10,000	10,000	15,000	0	3,000
Sums†	53,909,701	16,820,080	8,747,240	10,950,072	4,947,966	3,865,124	6,385,513

*PPE, personal protective equipment.
†Summarized data were collected through self-report by individual treatment centers through an electronically administered survey.

facilities to treat EVD (*9*,*10*). A tiered nationwide network of healthcare facilities that can rapidly identify, isolate, and treat patients with EVD has been established to improve the nation's preparedness for EVD and can serve as a valuable resource for future outbreaks of other highly infectious diseases. Ongoing resources will be needed to sustain the readiness of such a network.

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Detection of Influenza D Virus among Swine and Cattle, Italy

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DOI: http://dx.doi.org/10.3201/eid2202.151439

To the Editor: Recent studies have identified a new genus of the *Orthomyxoviridae* family (*1*–*5*). The virus, distantly related to human influenza C virus, has been provisionally designated as influenza D virus. This novel virus was identified for the first time in pigs with influenza-like illness (*1*), but subsequent serologic and virologic surveys have suggested cattle as a possible reservoir (*2*–*4*). Moreover, the virus was shown to infect ferrets used in laboratories as surrogates for humans when investigating influenza infection (*1*). In a serologic study conducted on 316 human samples, low antibody titers and a low level of positive samples (1.3%) were detected (*1*), suggesting that humans are a possible host to be studied in depth. To investigate the circulation of influenza D viruses among pigs and cattle in Italy, we performed biomolecular and virological tests on clinical samples collected from respiratory outbreaks in Po Valley, the area in Italy with the highest density of swine and cattle farms.

We screened clinical specimens from swine (n = 150) and cattle (n = 150) for influenza D virus by reverse transcription quantitative PCR (*1*). Three nasal swab samples were found positive: 1 from a sow and 2 from cattle, collected from 3 farms located at linear distances ranging from 47 to 80 km. All positive samples were confirmed by partial polymerase basic 1 gene sequencing and submitted to viral isolation in cell cultures as previously described (*5*,*6*). The virus was isolated on CACO-2 and HRT18 cell cultures only from the sow sample (D/swine/Italy/199723-3/2015). Cell cultures were tested by using reverse transcription quantitative PCR. Viral RNA was isolated from clinical samples or cell culture by using One-For-All Vet Kit (QIAGEN, Milan, Italy). Full-genome amplification from influenza D virus–positive samples was achieved as previously described (*3*). A sequencing library of the purified amplicons was prepared by using NEXTERA-XT kit and

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sequenced by using a Miseq Reagent Kit v2 in a 250-cycle paired-end run (both from Illumina Inc., San Diego, CA, USA). Sequencing reads were assembled de novo or by using D/swine/Oklahoma/1334/2011 (GenBank accession nos. JQ922305–JQ922311) as a template by Seqman NGen DNASTAR version 11.2.1 (DNASTAR, Madison, WI, USA). Gene sequences from the 3 influenza D viruses isolated in Italy and all the available influenza D virus sequences retrieved from GenBank were aligned with ClustalW by using MEGA5 (*7*). We analyzed the predicted amino acid sequences for each gene.

Phylogenetic trees of the individual segments were inferred by using the maximum-likelihood method implemented in the IQ-TREE package 0.9.6 (*8*). The robustness of the maximum-likelihood trees was evaluated by bootstrap analysis by comparison to 1,000 bootstrap samples. The swine isolate D/swine/Italy/199723-3/2015 was fully sequenced (GenBank accession nos. KT592530–KT592536). Full genome sequences of D/bovine/Italy/1/2014 (GenBank accession nos. KT592516–KT592522) and D/bovine/Italy/46484/2015 (GenBank accession nos. KT592523–KT592529) were obtained directly from the nasal swab samples. The 7 genomic segments of each of the 3 influenza D virus genomes encode the proteins of polymerase basic subunits 1 and 2, polymerase 3, glycoprotein, nucleoprotein, matrix 1, matrix 2, and nonstructural proteins 1 and 2. These segments contain 772, 755, 710, 664, 552, 387, 246, 243, and 184 aa residues, respectively, similar to viruses of their counterparts of the isolates documented in Asia and America. The predicted amino acid sequence of the hemagglutinin gene shows unique features for the strains isolated in Italy: V in position 289, K409R, I563L, and A652V. In the apex of the hemagglutinin 1 receptor-binding domain of the glycoprotein-predicted proteins, position 212 is occupied by K, as previously observed for D/swine/Oklahoma/1334/2011 (*5*). Moreover, the 3 isolates from Italy share unique mutations in the polymerase basic 1 gene (R191G, F278S, R444G) and in the polymerase 3 predicted proteins (I194V, M596V). D/swine/Italy/199723-3/2015 shows no unique amino acid difference to bovine strains, and its gene segments cluster with influenza D viruses isolated from cattle, suggesting the circulation of this virus among cattle and swine in Italy. Phylogenetically, all 7 segments of the strains isolated in Italy clustered with D/swine/Oklahoma/1334/2011, showing no sign of reassortment (Figure).

Our findings show that influenza D viruses circulate among swine and bovine herds in Italy affected by respiratory disease. Genetic analysis highlights that the swine and bovine influenza D viruses are very closely related, both belonging to the D/swine/Oklahoma/1334/2011 cluster. Further studies are ongoing to better understand the epidemiology, virology, and pathobiology of influenza D virus in swine and cattle, especially concerning the evidence that Koch's postulates are fulfilled for this agent. Implications in zoonotic aspects of influenza D virus infections will be also considered in ongoing research.

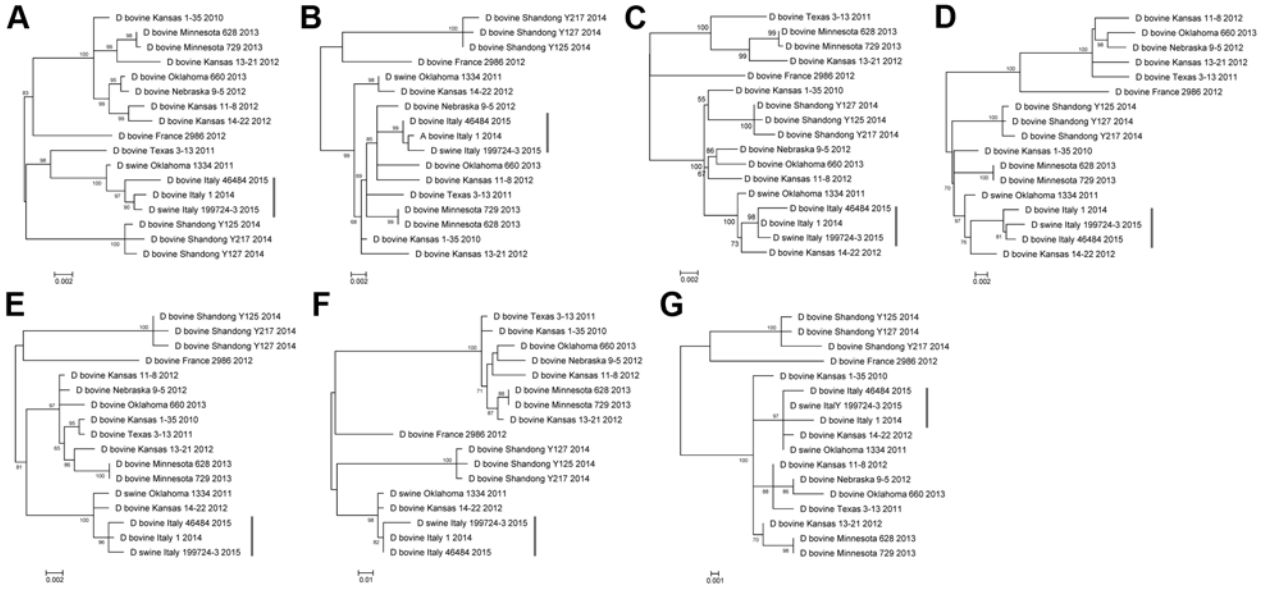

Figure. Phylogenetic trees of the 7 genes of influenza D viruses obtained from 1 sow and 2 cattle in Italy (vertical bars) and comparison isolates retrieved from GenBank. A) Polymerase basic (PB) 2: 2,319 nt; B) PB1: 1,434 nt; C) P3: 2,133 nt; D) glycoprotein hemagglutinin-esterase: 1,995 nt; E) nucleoprotein: 1,659 nt; F) polymerase 42: 1,164 nt; G) nonstructural: 732 nt. Genes were trimmed and aligned, then phylogenetically analyzed by using the maximum-likelihood method. Sequences are listed by their host, country, strain name, and collection year. Scale bars indicate nucleotide substitutions per site.

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AP92-like Crimean-Congo Hemorrhagic Fever Virus in *Hyalomma aegyptium* Ticks, Algeria

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DOI: http://dx.doi.org/10.3201/eid2202.151528

To the Editor: Crimean-Congo hemorrhagic fever virus (CCHFV) (Nairovirus, *Bunyaviridae*), the causative agent of Crimean-Congo hemorrhagic fever, has been detected in sub-Saharan Africa, southeastern Europe, the Middle East, and central Asia. The virus has been detected in >31 species of ticks and is transmitted to humans by bite of infected ticks (mainly of the genus *Hyalomma*) or by contact with body fluids or tissue of viremic patients or livestock. The disease is characterized by fever, myalgia, headache, vomiting, and sometimes hemorrhage; reported mortality rate is 10%–50% (*1*).

CCHFV strains currently constitute 7 evolutionary lineages, 1 of which (Europe 2) contains the prototype strain AP92, which was isolated in 1975 from *Rhipicephalus bursa* ticks collected from goats in Greece (*2*). This strain seems to have low or no pathogenicity for humans; only a few mild cases have been reported (*3*). This observation is supported by the relatively high (14.4%) seroprevalence but no clinical cases in humans in northwestern Greece (*4*). The documented tick carriers of this strain are *R. bursa* and *Hyalomma marginatum* (*5*).

Hyalomma aegyptium ticks are highly host specific; adults feed almost entirely on tortoises of the genus *Testudo* (*6*) and occassionally on hedgehogs and hares. Unlike adult ticks, the larvae and nymphs are less host specific and feed on a wide spectrum of hosts (e.g., other reptiles, birds, and mammals [including humans]) (*7*). This trait elevates the epidemiologic role of the tick as a possible bridge vector connecting wildlife, domestic animals, and humans.

To determine the biological and epidemiological role of *H. aegyptium* ticks, during 2009–2010, we collected 56 adult ticks from 12 *Testudo graeca* tortoises at a locality near the city of Aflou in Laghouat Province, Algeria. We tested the ticks for probable CCHFV infection by using nested reverse transcription PCR (*8*), which amplifies a partial fragment of the CCHFV small RNA segment. We slightly modified the assay: reverse transcription time was 60 minutes and annealing temperature was 52°C (*9*).

In total, 16 (28.6%) ticks were positive for CCHFV. The PCR products of 15 (26.8%) positive samples were sequenced. BLAST (http://blast.ncbi.nlm.nih.gov//Blast.cgi) analysis identified all 15 sequences as CCHFV with 98%–100% identity to the AP92 strain (GenBank accession no. DQ211638). Two variants of AP92 were detected and differed by 0.6%. A phylogenetic tree was constructed by Bayesian inference, using MrBayes version 3.1.2 (http://mrbayes.sourceforge.net/index/php) under a general time-reversible plus gamma distribution plus invariable site model with 10^7 generations setup (Figure). Sequences are available in GenBank accession nos. KT99097 and KT99098).

Our findings demonstrate the presence of CCHFV in Algeria, either recently introduced or overlooked. The nearest location where CCHFV has been reported is the Zouala region in Morocco, where the virus was detected in *H. marginatum* tick larvae and nymphs collected from

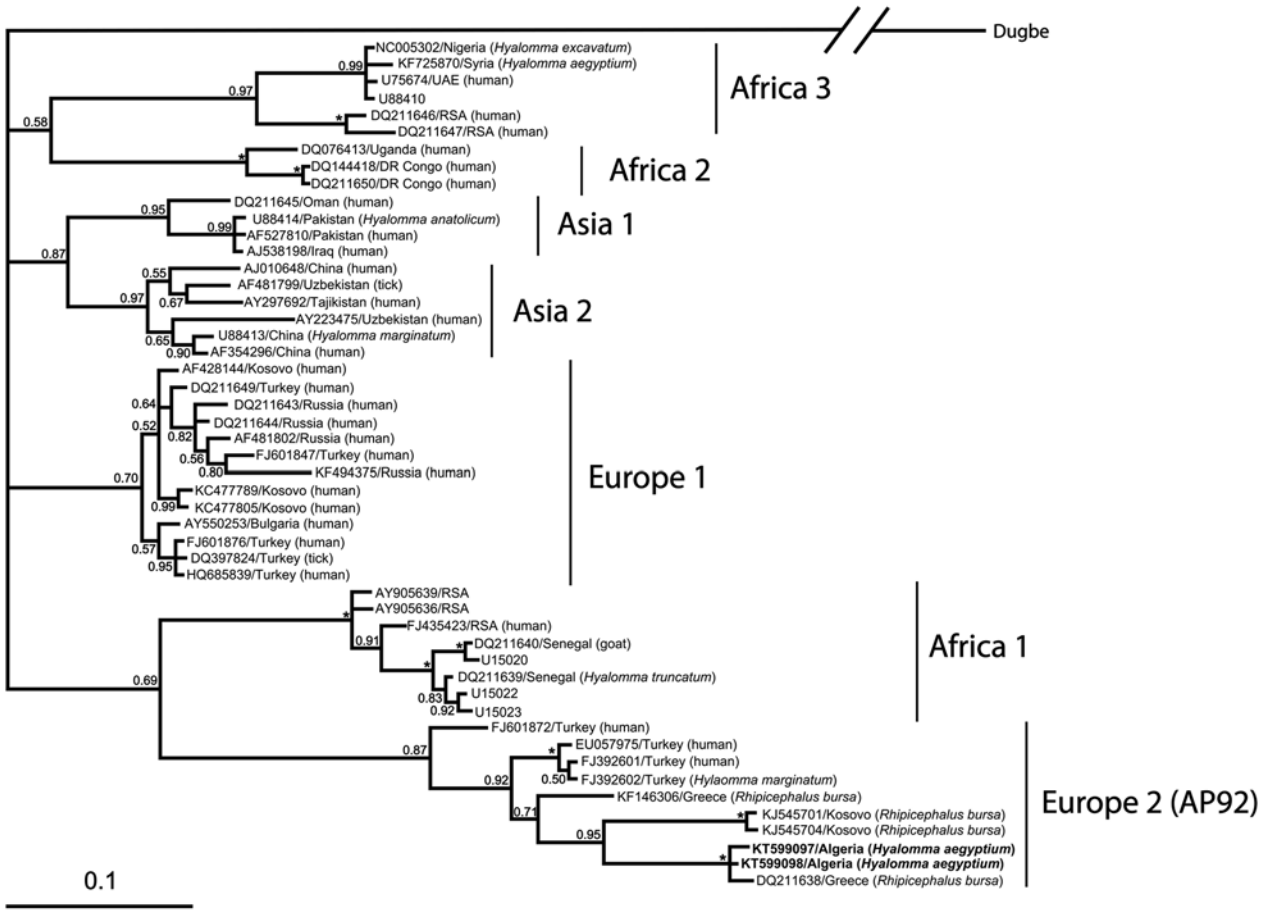

Figure. Phylogenetic analysis of Crimean-Congo hemorrhagic fever virus small RNA segment sequences, performed by using Bayesian inference in MrBayes version 3.1.2. (http://mrbayes.csit.fsu.edu/) under a general time-reversible plus gamma distribution plus invariable site model with 10^7 generations setup. Bootstrap values (>50%) are shown at nodes. Asterisks (*) indicate 1.00 bootstrap value. Scale bar represents the estimated number of substitutions per site. Individual sequences are named with GenBank accession number/country of origin and the host, if available, in parentheses. Boldface indicates sequences of virus isolated from ticks collected from 12 *Testudo graeca* tortoises in Algeria, 2009–2010.

migratory birds (*10*). It also confirms association of AP92-like sequences with *H. aegyptium* ticks.

This study shows that the Europe 2 lineage is not restricted to the Balkan region and Turkey. The role of *H. aegyptium* ticks as CCHFV vectors should be further tested. Further investigation of the distribution of CCHFV in ticks in Algeria is also needed. To date, CCHFV strains of lineage Europe 2 have not been associated with severe disease in humans. However, physicians in Algeria should be aware of potential Crimean-Congo hemorrhagic fever cases.

This study was supported by the Internal Grant Agency of the University of Veterinary and Pharmaceutical Sciences Brno (project no. 15/2015/FVHE). A.P. is supported by the ANTIGONE EU project (grant no. 278976).

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Correction: Vol. 22, No. 1

Some references were cited incorrectly in the print and initial PDF editions of Epidemiology of *Haemophilus ducreyi* Infections (C. González-Beiras et al.). The online edition of the article is correct, and the online PDF has been corrected (http://wwwnc.cdc.gov/EID/article/22/1/14-0425_article).

Edward Epp (b. 1950), ***From the Randal Street Apartment—to the South East—Monrovia***, 1981. Watercolor on paper, 22 in x 30 in/55.8 cm x 76.2 cm. Digital image courtesy of Marion Scott Gallery/Kardosh Projects, Vancouver, British Columbia, Canada.

Responding to Ebola through Visual Poetry

Byron Breedlove

In 1981, when Canadian artist Edward Epp and his wife Leanne Boschman moved to Liberia to work as teachers, they initially lived in an apartment on Randal Street in downtown Monrovia. From there, Epp noted, "the sights and sounds from the community were unlike anything that I had experienced before in North American or European cities: the architecture, the flora, the street noises and outdoor activities, such as football games in a field visible beyond the cement block businesses and private apartments, were all new to us."[1]

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DOI: http://dx.doi.org/10.3201/eid2202.AC2202

Epp is best known for his light-filled landscapes, and his works have been shown in exhibitions across Canada and in the United States and Botswana. Before traveling to Liberia, he experimented with watercolors to paint scenes from the dense forests of British Columbia, "making abstract patterns and rhythms, very much like jazz-improvisation, suspending judgment, and being open to new environments."[1] An advocate of painting en plein air, which stresses attention to color, light, and movement, Epp states in the introduction to his Marion Scott Gallery exhibit that "In Liberia, I was experiencing a new world of light and colour, and the only way for me to make these experiences meaningful was to begin painting what I was seeing and perceiving."

[1]Personal communication with Edward Epp, December 7, 2015.

Explaining that "Painting was and still is a way for me to ground myself in new spaces,"[1] Epp completed this month's cover image, *From the Randal Street Apartment—to the South East—Monrovia,* soon after arriving in Liberia. It offers an impressionistic snapshot of the shimmering shapes and colors that spilled out from his vantage point in the brilliant tropical heat and light. The artist's brush strokes telegraph a sense of bustle, energy, and heat from the city. Edifices, rooftops, and streets are sketched mostly in reds and grays, whereas white spaces define and suggest structure and direction. The green and gold fronds splay out like fireworks from the trunk of a large palm tree, commanding the center of the painting.

During his time in Liberia, Epp created a colorful body of "visual poetry" featuring both architectural studies and urban landscapes and images from what he has called the "green chaos" of the West African jungle. In an interview in the *Vancouver Sun*, Epp recounted that when he worked in Liberia in the 1980s, he had heard about periodic small outbreaks of Ebola among humans in central Africa. The news of the 2014 Ebola outbreak in multiple West African countries motivated him to find a way to help a country and a region important to him as an artist and to his family. In November 2014, Epps worked with the Vancouver-based Marion Scott Gallery/Kardosh Projects on an exhibition and sale of nearly 40 his paintings from Liberia. All proceeds from the gallery's sale of his works and a portion of the artist's proceeds were subsequently donated to support the work of Medécins Sans Frontières in responding to the Ebola epidemic.

Since that time, the situation in West Africa has evolved. The World Health Organization (WHO) declared Sierra Leone free of Ebola virus transmission on November 7, 2015, and Guinea free of Ebola virus transmission on December 29, 2015. On January 14, 2016, WHO declared that human-to-human transmission of Ebola had also ended in Liberia—the country that has experienced the highest number of deaths from Ebola. Researchers now know that Ebola virus can persist in certain parts of the body in some persons who have recovered from the disease. Continued vigilance in West Africa remains a public health priority.

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- Methylotroph Infection in Patients with Chronic Granulomatous Disease
- Tuberculosis Caused by *Mycobacterium africanum*, United States, 2004–2013
- Improved Detection of Tuberculosis and Multidrug-Resistant Tuberculosis among Tibetan Refugees, India
- Mortality Rates during 2010–2011 Cholera Epidemic, Haiti
- Decreased Time to Treatment Initiation for Multidrug-Resistant Tuberculosis after Use of Xpert MTB/RIF Test, Latvia
- Factors Associated with Loss to Follow-up during Treatment for Multidrug-Resistant Tuberculosis, the Philippines, 2012
- Whole-Genome Sequencing to Determine Origin of Multinational Outbreak of *Sarocladium kiliense* Bloodstream Infections
- Faster Detection of Poliomyelitis Outbreaks to Support Polio Eradication
- Encephalitis, Ontario, Canada, 2002–2013
- Preliminary Favorable Outcome for Medically and Surgically Managed Extensively Drug-Resistant Tuberculosis, France, 2009–2014
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- Treatment of *Mycobacterium abscessus* Infection
- Middle East Respiratory Syndrome Coronavirus during Pregnancy, Abu Dhabi, United Arab Emirates, 2013
- Lyme Disease in Hispanics, United States, 2000–2013
- Human Lymphadenopathy Caused by Ratborne *Bartonella*, Tbilisi, Georgia
- Tuberculosis, Fiji, 2002–2013
- Absence of Middle East Respiratory Syndrome Coronavirus in Camelids, Kazakhstan, 2015
- *Borrelia miyamotoi* and *Candidatus* Neoehrlichia mikurensis in Questing *Ixodes ricinus* Ticks, Romania
- Generalized Cowpox Virus Infection in a Patient with HIV, Germany, 2012
- *Mycobacterium microti* Infection in Dairy Goats, France

Complete list of articles in the March issue at
http://www.cdc.gov/eid/upcoming.htm

Upcoming Infectious Disease Activities

March 2–5, 2016
ISID
17th International Congress on Infectious Diseases
Hyderabad, India
http://www.isid.org/icid/

April 18–20, 2016
19th Annual Conference on Vaccine Research
Baltimore, MD, USA
http://www.cvent.com/events/19th-annual-conference-on-vaccine-research/event-summary-9c2a6b5301a64921afbd9c07a4cffa14.aspx?refid=spcoc

May 18–21, 2016
The Society for Heathcare Epidemiology of America
Atlanta, GA, USA
http://www.shea-online.org/Education/SHEASpring2016Conference.aspx

June 16–20, 2016
American Society for Microbiology
Boston, MA, USA
http://www.asmmicrobe.org/

July 18–22, 2016
21st International AIDS Conference
Durban, South Africa
http://www.aids2016.org/

October 29–November 2, 2016
American Public Health Association
Denver, Colorado, USA
https://www.apha.org/events-and-meetings/annual/past-and-future-annual-meetings

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Article Title

Sustained Transmission of Pertussis in Vaccinated, 1–5-Year-Old Children in Preschool, Florida, USA

CME Questions

1. You are evaluating a 2-year-old girl for a cough that she has had for the past 2 weeks. The cough is described as a bark, and it may be paroxysmal. The child has had no fever and had some nasal congestion 10 days ago. You consider that this patient may have pertussis. What should you consider regarding the epidemiology and prevention of pertussis?

A. Pertussis is most common among children younger than 4 years, but it is rarely found in school-aged children
B. The prevalence of pertussis increased more than 6-fold from 2000 to 2012
C. Pertussis vaccination is associated with long-term immunity without a waning phenomenon
D. Vaccine efficacy for the pertussis vaccine is 99% to 100%

2. Your patient is up to date with her pertussis vaccine. What should you consider regarding pertussis vaccine data in the current study?

A. Nearly half of children in the affected preschool were not up to date for pertussis vaccination
B. All unvaccinated children had clinical cases of pertussis
C. Less than 5% of children in the affected preschool were vaccinated in the past year
D. The highest attack rate was encountered in the classroom of a teacher who had not received a tetanus-diphtheria-acellular pertussis (Tdap) booster

3. The patient's mother is concerned that her daughter acquired pertussis at her daycare facility. What should you consider regarding the attack rate for pertussis in the current study?

A. The attack rate was less than 20% in all classrooms
B. The highest attack rate was found in the classrooms with 3-year-old students
C. The attack rate was 0% in the classroom with all students up to date on pertussis vaccination
D. The greatest risk for pertussis infection was among 4- to 6-year-old children

4. You initiate testing for pertussis as well as treatment, but the diagnosis is not confirmed. What was the most common symptom besides a prolonged cough in the current study?

A. Paroxysmal cough
B. Apnea
C. Inspiratory whoop
D. Post-tussive vomiting

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

Molecular Characterization of Invasive *Streptococcus dysgalactiae* subsp. *equisimilis*, Japan

CME Questions

1. Your patient is an 81-year-old Japanese man with invasive *Streptococcus dysgalactiae* subsp. *equisimilis* (iSDSE) infection. According to the study by Wajima and colleagues regarding isolates from Japanese patients with invasive streptococcal infections, which of the following statements about the clinical characteristics of SDSE infections is correct?

A. Most patients with iSDSE infection were 50 to 60 years old
B. Mean age of patients with iSDSE infections was younger than for those with *S. pyogenes* and *S. agalactiae* infections
C. Pneumonia was the most common type of infection
D. Mortality rate was significantly higher in elderly patients, especially those with pneumonia, severe sepsis, septic shock, or disseminated intravascular coagulation

2. According to the study by Wajima and colleagues regarding isolates from Japanese patients with invasive streptococcal infections, which of the following statements about *emm* and multilocus sequence typing (MLST) of SDSE is correct?

A. stG245 was the most prevalent SDSE isolate
B. Mortality rates varied significantly among *emm* types
C. Genomic analysis suggests that SDSE obtained several virulence genes from *S. pyogenes* by horizontal transfer
D. MLST identified 34 sequence types and 8 novel types

3. According to the study by Wajima and colleagues regarding isolates from Japanese patients with invasive streptococcal infections, which of the following statements about antibiotic resistance genes of SDSE isolates from Japanese patients with invasive streptococcal infections would most likely be correct?

A. Macrolide-resistance genes were present in 9% of SDSE isolates
B. β-lactam resistance was present in 15%
C. Quinolone-resistance genes were present in 19% of SDSE isolates
D. Quinolone resistance is likely to increase with increasing quinolone administration

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.				
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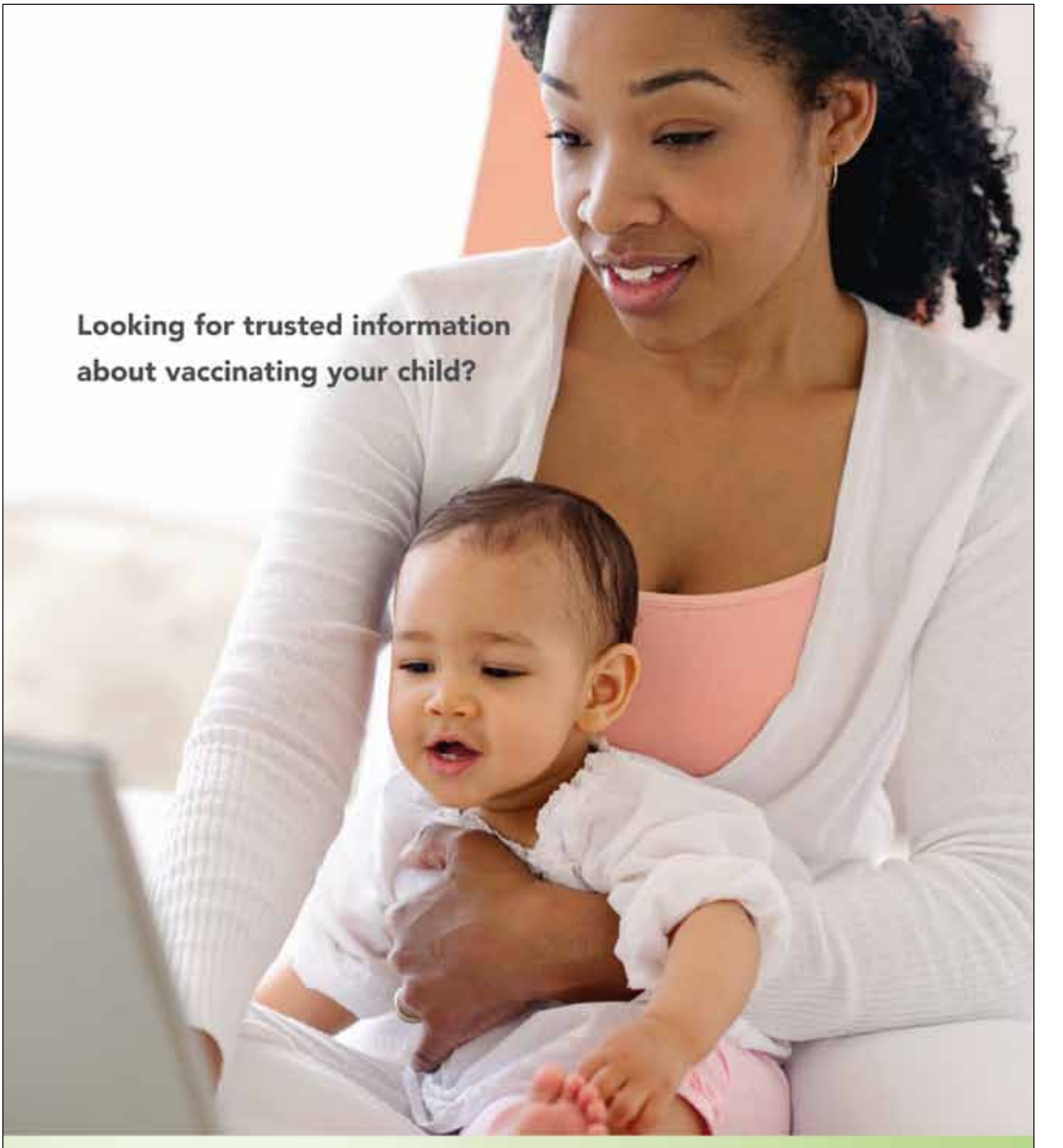
1	2	3	4	5



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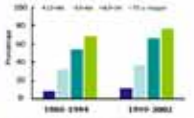

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Books, Other Media. Reviews (250–500 words) of new books or other media on emerging disease issues are welcome. Title, author(s), publisher, number of pages, and other pertinent details should be included.

Conference Summaries. Summaries of emerging infectious disease conference activities (500–1,000 words) are published online only. They should be submitted no later than 6 months after the conference and focus on content rather than process. Provide illustrations, references, and links to full reports of conference activities.

Online Reports. Reports on consensus group meetings, workshops, and other activities in which suggestions for diagnostic, treatment, or reporting methods related to infectious disease topics are formulated may be published online only. These should not exceed 3,500 words and should be authored by the group. We do not publish official guidelines or policy recommendations.

Photo Quiz. The photo quiz (1,200 words) highlights a person who made notable contributions to public health and medicine. Provide a photo of the subject, a brief clue to the person's identity, and five possible answers, followed by an essay describing the person's life and his or her significance to public health, science, and infectious disease.

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.

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ISSN 1080-6040